รายงานวิจัยฉบับสมบูรณ์

เทคโนโลยีชีวภาพทางทะเลเพื่อการพัฒนาที่ยั่งยืน : การปรับปรุงกระบวนการผลิตพันธุ์กุ้งที่มีคุณภาพ

โดย

เปี่ยมศักดิ์ เมนะเศวต และคณะ

29 กันยายน 2542

สัญญาเลขที่ RTA/06/2538

รายงานวิจัยฉบับสมบูรณ์

เทคโนโลยีชีวภาพทางทะเลเพื่อการพัฒนาที่ยั่งยืน : การปรับปรุงกระบวนการผลิตพันธุ์กุ้งที่มีคุณภาพ

โดย

ศาสตราจารย์ ดร. เปี่ยมศักดิ์ เมนะเศวต เมธีวิจัยอาวุโส สกว. และคณะ*

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

ชุดโครงการทุนส่งเสริมกลุ่มวิจัย

---

* รายชื่อคณะผู้วิจัยได้แสดงไว้ในตารางหน้า 26

กิตติกรรมประกาศ

ผู้วิจัยและคณะขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยที่ได้ให้การสนับสนุนเงินทุนวิจัยตลอดระยะเวลา 4 ปี ที่ผ่านมา

ผลงานทั้งหมดเป็นความร่วมมือของบุคลากรจากหลายหน่วยงาน กล่าวคือ สถาบันวิจัยทรัพยากรทางน้ำ ภาควิชาวิทยาศาสตร์ทางทะเล และภาควิชาจุลชีววิทยา จุฬาลงกรณ์มหาวิทยาลัย, หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเล ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ, ภาควิชาชีววิทยา มหาวิทยาลัยศรีนครินทรวิโรฒ (ประสานมิตร), Hawaii Institute of Marine Biology - University of Hawaii, Marine Biomedical Research Institute – University of Texas, USA.

ขอขอบคุณ คุณรุจีพัชร โรจนกุล, คุณทิพยวรรณ หงษ์เจ็ด, คุณทิพวรรณ มัทธวรัตน์ ที่ได้ให้ความร่วมมือในเรื่องธุรการ และการจัดทำรายงานฉบับสมบูรณ์

บทคัดย่อ

โครงการฯ มีวัตถุประสงค์ในการศึกษาวิจัยในเรื่อง การปรับปรุงกระบวนการผลิตพันธุ์กุ้งกุลาดำที่มีคุณภาพ โดยการประยุกต์ใช้เทคโนโลยีชีวภาพ ทั้งนี้เพื่อทำให้การเพาะเลี้ยงกุ้งมีความยั่งยืนและเป็นมิตรกับสิ่งแวดล้อมมากขึ้น นอกจากนี้โครงการฯ ยังมีวัตถุประสงค์และเป้าหมายอื่นๆ อีก 4 ประการคือ 1. เพื่อสร้างกลุ่มวิจัยที่มีลักษณะเป็นหน่วยปฏิบัติการเชี่ยวชาญเฉพาะทาง 2. ดำเนินการวิจัยเพื่อสร้างองค์ความรู้และเทคโนโลยีโดยเน้นเทคโนโลยีชีวภาพกุ้ง 3. การผลิตมหาบัณฑิตและดุษฎีบัณฑิตและสร้างประสบการณ์และผลงานวิจัยให้คณาจารย์ผู้ร่วมวิจัย และ 4. สร้างความเชื่อมโยงกับนักวิชาการของหน่วยงานทั้งภายในและภายนอกประเทศ

โครงการฯ ได้ดำเนินการวิจัยครอบคลุมในหลายหัวข้อ ซึ่งทั้งหมดเกี่ยวข้องกับแง่มุมต่างๆ ของการปรับปรุงกระบวนการผลิตพันธุ์กุ้งที่มีคุณภาพ โดยพยายามหลีกเลี่ยงความซ้ำซ้อนกับงานวิจัยที่กำลังดำเนินการอยู่แล้วหรือได้ดำเนินการไปแล้ว ผลการดำเนินงานวิจัยได้ครอบคลุมหัวข้อวิจัย 9 ประเด็นหลัก คือ 1. ศึกษาสถานะภาพของการเพาะเลี้ยงกุ้งในอดีตถึงปัจจุบัน 2. โภชนาการของลูกกุ้ง 3. การใช้แบคทีเรียเป็นโพรไบโอติก 4. การใช้ DNA marker เพื่อประโยชน์ในการจำแนกสายพันธุ์กุ้ง 5. การประยุกต์วิทยาอิมมูนเพื่อตรวจสอบการเจริญพันธุ์ของกุ้งและการวินิจฉัยโรคกุ้งที่เกิดจากไวรัส 6. การประยุกต์ใช้เทคนิคทางอนูพันธุศาสตร์เพื่อศึกษา neurohormones ในก้านตากุ้ง 7. ผลของความเค็มระดับต่าง ๆ ต่อสรีรวิทยาของกุ้ง 8. การพัฒนาระบบหมุนเวียนน้ำแบบปิดเพื่อเลี้ยงพ่อแม่พันธุ์กุ้ง และ 9. นวัตกรรมของระบบการเลี้ยงกุ้งทะเล : บทความปริทัศน์

การศึกษาวิจัยดังกล่าวข้างต้นได้เสริมสร้างความแข็งแกร่งให้แก่หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเลในเชิงการผลิตผลงานวิจัยและการสร้างองค์ความรู้ในด้านเทคโนโลยีชีวภาพกุ้ง โดยมีการผลิตผลงานทางวิชาการ 20 เรื่อง วิทยานิพนธ์ 13 เรื่อง ผลิตมหาบัณฑิต 12 คน และดุษฎีบัณฑิต 1 คน และสร้างเสริมประสบการณ์วิจัยให้คณาจารย์ผู้ร่วมวิจัย 2 คน ผลิตผลงานวิจัยให้แก่นักวิจัยหลังดุษฎีบัณฑิต 4 คน สร้างความเชื่อมโยงกับหน่วยงานภายในประเทศ 1 แห่ง และระหว่างประเทศอีก 2 แห่ง รวมทั้งได้มีการถ่ายทอดเทคโนโลยีให้แก่บุคคลทั่วไปในรูปของการจัดฝึกอบรมให้แก่บัณฑิตที่ว่างงาน 2 รุ่น จำนวน 160 คน โครงการฯ ได้มีส่วนร่วมในการจัดประชุมทางวิชาการนานาชาติ 1 ครั้ง มีผู้เข้าร่วมประชุมมากกว่า 800 คน

Keywords : *Penaeus monodon*, shrimp biotechnology, shrimp culture systems.

Executive Summary

The research objective of the project was to improve all aspects of methodology related to the production of *Penaeus monodon* quality seed. This in turn would make shrimp culture more sustainable and more environmental friendly. Other targets and objectives include, 1.establishing a core group specializing in this field, 2. build up of knowledge in the area of shrimp biotechnology, 3. producing M.S. and Ph.D. graduates including expertise build up of research colleagues, and 4. creating the linkages between laboratories nationally and internationally.

The project carried out several research topics. All were related to different aspects and steps of the improved shrimp quality seed stocks. Overlaping with other on-going research were avoided. The topics could be grouped into 9 categories, i.e. 1. status of shrimp culture development from past to present, 2. nutritional aspects of juvenile shrimps, 3. use of bacteria as probiotic, 4. use of DNA marker for shrimp racial study, 5. application of immunology for checking matuation development and viral disease diagnosis, 6. application of molecular biology for studying neurohormones in shrimp eye-stalks, 7. effect of different salinities on the physiology of shrimp, 8. evelopment of the closed recirculating seawater systems, and 9. innovation of shrimp pond cultures : the review.

The above researches resulted in strengthening a marine biotechnology research unit in term of producing research papers and knowledge build–up. Twenty research papers and 13 thesis were produced. Thirteen M.S. graduates and one Ph.D. graduate were produced. Two of research colleagues gained more expertise and received higher promotion. Four post-doctoral research associates gained more expertise. One linkage was built nationally, and two linkages were built internationally. Transfer of technology were performed by organizing 2 training courses for 160 graduated un-employs. Besides the project was also involved in organizing an international conference which more than 800 people attended.

Keywords : *Penaeus monodon*, shrimp biotechnology, shrimp culture systems.

บทนำ

การเพาะเลี้ยงกุ้งของไทยมีผลผลิตสูงเป็นอันดับหนึ่งของโลกตั้งแต่ พ.ศ. 2536 เป็นต้นมา อย่างไรก็ดีผลผลิตกุ้งจากการเลี้ยงมีแนวโน้มลดลงอย่างเห็นได้ชัดตั้งแต่ปี พ.ศ. 2538 ทั้งนี้เนื่องจากปัญหาในเรื่องโรคไวรัส เหตุการณ์เหล่านี้ทำให้เกิดคำถามในหมู่นักวิชาการและผู้ประกอบการว่าการเพาะเลี้ยงกุ้งจะเป็นอุตสาหกรรมที่ยั่งยืนหรือไม่?

โดยหลักการทางทฤษฎีนั้น การเพาะเลี้ยงกุ้งถ้าจะทำให้ประสบความสำเร็จและยั่งยืนขึ้นอยู่กับปัจจัยหลักสามประการคือ พันธุ์กุ้ง อาหารกุ้ง และการจัดการฟาร์ม จากประสบการณ์ที่ผ่านมาอาหารกุ้งและการจัดการฟาร์มไม่ค่อยเป็นปัญหา ในขณะที่การผลิตพันธุ์กุ้งในด้านปริมาณก็ไม่เป็นปัญหา แต่ที่เป็นปัญหาที่สำคัญคือคุณภาพของลูกกุ้ง พันธุ์กุ้งที่คุณภาพควรมีคุณสมบัติดังต่อไปนี้คือ เจริญเติบโตเร็ว มีกำลังต้านทานโรคสูงและเป็นพันธุ์กุ้งที่ผลิตจากพ่อแม่ที่เป็นพันธุ์บ้าน (domestication) และมีการคัดพันธุ์

ผลการตรวจพ่อแม่พันธุ์ที่จับจากทะเลในปัจจุบันพบว่า มีการติดเชื้อไวรัสตัวแดงดวงขาวสูงถึงร้อยละ 25 และเชื้อไวรัสดังกล่าวสามารถถ่ายทอดไปยังลูกพันธุ์ได้ ทำให้เพิ่มปัจจัยเสี่ยงต่อกิจการการเลี้ยงกุ้งในปัจจุบัน

ด้วยเหตุผลดังกล่าวข้างต้น จึงควรที่จะมีการศึกษาวิจัยในเรื่องการปรับปรุงกระบวนการผลิตพันธุ์กุ้งที่มีคุณภาพโดยการประยุกต์ใช้เทคโนโลยีชีวภาพ ทั้งนี้เพื่อทำให้การเพาะเลี้ยงกุ้งมีความยั่งยืนและเป็นมิตรกับสิ่งแวดล้อม การปรับปรุงกระบวนการผลิตดังกล่าวจะครอบคลุมการวิจัยและพัฒนาในหลายหัวข้อ อาทิเช่น การจัดตั้งกลุ่มพ่อแม่พันธุ์ (Broodstocks) ที่มีการคัดพันธุ์ การปรับปรุงในเรื่องโภชนาการ การปรับปรุงระบบที่ใช้เลี้ยงกุ้งเพื่อให้มีคุณภาพน้ำที่ดีเหมาะสมแก่การอยู่อาศัยของกุ้ง การเพิ่มอัตรารอดโดยวิธีโพรไบโอติก การนำเอาวิธีการทางด้านอิมโมโนวิทยา เพื่อใช้ในการตรวจสภาพการเจริญพันธุ์และการวินิจฉัยโรคกุ้ง ฯลฯ

ผลของการวิจัยในหัวข้อต่างๆ ดังกล่าวข้างต้นจะมีผลในทางอ้อมในเรื่องการสร้างกลุ่มวิจัยที่มีลักษณะเป็นหน่วยปฏิบัติการที่มีความเชี่ยวชาญเฉพาะทาง สร้างองค์ความรู้และเทคโนโลยีที่เกี่ยวกับการผลิตพันธุ์กุ้งที่มีคุณภาพ สร้างเสริมประสบการณ์และความชำนาญให้แก่คณาจารย์และนักวิจัยประจำหน่วยปฏิบัติการ โดยมีความเชื่อมโยงกับบัณฑิตศึกษา (ระดับปริญญาโทและเอก) และสร้างความเชื่อมโยงกับนักวิชาการของหน่วยงานทั้งภายในและภายนอกประเทศ

วิธีการดำเนินงาน

ในเรื่องการเตรียมสิ่งอำนวยความสะดวกในการวิจัยนั้น โครงการฯ ได้ดำเนินการออกแบบและก่อสร้างโรงเพาะเลี้ยงสัตว์น้ำหลังใหม่แทนหลังเก่าซึ่งถูกรื้อถอน การก่อสร้างอาคารโรงเพาะเลี้ยงสัตว์น้ำหลังใหม่นี้ได้เริ่มขึ้นเมื่อเดือนมกราคม พ.ศ. 2539 นอกจากนี้ยังได้มีการเตรียมการที่จะติดตั้งอุปกรณ์สิ่งอำนวยความสะดวกในการวิจัยภายในอาคาร อาทิเช่น ห้องปฏิบัติการชีววิทยาโมเลกุล บ่อทดลอง ระบบหมุนเวียนน้ำแบบปิด การตรวจคุณภาพน้ำแบบ data–logging

สถานปฏิบัติการดังกล่าวเป็นหน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเล ซึ่งเป็นหน่วยปฏิบัติการร่วมระหว่างศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติและจุฬาลงกรณ์มหาวิทยาลัย

การดำเนินการวิจัยได้ใช้สถานปฏิบัติการอื่นๆ อีกหลายแห่ง เช่น ที่สถานีวิจัยวิทยาศาสตร์ทางทะเลฯ เกาะสีชัง, ฟาร์มกุ้งคลอง 8, และห้องปฏิบัติการวิทยาอิมมูน คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

โครงการฯ ได้ดำเนินการวิจัยครอบคลุมในหลายหัวข้อ ซึ่งทั้งหมดเกี่ยวข้องกับแง่มุมต่างๆ ของการปรับปรุงกระบวนการผลิตพันธุ์กุ้งที่มีคุณภาพ โครงการฯ ได้พยายามดำเนินการในหัวข้อต่างๆ เท่าที่จำเป็น และหลีกเลี่ยงความซ้ำซ้อนกับงานวิจัยที่ดำเนินการอยู่แล้ว หรือได้ดำเนินการไปแล้ว โดยการสนับสนุนของทุนวิจัยอื่นๆ ในระยะเวลา 4 ปีที่ผ่านมาโครงการฯ ได้ดำเนินการวิจัยครอบคลุมหัวข้อวิจัย 9 ประเด็นหลัก ทั้งหมดเกี่ยวข้องกับกุ้งกุลาดำ (*Penaeus monodon*) ดังนี้

1. ศึกษาสถานะภาพของการเพาะเลี้ยงกุ้งในอดีตถึงปัจจุบัน
2. โภชนาการของลูกกุ้ง
3. การใช้แบคทีเรียเป็นโพรไบโอติก
4. การใช้ DNA marker เพื่อประโยชน์ในการจำแนกสายพันธุ์กุ้ง
5. การประยุกต์วิทยาอิมมูน เพื่อตรวจสอบการเจริญพันธุ์ของกุ้ง และการวินิจฉัยโรคกุ้งที่เกิดจากไวรัส
6. การประยุกต์ใช้เทคนิคทางอนูพันธุศาสตร์เพื่อศึกษา neurohormones ในก้านตากุ้ง
7. ผลของความเค็มระดับต่างๆ ต่อสรีรวิทยาของกุ้ง
8. การพัฒนาระบบหมุนเวียนน้ำแบบปิดเพื่อเลี้ยงพ่อแม่พันธุ์กุ้ง และ
9. นวัตกรรมของระบบการเลี้ยงกุ้งทะเล : บทความปริทัศน์

ผลการดำเนินงาน

1. การศึกษาสถานะภาพของการเพาะเลี้ยงกุ้งในอดีตถึงปัจจุบัน

โครงการฯ ได้ศึกษาสถานะภาพของการเพาะเลี้ยงสัตว์น้ำในประเทศไทยจากอดีตจนถึงปัจจุบัน ทั้งนี้เพื่อเป็นข้อมูลพื้นฐาน และเพื่อวิเคราะห์ดูว่าการเพาะเลี้ยงกุ้งทะเลในประเทศไทยมีบทบาทสำคัญอย่างไรต่อการเพาะเลี้ยงสัตว์น้ำในประเทศไทยโดยรวม ซึ่งผลการศึกษามีข้อมูลชี้ชัดว่า การเพาะเลี้ยงกุ้งทะเล โดยเฉพาะอย่างยิ่งกุ้งกุลาดำนั้นประสบความสำเร็จเป็นอย่างดี มีผลผลิตเพิ่มขึ้นอย่างรวดเร็ว เมื่อเปรียบเทียบกับการเพาะเลี้ยงสัตว์น้ำชนิดอื่นๆ ทั้งนี้เพราะมีการนำเอาเทคโนโลยีที่ทันสมัยมาใช้ในการผลิต (Menasveta, 1997)

อย่างไรก็ดี ถึงแม้ว่าการเพาะเลี้ยงกุ้งทะเลจะประสบความสำเร็จในเรื่องผลผลิต แต่ก็มีปัญหาและคำถามที่ตามมา เช่น การเพาะเลี้ยงกุ้งมีความยั่งยืนจริงหรือไม่ และมีผลกระทบมากน้อยแค่ไหนของสิ่งแวดล้อมในบริเวณชายฝั่ง การเพาะเลี้ยงกุ้งได้ถูกกล่าวหาบ่อยครั้งจากกลุ่ม

องค์การอิสระว่าเป็นต้นเหตุสำคัญที่ทำลายป่าชายเลน โครงการฯ จึงได้ทำการศึกษาเรื่องนี้ โดยการนำข้อมูลต่างๆ เกี่ยวกับการใช้ป่าชายเลน และการพัฒนาการเลี้ยงกุ้งทะเลในอดีตถึงปัจจุบันมาประมวลและวิเคราะห์ ผลการศึกษาได้ผลชี้ชัดว่า การเพาะเลี้ยงกุ้งในบริเวณชายฝั่งไม่ได้เป็นสาเหตุเดียวที่ทำให้จำนวนป่าชายเลนลดลง ในช่วง ค.ศ. 1961 ถึง 1993 ป่าชายเลนจำนวน 203,606 ha ได้ถูกทำลายลง ในจำนวนนี้การเพาะเลี้ยงกุ้งมีส่วนประมาณ 32% ส่วนที่เหลือเกิดจากหลายสาเหตุ เช่น การตัดฟันไม้มาทำฟืน การทำนาเกลือ การทำเหมืองแร่ การสร้างถนนหนทาง และการขยายของตัวเมือง ส่วนใหญ่ของป่าชายเลนที่ถูกทำลายโดยการเพาะเลี้ยงกุ้งเกิดขึ้นในช่วงก่อนปี ค.ศ. 1987 ทั้งนี้เพราะการเพาะเลี้ยงกุ้งในช่วงดังกล่าวใช้วิธีพื้นบ้าน ต้องการพื้นที่กว้างใหญ่ติดทะเล และอาศัยธรรมชาติ เช่น การขึ้นลงของน้ำเป็นหลัก ดังนั้นป่าชายเลนจึงถูกเลือกใช้ หลังปี 1987 ได้มีนวัตกรรมของการเลี้ยงกุ้งที่ไม่ต้องการเนื้อที่มากนัก ได้มีการนำเอาวิธีการเลี้ยงกุ้งแบบเข้ม (intensive) มาใช้ การเลี้ยงกุ้งดังกล่าวต้องการพื้นที่ที่มีระดับ (land elevation) สูงกว่าบริเวณป่าชายเลน ดังนั้นจึงไม่ต้องการพื้นที่ในบริเวณป่าชายเลนอีกต่อไป อัตราการทำลายป่าชายเลนจึงลดลงอย่างมากตั้งแต่ปี ค.ศ. 1987 เป็นต้นมา จึงเห็นได้ว่าการพัฒนาการเลี้ยงกุ้งในระยะหลังนี้ประเทศไทยได้ใช้วิธีที่เหมาะสมดีแล้ว จึงน่าจะมีความยั่งยืนและเป็นมิตรต่อสิ่งแวดล้อมมากกว่าในอดีต (Menasveta, 1997)

2. โภชนาการของลูกกุ้ง

เลซิทินและคอเลสเทอรอลจัดอยู่ในกลุ่มของลิปิดที่มีความสำคัญต่อการเจริญเติบโตและอัตรารอดของกุ้ง โครงการวิจัยเรื่องนี้มีวัตถุประสงค์เพื่อให้ทราบว่าลิปิดทั้งสองชนิดนี้มีปฏิสัมพันธ์ (interaction) กันหรือไม่ และระดับต่างๆ ของลิปิดแต่ละชนิดจะมีผลในการเพิ่มขึ้นหรือลดลงอย่างไรต่อการเจริญเติบโตและอัตรารอด โครงการฯ ได้ทำการทดลองกับกุ้งกุลาดำวัยอ่อน 3 ระยะ กล่าวคือ zoea, mysis, และ postlarvae โดยใช้เลซิทิน 4 ระดับ คือที่ 0, 0.5, 1.0 และ 1.5% ของอาหาร และระดับคอเลสเทอรอล 3 ระดับ คือที่ 0, 0.5, และ 1.0% ของอาหาร ผลการทดลองพบว่าเลซิทินและคอเลสเทอรอลไม่มีปฏิสัมพันธ์ต่อกันในทุกระดับและทุกระยะของกุ้ง ผลของเลซิทินในกุ้งทุกระยะไม่ค่อยเด่นชัดเท่ากับคอเลสเทอรอล แต่ก็อาจสรุปได้ว่าอาหารกุ้งกุลาดำวัยอ่อนควรมีเลซิทินและคอเลสเทอรอลในอัตราส่วน 1.0% เท่ากันเพื่อช่วยส่งเสริมการเจริญเติบโตและอัตรารอด (Paibulkichakul et al., 1998)

ในเรื่องอัตราส่วนระหว่างโปรตีนต่อพลังงาน โครงการฯ มีวัตถุประสงค์ในการศึกษาอัตราส่วนระหว่างโปรตีนต่อพลังงานที่เหมาะสมที่สุดในอาหารกุ้งกุลาดำวัยรุ่น ทั้งนี้เพื่อก่อให้เกิดการประหยัดในการผสมวัสดุอาหารที่เป็นแหล่งของโปรตีนซึ่งมีราคาแพงลงในอาหารกุ้ง ทำให้การอนุบาลและการเลี้ยงกุ้งมีผลลัพธ์ทางเศรษฐศาสตร์ที่ดีกว่าในอดีต การทดลองได้ดำเนินการกับกุ้งวัยรุ่น โดยแบ่งการทดลองออกเป็น 3 ตอน ตอนที่ 1 เพื่อวิเคราะห์ระดับโปรตีนที่เหมาะสมเมื่อพลังงานคงที่ ๆ 330 kcal/100 g ตอนที่ 2 นำผลระดับโปรตีนจากตอนที่ 1 มาเป็นเกณฑ์แปรผันระดับไขมันต่อคาร์โบไฮเดรตที่พลังงานคงที่ ที่ 330 kcal/100 g ตอนที่ 3 นำเอาอัตราส่วนไขมันต่อคาร์โบไฮเดรตที่เหมาะสมจากการทดลองในตอนที่ 2 มาเป็นบรรทัดฐานโดยแปรผันโปรตีนและ

พลังงาน ผลการทดลองพบว่าอาหารกุ้งวัยรุ่นควรมีระดับโปรตีน 35% พลังงานอยู่ในช่วง 223 – 459 kcal/100 g อัตราส่วนโปรตีนต่อพลังงานที่เหมาะสม (P/E ratio) คือ 150 g/kcal (Chuntapa et al., 1998)

3. การใช้แบคทีเรียเป็นโพรไบโอติก

โครงการฯ นี้มีวัตถุประสงค์เพื่อศึกษาผลของแบคทีเรียชนิดที่แยกได้จากลำไส้ของกุ้งจากแหล่งต่างๆ และได้ทดสอบในห้องปฏิบัติการแล้วว่ามีฤทธิ์ในการเป็นโพรไบโอติก เมื่อผสมอาหารกุ้งแล้วใช้เลี้ยงกุ้งว่ามีผลต่อการเจริญเติบโต อัตรารอดและการต้านทานโรคได้มากน้อยเพียงไร การดำเนินการแบ่งเป็น 2 ขั้นตอน ขั้นแรกได้แก่ การแยกแบคทีเรียชนิดต่างๆ จากลำไส้กุ้งจากบ่อเลี้ยงและทะเล ทำการทดสอบว่ามีผลทางโพรไบโอติกหรือยับยั้งเชื้อทดสอบหรือไม่โดยวิธีซึมผ่านวุ้น (well diffusion assay) ผลการศึกษาพบเพียง 1 สายพันธุ์ คือ Bacillus S11 ที่มีผลดีที่สุด จึงได้ทำการเพิ่มขยายแบคทีเรียสายพันธุ์นี้ให้ได้ปริมาณมากๆ แล้วนำมาทำการทดลองในขั้นที่สองกับกุ้งกุลาดำวัยรุ่น ผลการทดลองพบว่า Bacillus S11 มีผลทำให้กุ้งมีการจริญเติบโตและอัตราการรอดดีขึ้นและมีอัตราการต้านทานโรคแบคทีเรียจาก *Vibrio harveyi* ได้อย่างมีนัยสำคัญทางสถิติ (Rengpipat et al., 1998)

การผสม *Lactobacillus* strain (ให้ชื่อว่า LP) ซึ่งได้จากการแยกเชื้อมาจากลำไส้ไก่ลงในอาหารกุ้งเพื่อเป็นโพรไบโอติกก็ได้ผลคล้ายกับ Bacillus S11 กล่าวคือ การเจริญเติบโต และอัตรารอดของลูกกุ้งกุลาดำ มีค่าสูงกว่าชุดควบคุม (Control) อย่างมีนัยสำคัญทางสถิติ (Phianphak et al., 1999)

การใช้ Bacillus S11 เป็นโพรไบโอติกในอาหารสำหรับเลี้ยงลูกกุ้งกุลาดำวัยอ่อนก็ได้ผลดีเช่นเดียวกัน การทดลองดังกล่าวได้ใช้ *Artemia* nauplii เป็นอาหารสำหรับลูกกุ้งวัยอ่อน โดยที่ *Artemia* ที่ใช้จะมีการกรองกิน Bacillus S11 เข้าไปไว้ในทางเดินอาหาร เมื่อลูกกุ้งกิน *Artemia* ก็จะได้รับ Bacillus S11 เข้าไปด้วย ผลการทดลองพบว่าอัตรารอดของลูกกุ้งวัยอ่อนที่ได้รับ Bacillus S11 โดยวิธีนี้มีอัตรารอดสูงกว่าชุดควบคุมอย่างมีนัยสำคัญทางสถิติ (Rengpipat et al., 1998)

อย่างไรก็ดี การเติมแบคทีเรียลงในน้ำที่ใช้เลี้ยงกุ้งได้ผลที่ไม่ชัดเจนนักในเรื่องโพรไบโอติก แบคทีเรียที่ใช้มี 4 ชนิด สามชนิดแรกเป็นสายพันธุ์ของ *Bacillus* อีกชนิดหนึ่งเป็นสายพันธุ์ของ *Vibrio* ที่ไม่ก่อโรคในกุ้ง

4. การใช้ DNA marker เพื่อประโยชน์ในการจำแนกสายพันธุ์กุ้ง

โครงการฯ นี้มีวัตถุประสงค์ในอันที่จะศึกษาความหลากหลายทางพันธุกรรมของกุ้งกุลาดำในน่านน้ำไทย โดยใช้เทคนิค RAPD-PCR ขั้นตอนของการดำเนินงานประกอบด้วยการเก็บตัวอย่างกุ้งจากห้าจังหวัด กล่าวคือ พังงา, สตูล, ตรัง, ชุมพร, และตราด แล้วทำการสกัด DNA จากขาว่ายน้ำ โดยที่กุ้งที่ได้จาก พังงา, สตูล และตรัง เป็นกุ้งที่เป็นผู้แทนของกุ้งในฝั่งทะเลอันดามัน

ส่วนกุ้งจากชุมพรและตราดเป็นกุ้งผู้แทนของอ่าวไทย ผลการศึกษาจากการวิเคราะห์ 3 primers พบว่ากุ้งจากอ่าวไทยเป็นคนละสายพันธุ์กับกุ้งจากฝั่งทะเลอันดามัน ส่วนกุ้งในอ่าวไทยแบ่งได้เป็นสองสายพันธุ์ คือ ตราด และชุมพร (Wudthijinda et al., 1999)

5. การประยุกต์ใช้วิทยาอิมมูนในการวินิจฉัยโรคไวรัสและการศึกษาการเจริญพันธุ์ในกุ้งกุลาดำ

แอนติบอดีหรือ Immunoglobulin เป็นโปรตีนในระบบภูมิคุ้มกันของสัตว์มีกระดูกสันหลัง ซึ่งสร้างขึ้นเพื่อใช้ต่อต้านสิ่งแปลกปลอมที่เข้าสู่ร่างกาย โดยแอนติบอดีมีคุณสมบัติที่จะจับกับสารที่ชักนำให้เกิดการสร้างแอนติบอดี อย่างจำเพาะเจาะจง จึงสามารถประยุกต์ใช้คุณสมบัติข้อนี้เพื่อการวินิจฉัยโรคต่างๆ ได้แม่นยำ มีความไวสูง และสามารถปรับเปลี่ยนเทคนิคการวินิจฉัยในรูปแบบต่างๆ ทำให้การวินิจฉัยทำได้ง่ายและรู้ผลในเวลาอันรวดเร็ว ข้อได้เปรียบที่สำคัญอีกประการหนึ่งได้แก่ แอนติบอดีสามารถผลิตได้ในรูป monoclonal antibody (MAb) ซึ่งคุณภาพของแอนติบอดีนี้มีความสม่ำเสมอ สามารถผลิตได้ในปริมาณไม่จำกัดในราคาที่ไม่แพงมากเพราะไม่จำเป็นต้องใช้แอนติเจนมากระตุ้นอีกต่อไป

สำหรับแนวทางการวิจัยในการผลิต MAb เพื่อใช้ในการวินิจฉัยโรคไวรัสที่ก่อให้เกิดโรคหัวเหลืองและตัวแดงจุดขาวในกุ้งกุลาดำนั้นจะใช้แอนติบอดีสำหรับพัฒนาวิธีตรวจสอบโดยใช้ Sandwich Dot-Blot Method โดยติดฉลากแอนติบอดีด้วยเอนไซม์หรือสารที่มีสี ทำให้การตรวจสอบไวรัสทั้งสองชนิดทำได้พร้อมกันในตัวอย่างเดียว และรู้ผลภายในเวลารวดเร็ว และมีความแม่นยำสูง ซึ่งแอนติบอดีจำเพาะต่อไวรัสนี้นอกจากจะมีประโยชน์ในแง่การวินิจฉัยแล้วยังมีประโยชน์ในแง่การศึกษาอุบัติการณ์ของโรค ปฏิกิริยาการติดเชื้อ ซึ่งจะเป็นแนวทางสำคัญในการป้องกันโรคให้มีประสิทธิภาพต่อไป (Sithigorngul et al., 1999)

การประยุกต์ใช้เทคนิคทางภูมิคุ้มกันเพื่อการศึกษาการเจริญพันธุ์ของกุ้งกุลาดำเพศเมียนั้นได้สร้าง MAb จำเพาะต่อไวเทลลินหน่วยย่อยต่างๆ เพื่อใช้แอนติบอดีเหล่านี้ในการตรวจวัดระดับไวเทลโลเจนินในเฮโมลิมฟ์ของกุ้งกุลาดำ ให้สามารถติดตามการเจริญของรังไข่ได้โดยที่ไม่จำเป็นต้องฆ่าแม่กุ้งเพื่อการศึกษา (Longyant et al., 1999 and Longyant et al., 1999) เพื่อใช้สำหรับการพัฒนาวิธีตรวจสอบการทำงานของฮอร์โมนที่เกี่ยวข้องกับพัฒนาการของรังไข่ ได้แก่ gonad inhibiting hormone (GIH assay) สำหรับโครงการที่จะดำเนินต่อไปได้แก่ การสร้าง MAb ต่อ GIH เพื่อใช้ในการทำให้บริสุทธ์ GIH เพื่อศึกษาโครงสร้าง และศึกษาแนวทางการทำงานของฮอร์โมน เพื่อปรับปรุงวิธีการชักนำการเจริญของรังไข่ เพื่อให้การใช้แม่พันธุ์กุ้งเป็นไปอย่างมีประสิทธิภาพคุ้มค่า เพื่อพัฒนาการใช้แม่พันธุ์กุ้งจากการเพาะเลี้ยง สำหรับการเลี้ยงกุ้งแบบครบวงจร และลดการพึ่งพาแม่พันธุ์กุ้งจากธรรมชาติต่อไป

6. การประยุกต์ใช้เทคนิคทางอนูพันธุศาสตร์เพื่อศึกษา Neurohormone ในก้านตากุ้ง

Neurohormone 3 ชนิด จากก้านตาของกุ้งได้แก่ crustacean hyperglycemic hormone (CHH), molt inhibiting hormone (MIH), และ gonad inhibiting hormone (GIH) มีความสำคัญกับระบบการควบคุมการเจริญเติบโต การลอกคราบและการเจริญพันธุ์ของกุ้ง ซึ่งการศึกษายีนที่ควบคุมการหลั่งฮอร์โมนเหล่านี้จะเป็นหนทางที่นำไปสู่การเพิ่มประสิทธิภาพของการเพาะเลี้ยงกุ้งทะเลได้ ในงานวิจัยนี้ได้ทำการหายีนทั้ง 3 ชนิดจากกุ้งทะเล 2 ชนิด คือกุ้งกุลาดำ *(P. monodon)* และกุ้งกุลาลาย *(P. semisalcatus)* ด้วยเทคนิค RT-PCR และ conventional PCR โดยใช้ mRNA ที่สกัดจากบริเวณก้านตาของกุ้งและ genomic DNA เป็นต้นแบบ โดยมี primers ที่ออกแบบมาจากลำดับนิวคลีโอไทด์ของยีนที่ผลิตฮอร์โมนทั้ง 3 ชนิดจากกุ้งชนิดต่างๆ ที่มีรายงานดังนี้คือ MIH จาก *P. vannamei* และ *P. japonicus;* CHH จาก *P. japonicus;* และ GIH จาก *Homarus americanus* ผลการทำ RT-PCR จาก mRNA ที่สกัดจากก้านตาของกุ้ง *P. monodon* และ *P. semisalcatus* พบว่าผลผลิตที่ได้จาก primer สำหรับสร้าง MIH (MIH I+II) ใน *P. monodon* มีขนาดประมาณ 400 bp เมื่อทำการหาลำดับนิวคลีโอไทด์และเปรียบเทียบกับ MIH ที่มีรายงานปรากฏว่าผลที่ได้ไม่มีความใกล้เคียงกันในลำดับนิวคลีโอไทด์ เมื่อทำ PCR โดยมี genomicDNA เป็นต้นแบบ template พบว่ามีผลผลิตที่มีขนาดแตกต่างกันทั้งใน *P. monodon* และ *P. semisalcatus* เมื่อนำผลผลิตที่ได้บางส่วนไปทำการหาลำดับนิวคลีโอไทด์และเปรียบเทียบกับฮอร์โมนที่มีรายงานพบว่าผลผลิตจาก *P. semisalcatus* ให้ลำดับนิวคลีโอไทด์ที่ใกล้เคียงกับ MIH ใน *P. vannamei* (มีความเหมือนของลำดับนิวคลีโอไทด์ %) จากความรู้ที่ได้จากการศึกษานี้สามารถนำไปใช้ในการค้นหาและศึกษาลักษณะทางโครงสร้างของยีนทั้ง 3 ชนิดอย่างสมบูรณ์ต่อไป (Puanglarp et al., 1999)

7. ผลของความเค็มระดับต่าง ๆ ต่อสรีรวิทยาของกุ้ง

กุ้งกุลาดำเป็นสัตว์น้ำที่สามารถอาศัยอยู่ในน้ำที่มีความเค็มในช่วงกว้าง (earyhaline) ดังนั้นจึงควรมีการศึกษาผลของความเค็มในระดับต่างๆ ต่อสรีรวิทยาของกุ้ง เช่น อิทธิพลของความเค็มและระดับโปรตีนต่อการจัดสรรพลังงานในกุ้งกุลาดำ ผลของการเปลี่ยนแปลงของความเค็มต่อการปรับสมดุลไอออน และผลของสารอาหารบางชนิด เช่น betaine ที่น่าจะมีต่อความสามารถในการปรับความเข้มข้นภายในตัวในระดับความเค็มที่ต่ำกว่าปกติ (osmoregulation)

ในเรื่องแรกได้ศึกษาผลของความเค็มและระดับของโปรตีนในอาหารต่อการจัดสรรพลังงานของกุ้งกุลาดำ *Penaeus monodon* ในระยะวัยรุ่น (น้ำหนัก 0.6 – 0.7 กรัม ความยาว 4.0 – 5.5 เซนติเมตร) โดยใช้อาหารสำเร็จรูปชนิดเม็ด ออกแบบการทดลองแบบ factorial design ที่มีความเค็ม 3 ระดับ (10, 20 และ 30 ppt) และโปรตีน 3 ระดับ (25, 35, และ 45%) ทุกชุดการทดลองทำ 3 ซ้ำ ก่อนการทดลองทำการปรับสภาพกุ้งให้เคยชินกับภาวะของการทดลองเป็นเวลา 1 เดือน ผลการทดลองพบว่า ความเค็มไม่มีปฏิสัมพันธ์กับระดับของโปรตีนต่อการจัดสรรพลังงานของกุ้งกุลาดำ เมื่อพิจารณาเฉพาะผลของความเค็มพบว่า มีผลต่อพลังงานที่สูญเสียไปในรูปของคราบ

แตกต่างอย่างมีนัยสำคัญ ($p<0.05$) โดยที่ความเค็ม 10 ppt มีพลังงานที่สูญเสียไปในรูปของคราบสูงสุดและลดลงตามความเค็มที่เพิ่มขึ้น แต่ไม่มีผลต่ออัตรารอด พลังงานจากการบริโภค พลังงานที่ใช้ในการเติบโต พลังงานที่ใช้ในการหายใจ พลังงานที่สูญเสียไปในรูปของแอมโมเนีย และพลังงานที่สูญเสียไปในรูปของอุจจาระ ส่วนระดับของโปรตีนนั้นพบว่า มีผลต่อพลังงานที่ใช้ในการเติบโตอย่างมีนัยสำคัญ ($p<0.05$) โดยที่ระดับโปรตีน 45% มีค่าพลังงานที่ใช้ในการเติบโตสูงสุดและลดลงตามระดับของโปรตีน แต่ระดับของโปรตีนไม่มีผลต่อพลังงานจากการบริโภค พลังงานที่ใช้ในการหายใจ พลังงานที่สูญเสียไปในรูปของแอมโมเนีย พลังงานที่สูญเสียไปในรูปของอุจจาระและพลังงานที่สูญเสียไปในรูปของคราบ จากผลการทดลองสามารถอธิบายการจัดสรรพลังงานในภาวะของความเค็ม (20 ppt) และระดับของโปรตีน (35%) ที่เหมาะสมต่อการเลี้ยงกุ้งกุลาดำในระยะวัยรุ่นได้ดังนี้ ใช้ในการเติบโต 15.39% การหายใจ 14.88% สูญเสียไปในรูปของแอมโมเนีย 0.98% สูญเสียไปในรูปของอุจจาระ 56.42% สูญเสียไปในรูปของคราบ 0.65% และสูญเสียไปในขั้นตอนของการบริโภค 11.68% ทั้งนี้สำหรับความเค็มที่เพิ่มขึ้น (30 ppt) หรือลดลง (10 ppt) และระดับโปรตีนในอาหารคงเดิม จะมีการสูญเสียพลังงานไปในรูปของแอมโมเนีย การหายใจและอุจจาระเพิ่มขึ้น ทำให้เหลือพลังงานสำหรับการเติบโตลดลง (พิพัฒน์ เวฬุคามกุล, 2541)

ในเรื่องที่สองได้ศึกษาอิทธิพลของความเค็มและการตัดก้านตาต่อการปรับสมดุลไอออนในกุ้งกุลาดำ *Penaeus monodon* โตเต็มวัยระยะต้น ที่ความเค็ม 4 ระดับ (5, 17, 30 และ 42 ppt) ผลการทดลองแสดงให้เห็นว่ากุ้งกุลาดำมีความสามารถในการปรับสมดุลไอออนได้อย่างมีประสิทธิภาพ โซเดียม, โปแตสเซียม และแคลเซียมไอออนมีจุด isoionic crossover ที่ 19.84, 23.45 และ 22.18 ppt โดยมีลักษณะ hyperionic regulation ที่ความเค็มต่ำกว่าจุด isoionic crossover และมีลักษณะ hypoionic regulation ที่ความเค็มสูงกว่าจุด isoionic crossover แมกนีเซียมไอออนมีลักษณะ hypoionic regulation ทุกความเค็ม การตัดตากุ้งไม่ได้มีผลทำให้ไอออนทั้งสี่ชนิดในตัวกุ้งมีการเปลี่ยนแปลง เมื่อเปรียบเทียบกับกลุ่มควบคุม การย้ายกุ้งจาก 17 ppt ไป 5 ppt และจาก 30 ppt ไป 42 ppt กุ้งปกติและกุ้งที่ถูกตัดก้านตาสามารถปรับค่าไอออนในตัวให้คงที่ได้ จึงอาจกล่าวได้ว่าก้านตาไม่มีฮอร์โมนที่เกี่ยวข้องกับการปรับสมดุลไอออน (จารุวรรณ มหิทธิ, 2541)

เรื่องที่สามเป็นการศึกษาผลของบีเทน (betaine) ต่อการเจริญเติบโตและอัตรารอดของกุ้งกุลาดำที่เลี้ยงด้วยอาหารที่แหล่งโปรตีนจากพืชสูง บีเทนเป็นสารที่มีคุณสมบัติในการควบคุมระบบออสโมซิสในสิ่งมีชีวิตในทะเล ในการทดลองครั้งนี้คาดหวังว่าบีเทนจะช่วยในการปรับตัวของกุ้งกุลาดำวัยรุ่นที่เลี้ยงในสภาพความเค็มที่แตกต่างกันและส่งเสริมให้กุ้งกุลาดำมีความอยากอาหารมากขึ้นโดยใช้อาหารที่ให้กุ้งกุลาดำ ในการทดลองนี้จะมีการทดแทนแหล่งโปรตีนจากสัตว์ในอาหารเลี้ยงกุ้งกุลาดำ (ปลาป่น) ด้วยแหล่งโปรตีนจากพืช (กากถั่วเหลือง) การทดลองใช้กุ้งกุลาดำวัยรุ่นอายุ 45 วัน ทำการเลี้ยงในบ่อทดลอง 25 ตัวต่อบ่อ นาน 15 สัปดาห์ อาหารที่ใช้ในการทดลองจะมีส่วนผสมของกากถั่วเหลือง 40% กลุ่มควบคุมจะไม่มีการเสริมบีเทน และในกลุ่มทดลองจะทำการเสริมบีเทน 1.5% เพื่อศึกษาผลของบีเทน (betaine) ต่อการเจริญเติบโตและอัตราการรอดของกุ้งกุลาดำทำการทดลองที่ 2 ระดับความเค็ม คือความเค็ม 8 ส่วนในพัน และ 25 ส่วนในพัน

เมื่อสิ้นสุดการทดลอง กุ้งกุลาดำที่เลี้ยงด้วยอาหารที่มีส่วนผสมของกากถั่วเหลือง 40% ในกลุ่มควบคุมที่ไม่มีการเสริมบีเทน และในกลุ่มทดลองที่มีการเสริมบีเทน 1.5% มีอัตราการเจริญเติบโตและอัตราการรอดของกุ้งกุลาดำ ไม่มีความแตกต่างทางสถิติ ($P>0.05$) ทั้งสองระดับความเค็มที่ทำการทดลอง แต่พบว่ากุ้งกุลาดำที่เลี้ยงในระดับความเค็ม 25 ส่วนในพัน มีการเจริญเติบโตและอัตราการรอดสูงกว่ากุ้งกุลาดำที่เลี้ยงในระดับความเค็ม 8 ส่วนในพัน ทั้งในกลุ่มทดลองและกลุ่มควบคุม (พุทธิ ช่วยชูวงศ์ และคณะ, 2542)

8. การพัฒนาระบบหมุนเวียนน้ำแบบปิดเพื่อใช้ในการเลี้ยงพ่อแม่พันธุ์กุ้ง

คุณภาพน้ำเป็นปัจจัยที่สำคัญสำหรับการเลี้ยงพ่อแม่พันธุ์กุ้งกุลาดำ การที่จะทำให้พ่อแม่พันธุ์กุ้งมีการเจริญพันธุ์ได้ดีนั้นคุณภาพน้ำในบ่อจะต้องมีคุณภาพใกล้เคียงกับน้ำในทะเลลึก (oceanic water) การที่จะปรับคุณภาพน้ำให้ได้คุณภาพดังกล่าวจะต้องอยู่ภายใต้ระบบการจัดการน้ำที่ดี โดยทั่วไปเรามักใช้ระบบหมุนเวียนน้ำแบบปิด (closed recirculating seawater systems) งานวิจัยที่เกี่ยวข้องกับหัวข้อนี้มีอยู่หลายเรื่อง

เรื่องแรกเป็นการเปรียบเทียบตัวกรองแบบชีวภาพสองแบบ กล่าวคือ ไบโอดรัม และตัวกรองใต้น้ำในระบบหมุนเวียนน้ำแบบปิดโดยใช้กุ้งกุลาดำและปลากระพงขาวเป็นสัตว์ทดลอง การทดลองแต่ละครั้งใช้เวลา 3 เดือน โดยระบบบ่อทดลองประกอบด้วยบ่อเลี้ยงทำด้วยคอนกรีตรูปทรงกลมที่มีปริมาตร 38 ลบ.ม. (เส้นผ่าศูนย์กลาง 7 เมตร ความลึกของน้ำ 1 เมตร) และบ่อบำบัดทำด้วยคอนกรีตรูปสี่เหลี่ยมผืนผ้า (2 x 4.6 เมตร) ซึ่งอัตราการหมุนเวียนน้ำในระบบการทดลองเท่ากับ 4 ครั้งต่อวัน

ในการทดลองเลี้ยงกุ้งกุลาดำ พบว่าระบบตัวกรองชีวภาพทั้งแบบไบโอดรัมและใต้น้ำสามารถควบคุมคุณภาพน้ำคือ แอมโมเนียรวม, ไนไตรท์ และไนเตรทให้อยู่ในเกณฑ์ปกติ แต่เนื่องจากในการทดลองครั้งนี้มวลชีวภาพของกุ้งกุลาดำมีปริมาณน้อยมากจึงไม่สามารถเปรียบเทียบประสิทธิภาพระหว่างระบบหมุนเวียนน้ำแบบปิดที่มีตัวกรองชีวภาพต่างกันได้ อัตรารอดของกุ้งกุลาดำมีค่าเท่ากับ 6.25% และ 7.03% และมีอัตราการเติบโตเท่ากับ 0.056 กรัม/วัน ในชุดการทดลองแบบไบโอดรัมและแบบใต้น้ำตามลำดับ (Kitimasak et al., 1999)

ส่วนการทดลองเลี้ยงปลากะพงขาว พบว่าระบบตัวกรองชีวภาพแบบไบโอดรัมสามารถควบคุมคุณภาพน้ำคือ แอมโมเนียรวม, ไนไตรท์ และไนเตรทให้อยู่ในเกณฑ์ปกติ ในขณะที่ระบบตัวกรองชีวภาพแบบใต้น้ำสามารถควบคุมคุณภาพน้ำคือ แอมโมเนียรวม และไนเตรทให้อยู่ในเกณฑ์ปกติแต่ไม่สามารถควบคุมปริมาณไนไตรท์ให้อยู่ในเกณฑ์ปกติได้ อัตรารอดของปลากะพงขาวมีค่าเท่ากับ 58.42% และ 57.00% และอัตราการเติบโตเท่ากับ 1.273 กรัม/วันและ 1.228 กรัม/วัน ในชุดการทดลองแบบไบโอดรัมและแบบใต้น้ำตามลำดับ (Kitimasak et al., 1999)

ข้อเสียเรื่องหนึ่งของระบบหมุนเวียนน้ำแบบปิดที่ใช้ในการเพาะเลี้ยงก็คือการสะสมของปริมาณไนเตรทซึ่งเป็นผลลัพธ์ขั้นสุดท้ายของกระบวนการไนตริฟิเคชั่น เมื่อไนไตรทมีปริมาณสะสมมากกว่า 50 mg/l ก็จะมีผลกระทบต่อกุ้ง ดังนั้นงานวิจัยในเรื่องที่สองของหัวข้อนี้เป็นการพัฒนาระบบหมุนเวียนน้ำแบบปิดซึ่งมีกระบวนการลดไนเตรท (Menasveta et al., 1999)

ได้มีการออกแบบระบบหมุนเวียนน้ำทะเลแบบปิดชุดทดลองซึ่งประกอบด้วยบ่อเลี้ยงทรงกลมขนาดความจุ 9 ลบ.ม. บ่อกรองแบบชีวภาพ (biological filter) ขนาด 1.98 x 1.88 x 1.70 ม.^{3} ซึ่งมีวัสดุเส้นใยสังเคราะห์ BIO-POLYMA เป็นตัวตรึงไนตริไฟอิ้งแบคทีเรียบรรจุอยู่ภายใน มีอัตราการหมุนเวียนน้ำประมาณ 7 ครั้งต่อวัน และมีระบบตัวกรองแบบชีวภาพในสภาวะไร้ออกซิเจน ระบบนี้ประกอบด้วยคอลัมน์สำหรับลดปริมาณออกซิเจนที่ละลายน้ำ คอลัมน์บรรจุวัสดุตรึงดีไนตริฟายอิ้งแบคทีเรีย และคอลัมน์เพื่อเพิ่มปริมาณการละลายของออกซิเจน โดยมีการควบคุมอัตราไหลของน้ำในระบบนี้ให้ผ่านคอลัมน์ทั้งสามตามลำดับ ในช่วง 40-110 มล./นาที ระบบดังกล่าวนี้ติดตั้งต่อจากบ่อกรองแบบชีวภาพเพื่อวัตถุประสงค์ในการลดปริมาณไนไตรทของมวลน้ำทั้งระบบของชุดทดลอง

ได้มีการทดสอบระบบหมุนเวียนน้ำทะเลแบบปิดชุดทดลองดังกล่าวข้างต้นกับระบบหมุนเวียนน้ำทะเลแบบปิดชุดควบคุม (control) ซึ่งไม่มีระบบกรองแบบชีวภาพในสภาวะไร้ออกซิเจน เพื่อทดสอบประสิทธิภาพในการควบคุมคุณภาพน้ำและการควบคุมการเพิ่มขึ้นของไนเตรตในชุดทดลอง โดยการเลี้ยงพ่อแม่พันธุ์กุ้งกุลาดำเป็นเวลา 400 วัน โดยแบ่งการทดลองเป็น 3 ช่วงติดต่อกัน ในช่วงที่หนึ่งได้ใช้ดินตะกอนจากป่าชายเลนเติมลงในระบบกรองแบบชีวภาพในสภาวะไร้ออกซิเจน ช่วงที่สองนำดีไนตริไฟอิ้งแบคทีเรียที่เพาะได้มาเติมลงในระบบกรองฯ โดยตรง ทั้งช่วงที่หนึ่งและช่วงที่สองได้ใช้เอทานอลเป็นแหล่งคาร์บอน ในช่วงที่สามมีการเปลี่ยนแหล่งของคาร์บอนเป็นเมทานอล

ผลการทดลองในช่วงที่หนึ่ง 155 วัน ได้แสดงให้เห็นว่าระบบหมุนเวียนน้ำทะเลแบบปิดทั้งชุดทดลองและชุดควบคุมสามารถควบคุมคุณภาพน้ำ กล่าวคือ ควบคุมแอมโมเนียม ไนไตรท์ ไนเตรท และปริมาณออกซิเจนให้อยู่ในเกณฑ์ปกติได้ อย่างไรก็ดีชุดทดลองไม่สามารถควบคุมให้ไนเตรทอยู่ในระดับต่ำได้ ในช่วงที่สองซึ่งมีการทดลอง 150 วัน การเติมดีไนตริไฟอิ้งแบคทีเรียลงในระบบกรองฯ โดยตรงมีผลทำให้ปริมาณไนเตรทในชุดทดลองลดลงอย่างมีนัยสำคัญทางสถิติ แต่ก็ลดลงได้ในอัตราที่ช้ามากและมีการกระเพื่อมสูง ในการทดลองในช่วงที่ 3 เป็นเวลาอีก 95 วัน พบว่าการเปลี่ยนแหล่งของคาร์บอนเป็นเมทานอลทำให้ปริมาณไนเตรทลดลงได้ตามวัตถุประสงค์ที่ตั้งไว้ (Menasveta et al., 1999)

ถึงแม้ว่าดีไนตริไฟอิ้งแบคทีเรียจะมีประสิทธิภาพที่ดีในการลดปริมาณไนเตรท แต่จะต้องอยู่ภายใต้ภาวะที่ไม่มีออกซิเจน การทดลองในครั้งที่แล้วต้องมีการใช้ไนโตรเจนเหลวเปลี่ยนเป็นก๊าซไนโตรเจนเพื่อไล่ออกซิเจนออกจากดีไนตริไฟอิ้งคอลัมน์ เป็นการสิ้นเปลืองมาก และอาจทำให้ไม่คุ้มกับค่าใช้จ่ายในการดำเนินการ ด้วยเหตุดังนี้เราจึงได้คิดที่จะลองใช้วิธีอื่นในการบำบัดไนเตรตซึ่งสาหร่ายน่าจะแสดงบทบาทนี้ได้

การใช้สาหร่ายเพื่อลดปริมาณของเสียไนโตรเจนในระบบหมุนเวียนน้ำแบบปิดสำหรับเลี้ยงสัตว์น้ำมีข้อแตกต่างจากระบบบำบัดที่ใช้แบคทีเรียคือ สาหร่ายสามารถนำไนโตรเจนที่อยู่ในหลายรูปแบบ เช่น แอมโมเนียและไนเตรทเข้าสู่เซลล์เพื่อเป็นธาตุอาหารสำหรับการเจริญโดยตรง ซึ่งการที่จะเลือกชนิดของสาหร่ายและการจัดการระบบอย่างมีประสิทธิภาพจะต้องคำนึงถึงปัจจัยที่

สำคัญก็คืออัตราการนำสารอาหารเข้าสู่เซลล์ (nutrient uptake rate) ของสาหร่ายในสภาพแวดล้อมที่เหมาะสม

การวิจัยนี้ได้ทำการทดลองเบื้องต้นเพื่อเปรียบเทียบอัตราการสังเคราะห์แสงและอัตราการนำไนเตรทเข้าสู่เซลล์ของสาหร่ายขนาดใหญ่สองชนิด คือ สาหร่ายเม็ดพริกไทย *(Caulerpa sp.)* และสาหร่ายหนาม *(Acanthophora sp.)* และได้ทดลองหาอัตราการนำไนเตรทเข้าสู่เซลล์ของสาหร่ายเซลล์เดียวดูนาลิเอลลา *(Dunaliella salina)* ในสภาพที่ถูกตรึงในเม็ดเจลแคลเซียมอัลจิเนต ผลการทดลองพบว่าสาหร่ายเม็ดพริกไทยและสาหร่ายหนามมีจุดอิ่มตัวของอัตราการสังเคราะห์แสงอยู่ที่ความเข้มแสงประมาณ 20,000 lux โดยเมื่อระดับความเข้มแสงเพิ่มสูงกว่านี้ก็ไม่ทำให้อัตราการปลดปล่อยออกซิเจนเนื่องจากการสังเคราะห์แสง (photosynthetic oxygen evolution) เพิ่มสูงขึ้นอีก ส่วนอัตราการนำไนเตรทเข้าสู่เซลล์ของสาหร่ายทั้งสองชนิดจะอยู่ในช่วงระหว่าง 0.006-0.13 mg NO_3/gfw/hr ส่วนสาหร่ายเซลล์เดียวดูนาลิเอลลาในสภาพที่ถูกตรึงและเลี้ยงที่ระดับความเข้มแสงประมาณ 6,000 lux มีอัตราการนำไนเตรทเข้าสู่เซลล์อยู่ระหว่าง 0.02-0.12 mg NO_3/gfw/hr ขึ้นกับความเข้มข้นของไนเตรทในน้ำ โดยมีอัตราการนำเข้าสูงสุด (Vmax) ที่ 6.73 mgNO_3/mg Chl-a/hr และความเข้มข้นของไนเตรทที่ทำให้อัตราการนำเข้าไนเตรทเป็นครึ่งหนึ่งของอัตราการนำเข้าสูงสุด (Km) อยู่ที่ 794.6 mg NO_3/l (Powtongsook et al., 1999)

ขั้นตอนหนึ่งของการบำบัดน้ำในระบบหมุนเวียนน้ำแบบปิดคือ การฆ่าเชื้อ (disinfection) ซึ่งเรามักจะทำหลังจากที่น้ำได้ผ่านการกรองแบบชีวภาพและการกรองใสมาแล้วเป็นการบำบัดขั้นสุดท้ายก่อนที่จะนำน้ำมวลเดิมไปใช้เลี้ยงสัตว์น้ำต่อไป สารฆ่าเชื้อฯ ในปัจจุบันมีหลายชนิด แต่ที่มีความสำคัญในระบบหมุนเวียนน้ำแบบปิด คือ โอโซน

การใช้โอโซนเพื่อกำจัดเชื้อโรคในน้ำดื่มน้ำใช้สำหรับมนุษย์มีการปฏิบัติมานาน เนื่องจากโอโซนมีประสิทธิภาพสูง, เสื่อมสลายได้ง่าย ไม่ทิ้งสารตกค้าง ทำให้ไม่เป็นอันตรายต่อสิ่งแวดล้อม เมื่อเปรียบเทียบกับสารฆ่าเชื้อตัวอื่นอย่างเช่น คลอรีน หรือฟอร์มาลิน ในสถานการณ์ปัจจุบันที่การเลี้ยงกุ้งประสบปัญหาบ่อยครั้งจากโรค โอโซนจึงอาจเป็นวิธีแก้ปัญหาที่เหมาะสม การศึกษาเรื่องการใช้โอโซนกับการเพาะเลี้ยงกุ้งแม้ว่าจะมีมานาน แต่ในทางปฏิบัติกลับไม่ค่อยแพร่หลาย อาจเป็นเพราะยังขาดรายละเอียดข้อมูลที่สามารถนำมาประยุกต์ใช้ได้ การศึกษาครั้งนี้จึงเน้นการทดลองในหลายด้านของโอโซน เช่น การวิเคราะห์ปริมาณโอโซนที่ผลิตได้ ความเป็นพิษของโอโซนต่อกุ้ง การใช้โอโซนเพื่อปรับปรุงคุณภาพน้ำ และการฆ่าเชื้อแบคทีเรียในน้ำเลี้ยงกุ้ง ท้ายสุดผลจากแต่ละการทดลองจะได้นำมาประมวลเพื่อสรุปหาวิธีการใช้โอโซนที่มีประสิทธิภาพสูงสุด และปลอดภัยต่อการเลี้ยงกุ้งต่อไป

ผลการวิจัยพบว่าความเข้มข้นของโอโซนที่เหลือในน้ำ (residue ozone) สูงสุดที่ไม่เป็นพิษต่อลูกกุ้งวัยอ่อน อยู่ในช่วง 0.20 – 0.40 mg/l ผลสรุปดังกล่าวได้จากการตรวจสอบสภาพของเหงือกกุ้งหลังจากที่อยู่ในน้ำที่มีความเข้มข้นของโอโซนในระดับนี้ประมาณ 8 ชั่วโมง ผลการตรวจสอบประสิทธิภาพในการฆ่าเชื้อ *Vibrio* พบว่าโอโซนที่เหลือในน้ำ 0.34 mg/l สามารถฆ่าเชื้อชนิดนี้ได้ 3 หน่วย log ภายใน 9 ชั่วโมง จึงอาจกล่าวได้ว่า โอโซนน่าจะถูกใช้เป็นสารเคมีสำหรับ prophylectic ได้ (Meunpol et al., 1999)

นอกเหนือจากการทดลองเพื่อปรับปรุงประสิทธิภาพของระบบหมุนเวียนน้ำแบบปิดในห้องปฏิบัติการแล้ว โครงการฯ ยังได้พัฒนาระบบหมุนเวียนน้ำในบ่อดินขนาดใหญ่เพื่อใช้เลี้ยงพ่อแม่พันธุ์กุ้งกุลาดำ

ได้มีการจัดเตรียมพ่อแม่พันธุ์กุ้งกุลาดำในระบบบ่อเลี้ยง ในเขตที่ห่างไกลจากการเลี้ยงกุ้งกุลาดำในเขตพื้นที่อำเภอหนองเสือ จังหวัดปทุมธานี โดยอาศัยพื้นที่น้ำจืดเป็นแนวป้องกันโรคที่อาจแพร่มากับน้ำทะเล หรือแหล่งเลี้ยงกุ้งอื่นๆ ระบบการจัดการพ่อแม่พันธุ์กุ้งกุลาดำของการศึกษาเป็นระบบน้ำหมุนเวียนน้ำแบบปิด ระบบประกอบด้วยหน่วยการผลิตกุ้งก่อนวัยเจริญพันธุ์ (ความเค็ม 5 ส่วนในพันส่วน) หน่วยพักกุ้งวัยเจริญพันธุ์ (ความเค็ม 15-25 ส่วนในพันส่วน) เป็นหน่วยปรับสภาพกุ้งให้เหมาะสมเพื่อการกระตุ้นให้เกิดไข่ หน่วยกระตุ้นการเจริญพันธุ์ (ความเค็ม 35 ส่วนในพันส่วน) และหน่วยสุดท้ายหน่วยการเพาะเลี้ยงกุ้งวัยอ่อน (ความเค็ม 30 ส่วนในพันส่วน)

หน่วยผลิตกุ้งวัยก่อนเจริญพันธุ์ เป็นบ่อดินประกอบด้วยบ่อเลี้ยงขนาด 1,000 ตารางเมตร ความลึก 1.5 เมตร เป็นบ่อเลี้ยงและบ่อบำบัดน้ำ 600 ตารางเมตร ความลึก 1.2 เมตร การเลี้ยงกุ้งแบ่งเป็น 2 ชุดการทดลอง โดยชุดแรกเลี้ยงกุ้งขนาด 35 กรัม ด้วยความหนาแน่น 1 ตัวต่อตารางเมตรและชุดที่ 2 เลี้ยงกุ้ง P-10 ด้วยความหนาแน่น 30 ตัวต่อตารางเมตร เพื่อใช้คัดกุ้งที่โตเร็ว (Top size) เพื่อเสริมในการเลี้ยงกุ้งชุด 1 รุ่นต่อไป การทดลองทั้ง 2 ชุดใช้เวลา 4 เดือน กุ้งแต่ละบ่อเลี้ยงใช้กุ้งครอบครัวเดียวกัน และแตกต่างกับกุ้งบ่ออื่น การเลี้ยงใช้อาหารที่ขายในท้องตลาดวันละ 3 มื้อ 7.00, 12.00 และ 18.00 น.

หน่วยพักปรับสภาพกุ้งวัยเจริญพันธุ์ เป็นระบบน้ำหมุนเวียนแบบปิดรูปทรงกลม ประกอบด้วยหน่วยบำบัดตรงกลางใช้ทรายและเปลือกหอยเป็นวัสดุสำหรับกรองและเป็นอาศัยของแบคทีเรีย ภายในบ่อกรองเติมสาหร่าย *Caulerpa* sp. หรือ *Acanthophora* sp. เพื่อกำจัดไนเตรทออกจากระบบ กุ้งจากหน่วยผลิตกุ้งวัยก่อนเจริญพันธุ์ที่มีขนาดใหญ่กว่า 100 กรัม จะถูกนำมาปรับสภาพ ทำเครื่องหมายเฉพาะตัวกุ้งเพื่อบันทึกประวัติ อัตราส่วนการปล่อยกุ้งใช้ 3 ตัวต่อตารางเมตร สัดส่วนเพศผู้ต่อเพศเมีย 1:2 ระยะพักกุ้งใช้เวลาประมาณ 1 เดือน เมื่อกุ้งลอกคราบเพศเมียจะถูกผสมเทียมด้วยเพศผู้จากต่างครอบครัว การให้อาหารดำเนินการเช่นเดียวกับหน่วยแรกแต่เสริมอาหารสด เช่น ปลาหมึก หอยกะพงหรือหอยลาย สัปดาห์ละ 1-2 ครั้ง

หน่วยกระตุ้นการเจริญพันธุ์ของเพศเมีย มีระบบเช่นเดียวกับหน่วยพักกุ้ง แต่ระบบควบคุมความเข้มของแสงไว้ที่ประมาณ 10% ของความเข้มแสงปกติ ภายในโรงเพาะเลี้ยงแบบปิด โดยนำกุ้งที่ได้รับการผสมจากเชื้อตัวผู้ที่สมบูรณ์ (2-3 วัน) มาตัดก้านตา 1 ข้าง เพื่อกระตุ้นให้กุ้งมีการพัฒนาของรังไข่ มีการตรวจการพัฒนาของรังไข่ทุก 2 วัน แม่กุ้งไข่ที่พัฒนาถึงขั้นที่ 4 จะถูกแยกตัวออกลงถังฟักไข่

หน่วยเพาะฟักตัวอ่อน ใช้ระบบปิด ขนาดบ่อ 1 x 2 x 0.8 $ม^3$ เป็นบ่อผ้าใบหรือถังไฟเบอร์ขนาด 2 ตัน การดำเนินงานทำเหมือนการเพาะเลี้ยงกุ้งวัยอ่อนของโรงเพาะฟักทั่วไป โดยดำเนินการที่สถานีวิจัยวิทยาศาสตร์ทางทะเลและศูนย์ฝึกนิสิต เกาะสีชัง

กุ้งที่นำเข้าสู่ระบบที่พัฒนานี้ต้องรับการตรวจ PCR ว่าปลอดเชื้อตัวแดงดวงขาวแล้ว โครงการขณะนี้อยู่ในระยะเริ่มต้นของการดำเนินงานทดลอง ผลการศึกษาเบื้องต้นพบว่า การพัฒนาเพื่อสร้างพ่อแม่พันธุ์ โดยวิธีที่กำลังศึกษามีความเป็นไปได้สูงในการจัดเตรียมพ่อแม่พันธุ์ปลอดเชื้อดังกล่าว และเป็นวิธีการหนึ่งที่ควรส่งเสริมให้มีการศึกษาวิจัยในแนวลึกต่อไป เพื่อการปรับปรุงสายพันธุ์ของกุ้งต่อไป

9. นวัตกรรมของระบบการเลี้ยงกุ้งทะเล : บทความปริทัศน์

โครงการฯ ได้มีการวิจัยเอกสารและรวบรวมผลงานทั้งหมดของโครงการฯ เขียนเป็นบทความปริทัศน์ เพื่อศึกษาเปรียบเทียบวิจารณ์ผลความก้าวหน้าในงานวิจัยและพัฒนาที่เกี่ยวกับระบบการเลี้ยงกุ้งทะเล บทความดังกล่าวนี้จะมีการลงพิมพ์ในหนังสือที่จะมีการวางจำหน่ายทั่วโลก

ผลผลิตกุ้งได้เพิ่มขึ้นจาก 0.4 ล้านตัน ในปี 1990 มาเป็น 0.8 ล้านตันในปี 1999 หรือประมาณ 25% ของผลผลิตกุ้งของโลกทั้งหมด (รวมที่ได้จากการจับ) ผลผลิตกุ้งที่เพิ่มขึ้นนี้ยังต่ำกว่าที่วงการได้วางเป้าไว้เมื่อ 10 ปีที่แล้ว (1.2 ล้านตัน) เหตุที่ทำให้ไม่สามารถทำได้ตามเป้าก็เพราะปัญหาในเรื่องโรคกุ้งเป็นหลักโดยเฉพาะอย่างยิ่งโรคที่เกิดจากไวรัส ด้วยเหตุดังกล่าวนี้แนวปฏิบัติของระบบการเลี้ยงกุ้งทะเลจึงได้มีการปรับปรุงเปลี่ยนแปลงไปจากเดิมหลายประการ อาทิ เช่น การผลิตพันธุ์กุ้งที่ปลอดเชื้อ พันธุ์กุ้งที่มีความสามารถในการต้านเชื้อโรค การใช้ระบบน้ำในบ่อเลี้ยงที่มีการหมุนเวียนแบบปิด และ/หรือไม่มีการเปลี่ยนน้ำ (zero water exchange) การเลี้ยงกุ้งในพื้นที่เขตน้ำจืด การเลี้ยงกุ้งในเขตที่ห่างไกลจากแหล่งเลี้ยงเพื่อป้องกันการติดเชื้อ การใช้โพรไบโอติกแบคทีเรียเพื่อเพิ่มภูมิคุ้มกันให้แก่กุ้ง การคัดพันธุ์กุ้ง นอกจากนี้สาธารณะชนก็ได้เข้ามามีบทบาทในการท้วงติงในเรื่องการเลี้ยงกุ้งกับปัญหาสิ่งแวดล้อมมากขึ้น ดังนั้นการเลี้ยงกุ้งจึงได้มีการปรับตัว และได้พิสูจน์ให้เห็นว่า การเลี้ยงกุ้งนั้นสามารถที่จะทำให้มีความยั่งยืนและเป็นมิตรกับสิ่งแวดล้อมได้ (Fast and Menasveta. 1999)

เอกสารอ้างอิง

Chuntapa, B., S. Piyatiratitivorakul, C. Nitithamyong, V. Viyakarn, and P. Menasveta. 1999. Optimal lipid:carbohydrate and protein:energy ratios in semi-purified diets for juvenile black tiger shrimp *Penaeus monodon.* Aquaculture Research 30:1-6

Fast, A.W. and P. Menasveta. 1999. Some recent innovation in marine shrimp pond culture. TRF Contribution No.14. Aquatic Resouces Research Institute, Chulalongkorn University, Bangkok. 80 p.

Kitimasak, N., P. Aranyakananda, and P. Menasveta. 1999. Comparisons of rotating bio-drum and submerged biofilters in closed, seawater recirculating systems with black tiger shrimp (*Penaeus monodon*) and sea base (*Lates calcarifer*). TRF Contribution No.15, Aquatic Resouces Research Institute, Chulalongkorn University, Bangkok. 15 p.

Longyant, S., P. Sithigorngul, N. Thampalerd, W. Sithigorngul, and P. Menasveta. 1999. Monoclonal antibodies production specific to vitellin and vitellogenin of giant tiger prawn *Penaeus monodon.* Invertebrate Reproduction and Development 35 (1): 9-17.

Longyant, S., P. Sithigorngul, N. Thammapalerd, W. Sithigorngul and P. Menasveta.1999. Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin sub-units. TRF Contribution No.16, Aquatic Resouces Research Institute, Chulalongkorn University. Bangkok. 20 p.

Menasveta, P. 1997. Progress of aquaculture development in Thailand. Pro. Second Int. Seminar of Fishenies Sci. in Tropical Area, Tokyo. Tokyo University of Fisheries: 65-69.

Menasveta, P. 1997. Mangrove destruction and shrimp culture systems. World Aquaculture 28 (4) : 36-42.

Menasveta, P., T. Panritdam, P. Sihanonth, S. Powthongsook, B. Chuntapa and P. Lee. 1999. Design and function of closed, recirculating seawater system with denitrification for culturing black tiger shrimp broodstocks. TRF Contribution No.17, Aquatic Resouces Research Institute, Chulalongkorn University, Bangkok. 15 p.

Meunpol, O., K. Lopinyosiri, and P. Menasveta. 1999. Use of ozone in prawn culture water treatment. TRF Contribution No.18, Aquatic Resources Research Institute, Chulalongkorn University, Bangkok. 15 p.

Paibulkichakul, C., S. Piyatiratitivorakul, P. Kittakoop, V. Viyakarn, A.W. Fast, and P. Menasveta. 1998. Optimal dietary levels of lecithin and cholesterol for black tiger prawn *Penaeus monodon* larvae and postlarvae. Aquaculture 167 : 273-281.

Phianphak, W., S. Rengpipat, S. Piyatiratitivorakul, and P. Menasveta. 1999. Probiotic use of *Lactobacillus* spp. for black tiger shrimp, *Penaeus monodon.* J. Sci. Res. Chula. Univ. 24(1) : 41-48.

Powtongsook, S., A. Chokwiwattanawanit, and P. Menasveta. 1999. Photosynthesis efficiency of two macroalgal species used in closed recirculating seawater system in Thailand. TRF Contribution No.19, Aquatic Resources Research Institute, Chulalongkorn University, Bangkok. 12 p.

Puanglarp, N., S. Klinbunga, and P. Menasveta. 1999. cDNA cloning of neurohormones from the eyestalk of *Penaeus monodon*, Fabricius. TRF Contribution No. 20, Aquatic Resources Research Institute, Chulalongkorn University, Bangkok. 20 p.

Rengpipat, S., W. Phianphak, S. Piyatiratitivorakul, and P. Menasveta. 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167 : 301-313.

Rengpipat, S., S. Rukpratanporn, S. Piyatiratitivorakul, and P. Menasveta. 1998. Probiotics in aquaculture : a case study of probiotics for larvae of the black tiger shrimp (*Penaeus monodon*). In Flegel, T.W. (ed), Advances in Shrimp Biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Sithigorngul, S., P. Chauychuwong, W. Sithigorngul, S. Longyant, P. Chaivisuthangkura, and P. Menasveta. 1999. Development of a monoclonal antibody specific to yellow head virus : establishing an immunological – basis for specific diagnosis of viral infection in *Penaeus monodon*. TRF Contribution No. 12, Aquatic Resources Research Institute, Chulalongkorn Univ. Bangkok : 12 p.

Wudthijinda, W., P. Jarayabhand, and P. Menasveta 1999. A genetic marker application with giant tiger shrimp (*Penaeus monodon*) for closed life-cycle culture. TRF Contribution No. 21, Aquatic Resources Research Institute, Chulalongkorn University, Bangkok. 12 p.

จารุวรรณ มหิทธิ 2541 ผลของการเปลี่ยนแปลงความเค็มต่อการปรับสมดุลของไอออนในกุ้งกุลาดำ *Penaeus monodon* Fabricius วิทยานิพนธ์ปริญญาโท สหสาขาวิชาวิทยาศาสตร์สภาวะแวดล้อม จุฬาลงกรณ์มหาวิทยาลัย 105 หน้า

พิพัฒน์ เวฬุคามกุล 2541 อิทธิพลของความเค็มและระดับของโปรตีนต่อการจัดสรรพลังงานของกุ้งกุลาดำ *Penaeus monodon* ระยะวัยรุ่น วิทยานิพนธ์ปริญญาโท ภาควิชาวิทยาศาสตร์ทางทะเล คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย 85 หน้า

พุทธิ ช่วยชูวงศ์, วรณพ วิยกาญจน์, สมเกียรติ ปิยะธีระธิติวรกุล และเปี่ยมศักดิ์ เมนะเศวต 2542 ผลของบีเทน (Betaine) ต่อการเจริญเติบโตและอัตรารอดของกุ้งกุลาดำ (*Penaeus monodon*) ที่เลี้ยงด้วยอาหารที่มีแหล่งโปรตีนจากพืชสูง TRF Contribution No.13 สถาบันวิจัยทรัพยากรทางน้ำ จุฬาลงกรณ์มหาวิทยาลัย 15 หน้า

ผลิตผล (output) จากโครงการ

ศาสตราจารย์ ดร. เปี่ยมศักดิ์ เมนะเศวต ได้รับการสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ให้ดำเนินการวิจัยในฐานะเมธีวิจัยอาวุโสในระหว่างปี พ.ศ.2538-2542 โครงการเทคโนโลยีชีวภาพทางทะเลเพื่อการพัฒนาที่ยั่งยืน : การปรับปรุงกระบวนการผลิตพันธุ์กุ้งที่มีคุณภาพ โครงการนี้มีวัตถุประสงค์และเป้าหมายหลัก 4 ประการ คือ

1. เพื่อการสร้างกลุ่มวิจัยที่มีลักษณะเป็นหน่วยปฏิบัติการเชี่ยวชาญเฉพาะทาง
2. ดำเนินการวิจัยเพื่อสร้างองค์ความรู้และเทคโนโลยี โดยเน้นเทคโนโลยีชีวภาพกุ้ง
3. ผลิตมหาบัณฑิต 12 คน ผลิตดุษฎีบัณฑิต 2 คน และสร้างประสบการณ์และผลงานวิจัยให้คณาจารย์ผู้ร่วมวิจัย 2 คน และ
4. สร้างความเชื่อมโยงกับนักวิชาการของหน่วยงานทั้งภายในและภายนอกประเทศ

ผลงาน

1. การสร้างกลุ่มวิจัย

ในระยะสามปีที่ผ่านมาผลการดำเนินงานของโครงการนี้ได้เสริมสร้างความแข็งแกร่งให้แก่หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเลในเชิงการผลิตผลงานวิจัย การขยายเครือข่ายของหน่วยปฏิบัติการฯ และการเสริมสร้างประสบการณ์และความเชี่ยวชาญให้แก่สมาชิกของหน่วยปฏิบัติการฯ ได้เป็นอย่างดี ทำให้หน่วยปฏิบัติการฯ เป็นที่รู้จักทั้งภายในและระหว่างประเทศ

2. การสร้างองค์ความรู้และเทคโนโลยี

โครงการฯ ได้ดำเนินการวิจัยเพื่อสร้างองค์ความรู้และเทคโนโลยีในเรื่องที่เกี่ยวกับเทคโนโลยีชีวภาพของกุ้งครอบคลุมหลายหัวข้อ อาทิเช่น การใช้เทคโนโลยีชีวภาพเพื่อการเพาะเลี้ยงกุ้งที่มีความยั่งยืนและเป็นมิตรกับสิ่งแวดล้อม โภชนาการของกุ้งทะเลวัยต่างๆ การใช้แบคทีเรียบางชนิดเป็น probiotics สำหรับการเลี้ยงกุ้ง โปรแกรมการคัดพันธุ์และการปรับปรุงพันธุ์กุ้ง การพัฒนาระบบหมุนเวียนน้ำแบบปิดเพื่อการเพาะเลี้ยงกุ้ง การใช้วิธีทางอิมูนโนวิทยาเพื่อการตรวจสอบการเจริญพันธุ์และวินิจฉัยโรคกุ้ง ผลการวิจัยได้มีการพิมพ์เผยแพร่ในรูปของบทความทางวิชาการและวิทยานิพนธ์ดังนี้

2.1 บทความทางวิชาการพิมพ์เผยแพร่ในวารสารต่างๆ และ proceedings (เรียงลำดับตามการพิมพ์ก่อนและหลัง)

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Menasveta, P. 1997. Intensive and efficient shrimp culture system-the Thai way-can save mangroves. Aquaculture Asia; Vol. 2 No. 1: 38-44.

Menasveta, P. 1997. Progress of aquaculture development in Thailand. Proceedings Second International Seminar on Fisheries Science. Tokyo Fisheries University. pp. 65-69.

Menasveta, P. 1997. Mangrove destruction and shrimp culture systems. World Aquaculture, December : 36-42.

Menasveta, P. 1998. The evolution of shrimp culture and its impact on mangrove. Infofish International 1/98 : 24-29.

Paibulkichakul, C., S. Piyatiratitivorakul, P. Kittakoop, V. Viyakarn, A.W. Fast, and P. Menasveta. 1998. Optimal dietary levels of lecithin and cholesterol for black tiger prawn *Penaeus monodon* larvae and postlarvae. Aquaculture 167 : 273-281.

Rengpipat, S., W. Phianphak, S. Piyatiratitivorakul, and P. Menasveta. 1998. Effects of a probiotics bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167 : 301-313.

Longyant, S., P. Sithigorngul, N., Thampalerd, W. Sithigorngul, and P. Menasveta. 1999. Monoclonal antibodies production specific to vitellin and vitellogenin of giant tiger prawn *Penaeus monodon*. Invertebrate Reproduction and Development 35 : 9-17.

Chuntapa, B., S. Piyatiratitivorakul, C. Nitithamyong, V. Viyakarn, and P. Menasveta. 1999. Optimal lipid:carbohydrate and protein:energy ratios in semi-purified diets for juvenile black tiger shrimp *Penaeus monodon*. Aquaculture Research 30: 1-6.

Phianphak, W., S. Rengpipat, S. Piyatiratitivorakul, and P. Menasveta. 1999. Probiotic use of *Lactobacillus* spp. for Black Tiger Shrimp, *Penaeus monodon*. J. Sci. Res. Chula. Univ., Vol. 24, No. 1. : 41-51.

ผลงานที่อยู่ระหว่างการตีพิมพ์ในวารสารวิชาการนานาชาติ

Longyant S., P. Sithigorngul, N. Thampalerd, W. Sithigorngul, and P. Menasveta. Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin subunits. Invertebrate Reproduction and Development (in press)

Fast, A.W. and P. Menasveta. 1999. Some recent innovation in marine shrimp pond culture.

Kitimasak, N., P. Aranyakananda, and P. Menasveta. 1999. Comparisons of rotating bio-drum and submerged biofilters in closed, seawater recirculating systems with black tiger shrimp (*Penaeus monodon*) and sea base (*Lates calcarifer*).

Menasveta, P., T. Panritdam, P. Sihamonth, S. Powthongsook, B. Chuntapa and P. Lee. 1999. Design and function of closed, recirculating seawater system with denitrification for culturing black tiger shrimp broodstocks.

Meunpol, O., K. Lopinyosiri, and P. Menasveta. 1999. Use of ozone in prawn culture water treatment.

Powtongsook, S., A. Chokwiwattanawanit, and P. Menasveta. 1999. Photosynthesis efficiency of two macroalgal species used in closed recirculating seawater system in Thailand.

Puanglarp, N., S. Klinbunga, and P. Menasveta. 1999. cDNA cloning of neurohormones from the eyestalk of *Penaeus monodon*, Fabricius.

Wudthijinda, W., P. Jarayabhand, and P. Menasveta. 1999. A genetic marker application with giant tiger shrimp (*Penaeus monodon*) for closed life-cycle culture.

หนังสือ

Fast, A. W. and P. Menasveta. 1998. Some recent innovations in marine shrimp pond recycling systems. pp. 87-91. In Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Rengpipat, S., S. Rukpratanporn, S. Piyatiratitivorakul, and P. Menasveta. 1998. Probiotics in aquaculture : a case study of probiotics for larvae of the black tiger shrimp (*Penaeus monodon*). pp. 177-181, ibid.

2.2 วิทยานิพนธ์

ระดับเลซิทินและคอเลสเทอรอลที่เหมาะสมในอาหารสำหรับกุ้งกุลาดำ
วิทยานิพนธ์ปริญญาโท พ.ศ. 2539
(นายชลี ไพบูลย์กิจกุล)

อัตราส่วนระหว่างโปรตีนและพลังงานที่เหมาะสมในอาหารสำหรับกุ้งกุลาดำวัยรุ่น
วิทยานิพนธ์ปริญญาโท พ.ศ. 2539
(นางสาวเบ็ญจมาศ จันทะภา)

การใช้แบคทีเรียเป็นโพรไบโอติกเสริมในอาหารกุ้ง
วิทยานิพนธ์ปริญญาโท พ.ศ. 2539
(นางสาววรรนิภา เพี้ยนภักตร์)

การใช้ *Bacillus* spp. เพื่อเสริมผลผลิตกุ้งกุลาดำ (*Penaeus monodon*)
วิทยานิพนธ์ปริญญาโท พ.ศ. 2540
(นางสาวนิภา เตโชดำรงสิน)

ระบบหมุนเวียนน้ำแบบปิดที่มีระบบดีไนตริฟิเคชั่นสำหรับการเลี้ยงกุ้งกุลาดำ (*Penaeus monodon*)
วิทยานิพนธ์ปริญญาโท พ.ศ. 2541
(นางสาวธัญญา พันธ์ฤทธิ์ดำ)

อิทธิพลของความเค็มและระดับของโปรตีนต่อการจัดสรรพลังงานของกุ้งกุลาดำ (*Penaeus monodon*) ในระยะวัยรุ่น
วิทยานิพนธ์ปริญญาโท พ.ศ. 2541
(นายพิพัฒน์ เวพุคามกุล)

การศึกษาเปรียบเทียบคุณภาพน้ำระหว่างระบบหมุนเวียนน้ำทะเลแบบปิดที่มีตัวกรองชีวภาพแบบไบโอดรัมและแบบใต้น้ำเพื่อการเพาะเลี้ยงสัตว์น้ำ
วิทยานิพนธ์ปริญญาโท พ.ศ. 2541
(นางสาวนภาพร กิติมศักดิ์)

ผลของการเปลี่ยนแปลงความเค็มต่อการปรับสมดุลของไอออนในกุ้งกุลาดำ *Penaeus monodon* Fabricius
วิทยานิพนธ์ปริญญาโท พ.ศ. 2541
(นางสาวจารุวรรณ มหิทธิ)

ความหลากหลายของพันธุกรรมของกุ้งกุลาดำในประเทศไทย
วิทยานิพนธ์ปริญญาโท พ.ศ. 2541
(นางสาววรรณลักษณ์ วุทธิจินดา)
การเสริมภูมิคุ้มกันโรคในกุ้งกุลาดำ *Penaeus monodon* ด้วย *Bacillus* S11
วิทยานิพนธ์ปริญญาโท พ.ศ. 2542
(นายสมบัติ รักประทานพร)
หมายเหตุ : ในขณะนี้ยังมีวิทยานิพนธ์ที่กำลังอยู่ในระหว่างการเขียนและพิจารณาอีก 2 เรื่อง

การเสริม *Bacillus* sp. สายพันธุ์ S11 ในการเลี้ยงกุ้งกุลาดำระดับบ่อดิน ของนายอรุณ ธัญญนันท์ นิสิตปริญญาโท คาดว่าจะจบปีการศึกษา 2543

การผลิตโมโนโคลนอลแอนติบอดีที่จำเพาะต่อไวเทลลินและไวเทลโลเจนิน สำหรับทดสอบระดับของฮอร์โมนยับยั้งพัฒนาการของรังไข่ในกุ้งกุลาดำ ของนายศิวาพร ลงยันต์ นิสิตปริญญาเอก คาดว่าจะจบปีการศึกษา 2543

3. การผลิตมหาบัณฑิตและดุษฎีบัณฑิต, นักวิจัยหลังดุษฎีบัณฑิต, และการสร้างประสบการณ์วิจัยและผลงานวิจัยให้แก่คณาจารย์ผู้ร่วมวิจัย

โครงการฯ ได้ผลิตมหาบัณฑิตออกมาแล้ว 9 ราย และกำลังอยู่ในระหว่างการผลิตอีก 3 ราย โครงการฯ กำลังอยู่ในระหว่างการผลิตดุษฎีบัณฑิต 1 ราย (ก้าวหน้าแล้ว 80%) ส่วนอีก 1 รายได้ลาออกไปเนื่องจากป่วย โครงการฯ จึงได้ตัดสินใจสร้างนักวิจัยหลังดุษฎีบัณฑิตเพื่อทดแทน โดยได้สนับสนุนโครงการวิจัยนำร่องให้แก่ อ. ดร. ศิราวุธ กลิ่นบุหงาและ อ. ดร. ณรงค์ ศักดิ์ พ่วงลาภ และในขณะนี้กำลังให้สนับสนุน อ. ดร. สรวิศ เผ่าทองศุขและ อ. อรพร หมื่นพล (Ph.D. Candidate) Post Doctoral Research 2 โครงการ คือ การตรึงเซลล์สาหร่ายและแบคทีเรียเพื่อบำบัดปริมาณไนเตรทในระบบหมุนเวียนน้ำแบบปิดสำหรับการเพาะเลี้ยงสัตว์น้ำ และการใช้โอโซนเพื่อการควบคุมคุณภาพน้ำเลี้ยงกุ้งกุลาดำ

โครงการฯ ได้มีส่วนสร้างประสบการณ์วิจัยให้แก่คณาจารย์ผู้ร่วมวิจัย 2 ราย ได้แก่ รศ. ดร. สมเกียรติ ปิยะธีรธิติวรกุลและ รศ. ดร. ศิริรัตน์ เร่งพิพัฒน์ โดยทั้งสองท่านมีผลงานเป็นที่ประจักษ์แก่วงวิชาการ และได้รับการพิจารณาเลื่อนตำแหน่งทางวิชาการจากผู้ช่วยศาสตราจารย์เป็นรองศาสตราจารย์โดยใช้ผลงานบางส่วนจากโครงการฯ

4. การสร้างความเชื่อมโยงกับนักวิชาการของหน่วยงานทั้งภายในและนอกประเทศ

โครงการฯ ได้สร้างความเชื่อมโยงในการวิจัยด้านเทคโนโลยีชีวภาพกุ้งกับ รศ. ดร. ไพศาล สิทธิกรกุล อาจารย์ประจำภาควิชาชีววิทยาของมหาวิทยาลัยศรีนครินทรวิโรฒ (ประสานมิตร) โดยเมธีวิจัยอาวุโสได้เป็นนักวิจัยพี่เลี้ยง (mentor) มาโดยตลอดในระยะ 4 ปีที่ผ่านมา และได้ให้การสนับสนุนงานวิจัยบางเรื่อง

โครงการฯ ยังได้มีความเชื่อมโยงกับนักวิจัยจากมหาวิทยาลัยในต่างประเทศและได้มีความร่วมมือในการวิจัยและผลิตผลงานร่วมกัน อาทิเช่น Dr. Arlo W. Fast แห่ง Hawaii Institute of Marine Biology, University of Hawaii และ Dr. Phillip Lee แห่ง University of Texas, Galveston ซึ่งกำลังร่วมมือวิจัยในเรื่องระบบหมุนเวียนน้ำแบบปิดเพื่อการเพาะเลี้ยงกุ้งกุลาดำ

โครงการฯ ได้มีส่วนสนับสนุนการจัดประชุมทางวิชาการนานาชาติ The 5th Asian Fisheries Forum ในระหว่างวันที่ 11-14 พฤศจิกายน 2541 ที่จังหวัดเชียงใหม่ โดยเมธีวิจัย อาวุโส สกว. เป็นประธานจัดการประชุม มีผู้เข้าร่วมประชุมทั้งหมด 800 คนจาก 41 ประเทศ และมีการเสนอผลงานวิจัยมากกว่า 500 เรื่อง

5. การถ่ายทอดเทคโนโลยี

โครงการฯ ได้มีส่วนร่วมจัดฝึกอบรมการสร้างงานเพาะเลี้ยงสัตว์น้ำให้แก่บัณฑิตที่กำลังว่างงาน 5 หลักสูตร ในระหว่างเดือนพฤษภาคม - กันยายน พ.ศ. 2542 โดยมีผู้เข้าฝึกอบรมหลักสูตรละ 80 คน การจัดฝึกอบรมในครั้งนี้นับว่าเป็นโอกาสดีของโครงการฯ ที่สามารถถ่ายทอดเทคโนโลยีได้ทันทีหลังการวิจัย เมธีวิจัยอาวุโสเป็นประธานคณะผู้วิจัยในการฝึกอบรมในครั้งนี้

6. การจดทะเบียนสิทธิบัตร ไม่มี

7. การเสนอผลงานในที่ประชุมวิชาการนานาชาติ

ปี 2539

(1) Menasveta P. Recent advance in reproductive biology of *Penaeus monodon.* 14-17 May 1996. Iloilo City, Philippines.

(2) Menasveta P. Shrimp Culture Systems and Mangrove Destruction. Fourth Fisheries Development Donor Consultation. 8-11 October 1996. Paris, France.

ปี 2540

(1) Menasveta P. Mangrove destruction and shrimp culture systems in Thailand. การประชุม World Aquaculture'97. Book of Abstracts. Washington State Convention Center Seattle, Washington, U.S.A. 1997: 326.

(2) Chuntapa B., S. Piyatiratitivorakul, V. Viyakarn and P. Menasveta. Optimal protein energy ratio in artificial diet for juvenile black tiger prawn *Penaeus monodon* larvae. Poster Session of the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology (APMBC'97 and APCAB'97). โรงแรมภูเก็ตอคาเดีย ภูเก็ต 1997: 20.

(3) Phianphak, W., Piyatiratitivorakul, S., Menasveta, P. and Rengpipat, S. Use of Probiotics in *Penaeus monodon*. Poster Session of the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology (APMBC'97 and APCAB'97). โรงแรมภูเก็ตออคาเดีย ภูเก็ต 1997: 18.

(4) Paibulkitchakul, C., Piyatiratitivorakul, S., Kittakoop, P. and Menasveta, P. 1997. Optimal levels of lecithin and cholesterol in diet for black tiger prawn *Penaeus monodon* Larvae. Poster Session of the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology (APMBC'97 and APCAB'97). โรงแรมภูเก็ตออคาเดีย ภูเก็ต 1997: 19.

(5) Phianphak, W., Piyatiratitivorakul, S., Menasveta, P. and Rengpipat, S. Use of probiotics in *Penaeus monodon*. Paper Presentation Oral of the 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology (APMBC'97 and APCAB'97). โรงแรมภูเก็ตออคาเดีย ภูเก็ต 1997: 165.

(6) Menasveta, P. Progress of aquaculture development in Thailand. การประชุม International Seminar on Aquaculture in Asia. The Nation Olympics Memorial Youth Center ประเทศญี่ปุ่น. 1997 : 39-53.

ปี 2541

(1) Fast, A.W. and P. Menasveta. 1998. Some recent innovations in Marine shrimp pond recycling systems. International Conference The Fifth Asian Fishreies Forum. Book of abstracts. Aquatic Resources Research Institute, Chulalongkorn Univ. : 52.

(2) Jarayabhand, P., S. Uraiwan, S. Klinbunga, A. Tassanakajon, P. Srimukda P. Pattanachan, R. Panakulchaiwit and P. Menasveta. 1998. Estimated heritabilities for early growth rate of the Black Tiger Prawn, *Penaeus monodon*, Fabricius. *ibid.* : 89.

(3) Rengpipat, S., S. Rukpratanporn, S. Piyatiratitivorakul, and P. Menasveta. 1998.Probiotics in aquacluture: a case study of probiotics for Larvae of the Black Tiger Shrimp (*Penaeus monodon). ibid.* : 193.

(4) Chuaychuwong, P., V. Viyakarn, S. Piyatiratitivorakul, and P. Menasveta. 1998. Effects of betaine of growth and survival rates of the Giant Tiger Shrimp (*Penaeus monodon). ibid.* : 326.

(5) Sithigorngul, P., S. Longyant, and N. Thampalerd. 1998. Production of monoclonal antibodies specific to vitellin and vitellogenin of *Penaeus monodon. ibid.* : 409.

(6) Panritdam, T., P. Sihanontn and P. Menasveta. 1998. Closed recirculating seawater system with denitrification for the culture of Black Tiger Prawns. *ibid.* : 439.

(7) Warukamkul, P., S. Piyatiratitivorakul, and P. Menasveta. 1998. Effect of salinity and protein levels on energetics of *Penaeus monodon* Juvenile. *ibid.* : 487.

ปี 2542

(1) Menasveta, P. Improved shrimp farming techniques for disease prevention and environmental sustainability. World Aquaculture'99. presentation at Sydney Convention & Exhibition Centre, 26 April - 2 May 1999.

6. การเสนอผลงานในที่ประชุมวิชาการในประเทศไทย

(1) ศิวาพร ลงยันต์. โมโนโคลนอลแอนติบอดีจำเพาะต่อไวเทลลินและไวเทลโลเจนนินของกุ้งกุลาดำ. การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 23. วันที่ 20-22 ต.ค. 2540. โรงแรมโลตัสปางสวนแก้ว เชียงใหม่ 2540

(2) Phianphak, W., Rengpipat, S. Piyatiratitivorakul, S. and Menasveta, P. Utilization of *Lactobacillus* spp. as probiotics for feeding *Penaeus monodon*, black tiger shrimp. การประชุมวิชาการ The 3rd Lactic Acid Bacteria in thai Industry. มหาวิทยาลัยเกษตรศาสตร์ 2540

(3) Menasveta, P. Progress of aquaculture development in Thailand. การประชุมเสนอผลงานวิจัยเฉลิมฉลอง 80 ปีแห่งการสถาปนาจุฬาลงกรณ์มหาวิทยาลัย. อาคาร 3 คณะครุศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย 2540: 39-53.

(4) ศิวาพร ลงยันต์. การผลิตโมโนโคลนอลแอนติบอดีต่อไวเทลลินสภาพปกติหรือถูกทำให้เสียสภาพของกุ้งกุลาดำ. เสนอผลงานวิชาการภาคโปสเตอร์. ศูนย์การประชุมแห่งชาติสิริกิติ์. กรุงเทพมหานคร 19 - 21 ต.ค. 2541

(5) รศ. ดร. ศิริรัตน์ เร่งพิพัฒน์. การเสริมภูมิคุ้มกันโรคในกุ้งกุลาดำ *Penaeus monodon* ด้วย *Bacillus* S11. การประชุมวิชาการกุ้งทะเลแห่งชาติ ครั้งที่ 1. โรงแรมบี.พี. สมิหลา จ. สงขลา 2542

7. ผลงานอื่นๆ

ปี 2539

- ศ. ดร. เปี่ยมศักดิ์ เมนะเศวตเป็นวิทยากรในการสัมมนาเรื่อง "ทางรอดของอนาคตกุ้งกุลาดำไทย" 13 มิถุนายน 2539 ณ ห้องบอลรูม 1 โรงแรมดิเอมเมอรัลด์ จัดโดย บริษัท เบ็ทเทอร์โพรดักส์ แอนด์ เทคโนโลยี จำกัด ร่วมกับหนังสือพิมพ์ประชาชาติธุรกิจ

- ศ. ดร. เปี่ยมศักดิ์ เมนะเศวตเป็นวิทยากรในการสัมมนาเรื่อง "ASCC Seminar and Discussion on Coastal Zone Management and Sustainable Shrimp Culture" 18 กรกฎาคม 2539 จัดโดย สมาพันธ์ผู้ประกอบการเลี้ยงกุ้งแห่งเอเชีย

- ศ. ดร. เปี่ยมศักดิ์ เมนะเศวตเป็นวิทยากรในการสัมมนาเรื่อง "การพิจารณาแนวทางการประเมินผลกระทบสิ่งแวดล้อมเบื้องต้น (IEE) สำหรับกิจกรรมการเพาะเลี้ยงกุ้งทะเลในพื้นที่ป่าชายเลน" 23-24 กันยายน 2539 ณ โรงแรมคลับอันดามันบีช รีสอร์ท จ. ภูเก็ต จัดโดย สำนักงานนโยบายและแผนสิ่งแวดล้อม

- จัดการสัมมนาเมธีวิจัยอาวุโส สกว. เรื่อง "การปรับปรุงกระบวนการผลิตพันธุ์กุ้งทะเลที่มีคุณภาพ : ผลการดำเนินงานระยะที่ 1" 15 ตุลาคม 2539 ณ ห้อง G.M. Hall อาคารศศนิเวศ จุฬาลงกรณ์มหาวิทยาลัย

ปี 2540

- ผศ. ดร. ศิริรัตน์ เร่งพิพัฒน์เป็นวิทยากรบรรยาย เรื่อง "จุลินทรีย์ในบ่อกุ้ง" ในการประชุมสัมมนางานวันกุ้งกุลาดำ ครั้งที่ 7 ณ โรงแรมวังใต้ จ. สุราษฎร์ธานี เมื่อวันที่ 21-23 กุมภาพันธ์ 2540

- ผศ. ดร. ศิริรัตน์ เร่งพิพัฒน์เป็นวิทยากรบรรยาย เรื่อง "Microorganism and Probiotics : A novel dream for shrimp business" ให้กับชมรมธุรกิจสัตว์น้ำ เมื่อวันที่ 2 สิงหาคม 2540

- ผศ. ดร. ศิริรัตน์ เร่งพิพัฒน์เป็นวิทยากรบรรยาย เรื่อง "การใช้โพรไบโอติกในการเพาะเลี้ยงกุ้ง" ให้กับศูนย์วิจัยการเลี้ยงกุ้งของบริษัทเจริญโภคภัณฑ์ เมื่อวันที่ 3 และ 10 มิถุนายน 2540

ปี 2541

- การให้สัมภาษณ์ในนิตยสาร "ดิฉัน" เรื่องเมธีวิจัยอาวุโส สกว. ศ. ดร. เปี่ยมศักดิ์ เมนะเศวต นิตยสารดิฉัน ปีที่ 21 ฉบับที่ 519 15 ตุลาคม 2541

- จัดแสดงนิทรรศการเนื่องในงานสัปดาห์วันวิทยาศาสตร์แห่งชาติ ประจำปี 2541 เรื่อง "ระบบหมุนเวียนน้ำแบบปิดเพื่อการเลี้ยงกุ้งกุลาดำที่ยั่งยืนและปลอดมลพิษ" ณ ศูนย์การประชุมแห่งชาติสิริกิติ์ วันที่ 18-22 สิงหาคม 2541

ปี 2542

- โครงการฯ ได้มีส่วนร่วมจัดฝึกอบรมการสร้างงานเพาะเลี้ยงสัตว์น้ำให้แก่บัณฑิตที่กำลังว่างงาน 5 หลักสูตร ในระหว่างเดือนพฤษภาคม - กันยายน พ.ศ. 2542 โดยมีผู้เข้าฝึกอบรมหลักสูตรละ 80 คน ที่สถานีวิจัยวิทยาศาสตร์ทางทะเลและศูนย์ฝึกนิสิต เกาะสีชัง จ. ชลบุรี, สถานีวิจัยสัตว์ทะเลอ่างศิลา จ. ชลบุรี, ฟาร์มเพาะเลี้ยงสัตว์น้ำ รังสิต จ. ปทุมธานีและคณะประมง มหาวิทยาลัยเกษตรศาสตร์ ได้แก่

หลักสูตรที่ 1 : การเพาะเลี้ยงหอยทะเล	15 พ.ค. - 4 มิ.ย. 2542
หลักสูตรที่ 2 : การเพาะเลี้ยงกุ้งและปลาทะเล	12 มิ.ย. - 2 ก.ค. 2542

หลักสูตรที่ 3 : การเพาะเลี้ยงปลาน้ำจืด 19 ก.ค. - 8 ส.ค. 2542
หลักสูตรที่ 4 : การเพาะเลี้ยงปลาสวยงาม 16 ส.ค. - 9 ก.ย. 2542
หลักสูตรที่ 5 : การเพาะเลี้ยงกุ้งน้ำจืดและกุ้งทะเล 28 ส.ค. -17 ก.ย. 2542

- จัดสัมมนาวิชาการเมธีวิจัยอาวุโส สกว. เรื่อง "เทคโนโลยีชีวภาพทางทะเลเพื่อการเพาะเลี้ยงกุ้งที่ยั่งยืน: สรุปผลการดำเนินงาน" 17 กันยายน 42 ที่ห้องประชุมอาคารสถาบัน 3 ชั้น 2 จุฬาลงกรณ์มหาวิทยาลัย

- เป็นวิทยากร

1) ศ. ดร. เปี่ยมศักดิ์ เมนะเศวต บรรยายเรื่อง "การเลี้ยงกุ้งกับการอนุรักษ์ป่าชายเลน" ที่ สถานีวิจัยวิทยาศาสตร์ทางทะเลและศูนย์ฝึกนิสิต เกาะสีชัง จ. ชลบุรี วันที่ 12 มิถุนายน 2542

2) รศ. ดร. สมเกียรติ ปิยะธีรธิติวรกุล

- บรรยายเรื่อง "อาหารสัตว์น้ำ" ที่ สถานีวิจัยวิทยาศาสตร์ทางทะเลและศูนย์ฝึกนิสิต เกาะสีชัง จ. ชลบุรี วันที่ 16 พ.ค. 42

- บรรยายเรื่อง "สารอาหารที่มีความสำคัญต่อภูมิต้านทานโรคของกุ้งทะเล" ที่ สถานีวิจัยวิทยาศาสตร์ทางทะเลและศูนย์ฝึกนิสิต เกาะสีชัง จ. ชลบุรี วันที่ 22 มิ.ย. 42 และ 9 ก.ย. 42

- บรรยายเรื่อง "การเจริญพันธุ์ของกุ้งกุลาดำ" ที่ สถานีวิจัยวิทยาศาสตร์ทางทะเลและศูนย์ฝึกนิสิต เกาะสีชัง จ. ชลบุรี วันที่ 29 ส.ค. 42

3) รศ. ดร. ศิริรัตน์ เร่งพิพัฒน์ บรรยายเรื่อง "การใช้โพรไบโอติกในการเพิ่มประสิทธิภาพการผลิตการเลี้ยงกุ้ง" ที่ สถาบันราชภัฏเพชรบุรีวิทยาลงกรณ์ จ. ปทุมธานี วันที่ 12 ก.ย. 42

4) น.ส. นภาพร กิติมศักดิ์ นิสิตปริญญาโทของโครงการฯ บรรยายเรื่อง "การเลี้ยงปลากะพงขาวในระบบปิด" ที่ สถานีวิจัยวิทยาศาสตร์ทางทะเลและศูนย์ฝึกนิสิต เกาะสีชัง จ. ชลบุรี วันที่ 20 มิ.ย. 42

รายชื่อคณะผู้วิจัย

ชื่อ-นามสกุล	ตำแหน่งทางวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
1. ดร. เปี่ยมศักดิ์* เมนะเศวต	ศ. 10	ศ. 11	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย และสถาบันวิจัยทรัพยากรทางน้ำ	หัวหน้าโครงการ	อยู่ในโครงการ
2. Dr. Arlo W. Fast	Assoc. Prof.	Full Prof.	-	Hawaii Institute of Marine Biology	University of Hawaii P.O. Box 1346, Kaneohe, Hawaii 96744 U.S.A.	ที่ปรึกษาโครงการ	อยู่ในโครงการ
3. Dr. Phillip Lee	Assoc. Prof.	Full Prof.	-	Marine Biomedical Institute	University of Texas at Galveston	ที่ปรึกษาโครงการ	อยู่ในโครงการ
4. ดร. สมเกียรติ* ปิยะธีรธิติวรกุล	ผศ. 8	รศ. 9	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย และสถาบันวิจัยทรัพยากรทางน้ำ	นักวิจัยและผู้ช่วยหัวหน้าโครงการ	อยู่ในโครงการ
5. ดร. ศิริรัตน์* เร่งพิพัฒน์	ผศ. 8	รศ. 9	จุลชีววิทยา	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	นักวิจัย	อยู่ในโครงการ

รายชื่อคณะผู้วิจัย

ชื่อ-นามสกุล	ตำแหน่งทางวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
6. ดร.เผดิมศักดิ์ จารยะพันธุ์	ผศ. 8	รศ. 9	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ร่วมวิจัย	อยู่ในโครงการ
7. ดร.ไพศาล สิทธิกรกุล	รศ. 9	รศ. 9	ชีววิทยา	วิทยาศาสตร์	มหาวิทยาลัยศรีนครินทร-วิโรฒ (ประสานมิตร)	ร่วมวิจัย	อยู่ในโครงการ
8. ดร.วรณพ วิยกาญจน์	อ. 5	อ. 6	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ร่วมวิจัย	ไม่อยู่ในโครงการ เนื่องจากติดราชการ ไม่สามารถร่วมทำการวิจัยได้
9. ดร.ณรงค์ศักดิ์ พ่วงลาภ	นักวิจัย 1	นักวิจัย 1	หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเล	ศช.	สวทช.	Post-Doc. Research Associate	อยู่ในโครงการ

รายชื่อคณะผู้วิจัย

ชื่อ-นามสกุล	ตำแหน่งทางวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
10. ดร.ศิราวุธ กลิ่นบุหงา	นักวิจัย 1	นักวิจัย 1	หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเล	ศช.	สวทช.	Post-Doc. Research Associate	อยู่ในโครงการ
11. ดร.สรวิศ เผ่าทองศุข	นักวิจัย 1	นักวิจัย 1	หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเล	ศช.	สวทช.	Post-Doc. Research Associate	อยู่ในโครงการ
12. น.ส.อรพร หมื่นพล	นักวิจัย 1	นักวิจัย 1	หน่วยปฏิบัติการเทคโนโลยีชีวภาพทางทะเล	ศช.	สวทช.	Post-Doc. Research Associate	อยู่ในโครงการ
13. นายศิวาพร ลงยันต์	-	-	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณมหาวิทยาลัย	นักศึกษาปริญญาเอก	อยู่ในโครงการ

รายชื่อคณะผู้วิจัย

ชื่อ-นามสกุล	ตำแหน่งทางวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
14. น.ส. เบ็ญจมาศ จันทะภา	-	-	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาโท	ไม่อยู่ในโครงการ กำลังศึกษาต่อปริญญาเอกที่คณะวิทยาศาสตร์ จุฬาฯ (โครงการปริญญาเอกกาญจนาภิเษก)
15. น.ส. วรรนิภา เพี้ยนภักตร์	-	นักวิจัย	จุลชีววิทยา	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาโท	ไม่อยู่ในโครงการ ทำงานสำนักงานพลังงานปรมาณูเพื่อสันติ กระทรวงวิทยาศาสตร์ เทคโนโลยีและสิ่งแวดล้อม
16. น.ส.นิภา เตโชดำรงสิน	-	-	สาขาวิชาเทคโนโลยีทางชีวภาพ	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	นักศึกษาปริญญาโท	ไม่อยู่ในโครงการ ทำงานบริษัทเอกชน
17. นายซลี ไพบูลย์กิจกุล	-	-	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย		ไม่อยู่ในโครงการ กำลังศึกษาต่อปริญญาเอกที่คณะวิทยาศาสตร์ จุฬาฯ (โครงการปริญญาเอกกาญจนาภิเษก)

รายชื่อคณะผู้วิจัย

ชื่อ-นามสกุล	ตำแหน่งทางวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
18. น.ส. ธัญญา พันธ์ฤทธิ์ดำ	-	อาจารย์	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	ไม่อยู่ในโครงการ เป็นอาจารย์สอนที่ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยทักษิณ อ. เมือง จ. สงขลา
19. นายพิพัฒน์ เวฬุคามกุล	-	-	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	ไม่อยู่ในโครงการ ประกอบกิจการส่วนตัว
20. น.ส.วรรณลักษณ์ วุทธิจินดา	-	-	หลักสูตรวิชาเทคโนโลยีทางชีวภาพ	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	ไม่อยู่ในโครงการ ประกอบกิจการส่วนตัว
21. น.ส.นภาพร กิติมศักดิ์	-	-	วิทยาศาสตร์ทางทะเล	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	ไม่อยู่ในโครงการ ทำงานที่สถานีตำรวจอำเภอบ้านสร้าง จ.ปราจีนบุรี
22. นายสมบัติ รักประทานพร	-	-	จุลชีววิทยา	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	ไม่อยู่ในโครงการ เมื่อพ.ค. 42 เนื่องจากได้รับทุนวิจัยจากแหล่งอื่น

รายชื่อคณะผู้วิจัย

ชื่อ-นามสกุล	ตำแหน่งทางวิชาการ		ต้นสังกัด			ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	ภาควิชา	คณะ	มหาวิทยาลัย/สถาบัน		
23. น.ส. จารุวรรณ มหิทธิ	-	-	สหสาขาวิชาวิทยาศาสตร์สภาวะแวดล้อม	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	SEAFDEC/SECRETARIAT ภายในบริเวณมหาวิทยาลัยเกษตรศาสตร์ บางเขน กทม.
24. น.ส. กัญญาจิต โล่ภิญโญสิริ	-	-	สหสาขาวิชาวิทยาศาสตร์สภาวะแวดล้อม	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	ไม่อยู่ในโครงการ เมื่อก.ค. 42 เนื่องจากได้รับทุนวิจัยจากแหล่งอื่น
25. น.ส. นันทิกา ปานจันทร์	-	-	เทคโนโลยีทางชีวภาพ	วิทยาศาสตร์	จุฬาลงกรณ์มหาวิทยาลัย	ผู้ช่วยวิจัยปริญญาโท	อยู่ในโครงการ

*ผู้วิจัยหลัก

ภาคผนวก

+Proc. Second Int. Seminar on Fisheries Sci.,
in Tropical Area, Tokyo Aug. 19-22, Japan, 1997, pp. 65-69

PROGRESS OF AQUACULTURE DEVELOPMENT IN THAILAND

Piamsak Menasveta

Aquatic Resources Research Institute
Chulalongkorn University
Bangkok 10330
Thailand

The paper reviews progress of aquaculture development in Thailand during the past two decades. Aquaculture activities could be categorized into three groups, i.e. freshwater culture, brackishwater culture, and mariculture. In general, progress of aquaculture has achieved beyond the target, especially the brackishwater shrimp culture. Success in shrimp culture is due to the introduction of a modern farming technology which is more intensive, and more environmental friendly.

I. INDRODUCTION

Aquaculture in Thailand has been developed for decades. The original objective was set as livestocks for household consumption in the rural areas. Nowdays, the fishery production by fishing could not be increased due to the limitation of the resources, aquaculture therefore play an important role in augmenting the overall fishery production. Thailand gave very high priority to aquaculture in the Sixth and Seventh National Social and Economic Development Plans (1988-1996). Progress of aquaculture during the past decade has achieved beyond the target. The current paper reviews progress of aquaculture development during the past two decades.

II. HISTORY AND STATUS OF AQUACULTURE

Aquaculture has developed considerably since the beginning of the century. Freshwater aquaculture was developed a long time ago, but coastal aquaculture is much more recent. Prior to the use of synthetic insecticides, substantial amounts of fish could be produced as a by-product of rice cultivation. Aquaculture production during the past decades in shown in Table 1.

Aquaculture contributed about 10 to 20 percent of the total fishery production of the country in the past years. Considering the data in Table 1, we could observe that freshwater and brackish water cultures have a tendency to increase constantly while mariculture production fell since 1978. It is generally believed that the declining production of mariculture is due to water quality degradation of farming areas, particularly in the The
area is subjected to runoff from the four main rivers of the Central Plain. Nevertheless, mariculture problems.

Table 1. Aquaculture production in Thailand

(MT)

Year	Aquaculture (Total production)	Freshwater culture	Brackishwater culture	Mariculture
1973	93,274	N.A.	N.A.	N.A.
1974	85,361	N.A.	N.A.	N.A.
1975	139,773	N.A.	N.A.	N.A.
1976	197,584	N.A.	N.A.	N.A.
1977	191,344	32,641	10,334	148,369
1978	159,345	39,367	10,049	109,929
1979	152,239	29,465	10,838	111,936
1980	138,153	34,634	11,901	91,618
1981	115,618	48,113	13,759	53,746
1982	82,719	45,829	12,988	23,902
1983	91,727	46,966	14,920	29,841
1984	111,945	50,411	16,346	45,188
1985	135,840	75,254	18,428	42,158
1986	128,417	89,325	20,217	18,875
1987	151,657	89,781	26,017	35,859
1988	211,048	102,128	57,512	51,408
1989	260,369	91,680	95,753	72,936
1990	296,952	103,754	120,216	72,982
1991	353,124	122,680	164,144	66,300
1992	371,475	142,105	188,757	40,613
1993	457,272	161,630	229,291	66,351

Note : N.A. = not available.
Source: Fishery Statistical Bulletin for South China Area, 1976-1995 SEAFDEC.[4]

The technical level of aquaculture in Thailand is fairly advanced, particularly in the seed production system, applying the induced spawning technique which has become popular in this country. At present, seed of all cultivated species could be produced from hatcheries.

In comparing aquaculture production of the countries in the South China Sea region (Table 2), it is evident that Thailand ranks the first.
production shows tendency to increase in recent years, dueing to better control on pollution of the Central Plain. Nevertheless, mariculture coastal area of the Upper Gulf of Thailand. problems.

Table 2. Comparison of aquaculture production among countries in South China Sea area in 1993

Country	Total land area (km^2)	Length of coastline (km)	Aquaculture production (ton)			
			Freshwater	Brackishwater	Marine	Total
Thailand	513,115	2,614	161,630	229,291	66,351	457,272
Malaysia	332,435	4,405	15,468	10,795	78,973	105,236
Philippines	299,404	17,460	113,663	234,651	423,768 [a]	772,082
Taiwan	36,000	1,566	185,904	64,267	35,105	285,276

Note : [a]. mostly seaweed.
Source: Fishery Statistical Bulletin for South China Area, 1995 SEAFDEC.

III. TYPES OF AQUACULTURE VENTURE

Aquaculture, although it contributes a relatively small portion, about 10 to 20 percent in tonnage to total fishery production, it has long term potential for increasing fishery production for either local consumption or export earning from high value shrimps and fish species. Aquaculture activities in Thailand can be divided into three categories, i.e., freshwater culture, brackishwater culture, and mariculture. The numbers of farms aquaculture farms, and rearing areas, and total production in 1993 are given in Table 3. The present cultured species of the three categories are listed in Table 4.

Table 3. Number of aquaculture farms, rearing area, and production in 1993

Category	Number of farms	Area (rai)	Production (ton)
Freshwater (Total)	**98,965**	**194,856**	**161,630**
Pond culture	88,463	178,011	131,186
Paddy field culture	979	1,435	1,227
Ditch culture	9,060	15,324	28,430
Cage culture	463	86	787
Brackishwater (Total)	**23,419**	**451,289**	**229,291**
Gulf of Thailand	19,735	424,783	194,833
Pond culture	18,138	424,700	193,309
Cage culture	1,597	83	1,524
West coast	3,684	26,506	34,458
Pond culture	2,206	26,462	33,969
Cage culture	1,478	44	489
Mariculture (Total)	**2,853**	**21,127**	**66,351**
Gulf of Thailand	2,747	20,570	62,081
Bloody cockle culture	476	11,668	20,555
Green mussel culture	358	1,481	24,391
Oyster culture	1,852	6,965	13,563
Horse mussel culture	61	456	3,572
West coast	106	557	4,270
Bloody cockle culture	1	500	22
Oyster culture	105	57	4,248
Total	**0**	**667,272**	**0**

Note : 6.25 rai = 1 ha.
Source: DOF, 1995.[5]

IV. FRESHWATER CULTUER

As mentioned elsewhere, freshwater fish culture has long been established and practiced in Thailand, and more than 20 species of fish and invertebrates are presently cultured. Freshwater culture consists of four main practices: pond culture, paddy field culture, ditch culture, and cage culture.

culture consists of four main practices: pond culture, paddy field culture, ditch culture, and cage culture.

Table 4. List of cultured species and production in 1993

Group and common name	Scientific name	Production (MT)
Mariculture	(Total production)	
Oyster	*Crassostrea* spp.	4,00
Mussels	*Mytilus smaragdinus*	20,000
Bloody cockles	*Anadara granosa*	20,131
Brackishwater culture		
Sea pearch	*Lates calcarifer*	2,806
Mullet fish[a]	*Mugil* sp.	171
Grouper	*Epinephelus* spp.	1,000
Banana prawn[b]	*Penaeus merguensis*	5,500
Tiger prawn[b]	*Penaeus monodon*	210,000
Marine prawn[a]	*Penaeus* spp.	100
Marine prawn[a]	*Metpenaeus* spp.	1,000
Mud crab	*Scylla serrata*	50
Freshwater culture		
Tawes	*Puntius gonionotus*	24,316
Rohu	*Labeo rohita*	1,160
Grass carp	*Ctenopharyngodon idellus*	
Bighead carp	*Aristichthys nobilis*	
Silver carp	*Hypopthalmichthys molitrix*	1,290
Nile tilapia	*Tilapia nilotica*	44,814
Marble goby	*Oxyeleotris marmorata*	40
River catfish	*Pangasius pangasius*	14,660
Catfish	*Clarias* spp.	24,250
Swamp eel	*Fluta alba*	
Eel	*Anguilla* spp.	
Gouramys	*Trichogaster pectoralis*	13,218
Gouramys	*Trichogaster* spp.	200
Snakehead	*Ophicephalus striatus*	4,809
Milkfish	*Chanos chanos*	
Small scale mud carp	*Cirrhinus mrigala*	310
Common carp	*Cyprinus carpio*	2,433
Jullien's golden-price carp	*Probarbus jullieni*	
Tilapia	*Oreochromis mossambicus*	125
Common climbing perch	*Anabus testudinus*	828
Giant snakehead fish	*Channa micropeltes*	923
Feathearback	*Notopterus* spp.	8
Giant gourami	*Osphronemus goramy*	8
Giant prawn	*Macrobrachium rosenbergii*	10,513
Frogs	*Rana* spp.	134
Total		392,383

Note : [a]. Some are exported.
: [b]. Mainly exported.
Source: Aquaculture Production Statistics 1984-1993, FAO.

1. *Pond Culture*

There were 194,856 rai (31,176 ha) of freshwater culture ponds in 1993, distributed among 98,965 farms with 88,465 ponds. Total production from pond culture in 1993 was 131,186 tons. The major production species were nile tilapia, gouramys, catfishes, carp-like fishes, and snakeheads.

2. *Paddy Field Culture*

Fish production from paddy fields totalled

1,227 tons in 1993. The area under production was recorded at 1,435 rai (229 ha). The species being cultured in this type of venture are carp-like fishes, especially tawes.

3. *Ditch Culture*

A variety of fish species are cultured in ditches throughout the country. Culture practices vary and are generally not a well developed subsector. Total production in 1993 was 28,430 tons, from a total area of 15,324 rai (245ha).

4. *Cage Culture*

About 463 cage culture farmers produced 787 tons of fish in 1993. The total production area was about 86 rai (13 ha). The fishes being cultured by this type of venture are marble goby and river catfish.

V. BRACKISHWATER CULTURE

Brackishwater culture is a type of aquaculture which is operated in the coastal area. It is the area where there is an interface between freshwater and seawater. Therefore, salinity in this area usually ranges from 5 to 25 ppt. Brackishwater culture concentrates on penaeid prawn culture. The cage culture of sea bass in the coastal area is also becoming popular.

1. *Shrimp Culture*

In 1994, the number of shrimp farms was 22,198 farms with a total area of 457,793 rai (73,246 ha, see also Table 5). Total production from the farms in 1996 was 230,000 tons. The shrimp farms are mainly concentrated in the eastern and southern parts of Thailand. During the past decade shrimp culture development in terms of production and area cultivated increased significantly (Table 5).

Table 5. Brackishwater shrimp culture development during 1987-1994

Year	Number of shrimp farms	Acreage (rai)	Production (ton)
1987	5,899	279,812	23,566.47
1988	10,246	342,364	55,632.84
1989	12,545	444,785	93,494.50
1990	15,072	403,787	118,227.05
1991	18,998	470,826	162,069.69
1992	19,403	454,975	184,884.32
1993	20,027	449,292	225,514.30
1994	22,198	457,793	263,445.96
1995	23,000	452,000	280,000.00*
1996	20,000	445,000	230,000.00*

Note :*. World shrimp Farming 1994 and 1995.
Source: DOF, 1995.[5]

Shrimp culture systems in Thailand have three levels of intensity: extensive, semi-intensive, and intensive. The characteristics of these culture systems are summarized in Table 6.

1.1. *Extensive system*

Extensive culture systems are characterized by low stocking densities and, therefore, low productivity. This system is characteristic of low income coastal communities. In addition to being a source of charcoal wood and fire wood, the mangrove is also a source of food and income for near shore communities. Through their long association with the mangrove ecosystem, these communities know that the mangrove is a concentration ground for small aquatic animals such as juvenile fish and crustaceans. The extensive aquaculture system, therefore, is an evolved form of hunting and gathering of food for these communities.

Table 6. Characteristics of shrimp culture at three levels of intensity

Characteristics	Level of intensity (Total production)	Semi - intensive	Intensive
Land elevation (m)	0 to +1.4 MSL*	0 to +1.4 MSL	> +2.0 MSL
Pond size (ha)	>5	1 to 2	1 or less
Aeration	Natural	Water exchange or mechanical	Continual mechanical and flushing
Stocking rate (PL m^{-2} $crop^{-1}$)	<5	5 to 15	20 or more
Feed	Natural (No supplement)	Natural + Supplement	Formulated
Production level (kg rai^{-1} yr^{-1})	16 to 48	96 to 288	>960

* MSL = Mean sea level.
Note : 6.25 rai = 1ha.

Early shrimp culture in Thailand was mostly extensive culture. Shrimp ponds were comparatively large, ranging from 31.25 to 62.5 rais, each with a peripheral canal around the inner dike. They are generally called "Wang Koong". Farmers may have used water elevators powered by wind mills for inundating the ponds. Shrimp seed entrained by the influent water grew in the ponds for 2 to 3 months before the ponds were drained and the shrimps, usually of small size, were harvested. During the culture period, some fertilizer might be added to the pond, but feed was not provided. Shrimp production by this extensive methods was rather low, with an average of only 24 kg rai-1 year-1 or less. This method is still used by some Thai shrimp culturists even today.

Mangrove areas in estuaries have been prime candidates for such pond construction since they typically have gentle slopes, adequate tidal range, abundant wild shrimp seed, and land ownership vested with the government. Such land was usually easy to use and constructing extensive culture ponds. In Thailand, which have a maximum tidal rage of -0.4 m mean sea level (MSL) to +2.0 m MSL, the most ideal elevation for the pond is between 0.0 m and +1.4 m MSL. Ponds built within this range can only be filled and drained twice monthly during ordinary high and low tides. Ponds with bottoms above +1.8 m MSL will normally require mechanical pumping to fill, while those with bottoms below -0.4 m LSL will require pumping to fully drain. Neither situation is desirable if the objective is to rely solely on tidal action for water exchange.

1.2. *Semi-intensive systems*

In the past, as shrimp demand rose and shrimp became a valuable commodity, extensive shrimp farmers started developing techniques to increase the yields. Developments towards monoculture of shrimp includes the active harvesting of juvenile shrimp from the wild for stocking. In 1980, the semi-intensive system was introduced into Thailand through the Aquaculture Development Project, a soft-loan from the Asian Development Bank. Semi-intensive culture ponds may be created through intensification of existing extensive ponds. This may include such things as clearing and leveling the pond bottom, and/or digging canals in the ponds bottom; converting from tidal water exchange to pumped water exchange; control stocking density; greater use of fish toxicants to control fish intruders; and provision of feed.[1)] Menasveta and Higuchi[2)] described a plan of staged intensification and shrimp postlarvae collection pond, 4 nursery ponds and 3 growout ponds with inlets and outlets on separate canals. Shrimp production was about 24 kg rai^{-1} $year^{-1}$ with the traditional configuration, but was expected to be 96 to 288 kg rai^{-1} $year^{-1}$ or more after intensification due to better use of space, better intruder control, less time for sediment removal, and better water quality management.

1.3. ***Intensive systems***

About a decade ago, some shrimp culturists began developing advance technologies for intensive culture systems. The Thai intensive culture system is very similar to the Taiwanese system. With these new technologies, shrimp production rate increased to 960-1600 kg rai^{-1} $year^{-1}$ taking 4 months per crop. The shrimp species used almost exclusively with this type of culture is black tiger shrimp *Penaeus monodon.* This type of culture system does not require mangrove anymore, since this system requires fast water exchange. In this regard, the pond bottom needs to be at higher elevation, preferably +2.0 MSL. These areas are behind the mangrove zone. Intensive farms are also liberated from harvesting wild caught juveniles as large numbers of shrimp fry are produced from hatcheries. Furthermore, certain requirements of intensive culture systems make mangrove areas entirely incompatible. Because of the high shrimp densities and the use of supplement feeding, there is large organic accumulation at the pond bottom after a culture period which requires complete drying to facilitate sanitation and removal of waste. Ponds constructed in the mangrove roots which are impossible to remove during the construction. Finally, alkaline water conditions are required for optimal shrimp culture, but in mangrove areas the soil is high in organic content (i.e., humus); therefore, it is acidic in nature and inappropriate for shrimp growth.

2. ***Sea Bass Culture***

In Samut Sakhon, and Samut Songkhram, *Lates calcarifers,* commonly called sea bass, is raised in ponds and in small-scale cages in Songkhla Lake. The net-cage commonly used is 3x5x2 meters and the mesh size can be increased gradually as the fish grows. The culture period from fingerlings (approximately 1 centimeter in total length) to marketable size (0.7 kg in body weight) is around six months. The stocking density at harvesting is 300-500 fish/cage.

VI. MARICULTURE

The mollusca which consist mainly of bivalves are cultivated in inshore and coastal areas of Thailand. In 1993 Thailand produced a total of 66,351 tons from cultivation, and made up as follows: approximately 20,577 tons of bloody cockles, 24,391 tons of green mussels, and 17,811 tons of oyster (Table 3).

1. ***Bloody Cockle Culture***

This species requires harder bottoms with a good mixture of sand for its habitat and is raised on the bottom is shallow waters. The culture site can be air-exposed during low tide, but preferably not exceeding four hours exposure. Small cockles used as generally collected from natural cockle grounds or imported from Malaysia. About 3.5 to 4 million seeds are used in hectare of cockle farm. Scattered seeds grow to marketable size within 1 to 1.5 years. Manual bottom dredges are used for harvesting, and the full-sized cockles are selected while undersized ones returned to the growing beds. Poaching has been a local problem. Although bloody cockles are presently produced in two provinces, Petchaburi and Satun, potential areas for this species are located in Chanthaburi, Rayong, Phangnga, Samut Songkhram, Chachoengsao, Chumphon and Trang.

Great demand for this species exists in domestic markets. The production, however, cannot satisfy the whole domestic demand and a certain amount of cockles is imported from Malaysia.

2. ***Mussel Culture***

Green mussels grow in water of about two to eight meters in depth. The culture method used in Thailand is exclusively the stake method. Palm stakes made from the trunk of the mangrove date palm (locally called "mai ping") and bamboo stakes are commonly used. At present, the use of the palm stakes is more widely practiced among farmers, because they are inexpensive and more durable (three years' operation) than bamboo (1 to 1.5 years). The only difference is that the farmer can get longer bamboo stakes (up to 10 meters) which can be used in deeper waters, while the palm stakes are generally from 3 to 6 meters in length. About 5,000 stakes at 0.25 meter intervals are set in very hectare of farm. The culture period is about eight months and the mussels grow up to 7 to 8 centimeters in shell length.

Recently, the hanging method of green mussel culture has been developed experimentally for the purpose of establishing economical culture

methods.[3] This technique should be extended to the mussel farmers. The major areas where green mussels are widely cultured are in Chachoengsao, Chonburi, Samut Sakhon, Samut Songkhram, Samut Prakan and Phetchaburi. Potential grounds for the culture method are extensive in the Gulf of Thailand, including Nakhon Si Thammarat and Surat Thani.

3. *Oyster Culture*

Natural rocks, artificial concrete blocks and wooden materials are used as a substratum for the attachment and growth of the oyster. These substratums are settled in the estuary area with a 500 centimeter interval between substratums which enables people to walk across for routine management. Once built, the farm lasts a long time with minor maintenance.

Spats of oyster occur in two seasons during the year, one in May to July and the other in October to November. Harvest is usually made from October to April, depending on the size of the meat. Small and medium-sized oysters are processed as a shell-less meat, and large sized ones, mainly produced in the Surat Thani bay area, are served as oysters on the half shell at restaurants and hotels.

Oyster farms are generally small-scale, about 0.6 ha on average. Although oyster farmers cultivate only small areas or a fraction of a hectare, they can get quite a profit as the price of oysters is comparatively high. Presently oysters are cultured in the province of Trat, Chonburi, Rayong, Surat Thani, Chumphon, Pattani, Prachuap Khiri Khan and Songkhla. Potential areas are still available in the above mentioned provinces and are newly found in Chanthaburi, Rayong, Phangnga, Narathiwat, Trang, Satun, Nakhon Si Thammarat, Krabi, and Phetchaburi.

VII. FUTURE TRENDS IN AQUACULTURE

Freshwater aquaculture is planned for fishes which are a staple food for Thai people. These fishes include pla salid (gouramy), pla nil (nile tilapia), pla dook (catfish), and pla chon (snakehead fish). Nile tilapia has a particularly good potential for future development, on the account of the availability of technological know-how and market acceptability. Nile tilapia has long been researched around the world. It is generally recognized as a fast growing and easily-propagated species, if properly managed.

The blackishwater culture, especially shrimp, has reached the maximum sustainable yield. Shrimp production from farming should be maintained at this level. Since the advent of modern shrimp farming techniques in 1986, intensive shrimp culture began the trend towards utilizing land away from the unsuitable low lying mangrove areas. Coupled with technologies such as formulated feed, water quality management, selective breeding, disease control and hatchery techniques, shrimp culture has become more efficient, producing more shrimp from less culture area. Farmers gradually have come to realize that intensive shrimp culture system is the sustainable system and that mangrove destruction for shrimp pond is contradictory to this. While shrimp farming was admittedly as a factor in destroying mangroves, the distinction must be made that this destruction was carried out during unsophisticated extensive culture system dating ten years ago. Ever since intensive culture came into practice, circa 1986, mangrove destruction by the shrimp industry has been minimal.

As for mariculture, future activities will be focused on the promotion and expansion of mollusk culture to other productive areas. As mentioned elsewhere, water pollution problems have caused a major setback of mollusk culture in the inner Gulf. The problem will be persistent and will not be solved unless wastewater treatment systems are set up in big cities, towns, and industrial plants situated above the inner Gulf. This is likely to be a large, expensive, and time consuming project. So mollusk should be promoted in other potential areas. It can be anticipated that the production from mariculture could easily achieve the same level recorded in 1977 (148,369 tons) within a short period, because the technology is already available.

Acknowledgments

This study is supported by the Thailand Research Fund (TRF).

REFERENCES

1) A.W. Fast : Penaeid semi-intensive growout systems. In A.W. Fast & L.J. Lester (eds.), Marine Shrimp Culture: Principle and Practices, Elsevier Science, Amsterdam, 1992, pp. 369-381.

2) P. Menasveta and M. Higuchi : Operation manual for aquaculture development projects in Thailand. Dept. of Marine Science, Chulalongkorn Univ., Thailand, 1983, 141 p. (mimeo).

3) N. Chaitanawisuti and P. Menasveta : Experimental suspended culture of green mussel, *Perna viridis* (Linn.), using spat transplanted from distant settlement ground in Thailand. *Aquaculture*, 66, 97-107 (1987).

4) SEAFDEC : Fishery Statistical Bulletin for South China Sea Area, 1974-1993. Southeast Asian Fisheries Development Center, Bangkok, 1976-1995.

5) Department of Fisheries (DOF) : Fisheries Record of Thailand 1993. Dept. of Fish., Ministry of Agriculture and Cooperatives, Bangkok, 1995.

Mangrove Destruction and Shrimp Culture Systems

Piamsak Menasveta

In our modern, environmentally-concerned atmosphere, mankind is becoming increasingly sensitive to environmental issues. With non-governmental organizations bringing issues to the forefront, the general public is becoming more receptive to environment-related information. One of the more popular issues is that of mangrove ecosystems. While socio-economic factors have always driven man to exploit natural areas of the earth for development, the world's mangroves have undergone significant decline in recent decades, and shrimp farming has been wrongfully accused of being the sole activity leading to the mangrove's demise.

Mangrove Destruction

Shrimp production comes from two principal sources in Thailand: the ocean-capture fishery and culture in ponds. In the past, ocean-capture contributed the greatest share, but production from pond culture now exceeds the capture production (Table 1).

The two most important shrimp fishing grounds in Thailand are the Gulf of Thailand and the Andaman Sea. In the past, Thai boats fished at sea beyond the 12 mile territorial sea limit of neighbouring countries. After Thailand ratified the 200-mile Exclusive Economic Zone (EEZ) in 1981, however, she lost about 50% of her historic fishing grounds. This is the main reason why shrimp production from the Thai capture fishery has not increased since 1981. This limitation on fishing grounds led Thailand to put special emphasis on shrimp culture development in coastal areas. Because of her 2600-km coastline, Thailand has many areas which are suitable for shrimp culture.

Coastal areas of Thailand have several conditions that are very favorable to shrimp culture. Thailand is seldom affected by typhoons or cyclones, water temperatures are ideal with small seasonal variations, and soils and terrain are often ideal for pond construction. These unique features give Thailand advantages over some other countries in Southeast Asia. Shrimp culture area in Thailand has increased steadily since 1980. Satellite imagery indicates that Thailand currently has about 406 198 rai[4] devoted to shrimp culture.

Shrimp culture development during the past decade has raised a major concern over mangrove destruction since many of the shrimp culture ponds were situated in areas which used to be mangrove. According to statistics disclosed by the Royal Forestry Department, about 54.7% (1 273 534 rai) of mangrove area was destroyed between 1961 and 1993 (Table 2), and the average mangrove destruction rate during 1961-1993 was 38 910 rai/year. The highest rate (81 136 rai/year) occurred between 1979 and 1986. Since 1987, however, the rate of destruction has steadily declined.

Recently, four national institutions decided to re-analyze the remote-sensing charts to determine the utilization of converted mangrove areas. The Joint Working Committee of the Royal Forestry Department, the Department of Fisheries, the Land Development Department and the National Research Council of Thailand, divided the total mangrove area of Thailand into four regions: Eastern, Central, Southern-Gulf of Thailand, and Southern-Andaman Sea. For each of the regions, mangrove utilization was categorized into either "shrimp farms," "community," or "others." Of the total 1 272 543 rai of mangrove area that had been destroyed between 1961 and 1993, community development accounted for 2.4%, shrimp farms for 32%, and "others" for 65.6%. The "others" category includes agriculture, road development, ports, salt farms, mining, charcoal fuel, etc. (Figure 1 and Table 3).

One of the problems with the surveys is the nature of the remote sensing charts. There is no way of determining from the charts whether certain shrimp ponds were developed from virgin mangrove area or from abandoned salt farms. Due to the multiple purpose of rectangular pond structures, the "ponds" can easily be converted for use as paddy fields, fish ponds, salt ponds or shrimp

ponds depending upon the economic outlook for that year for that pond owner.

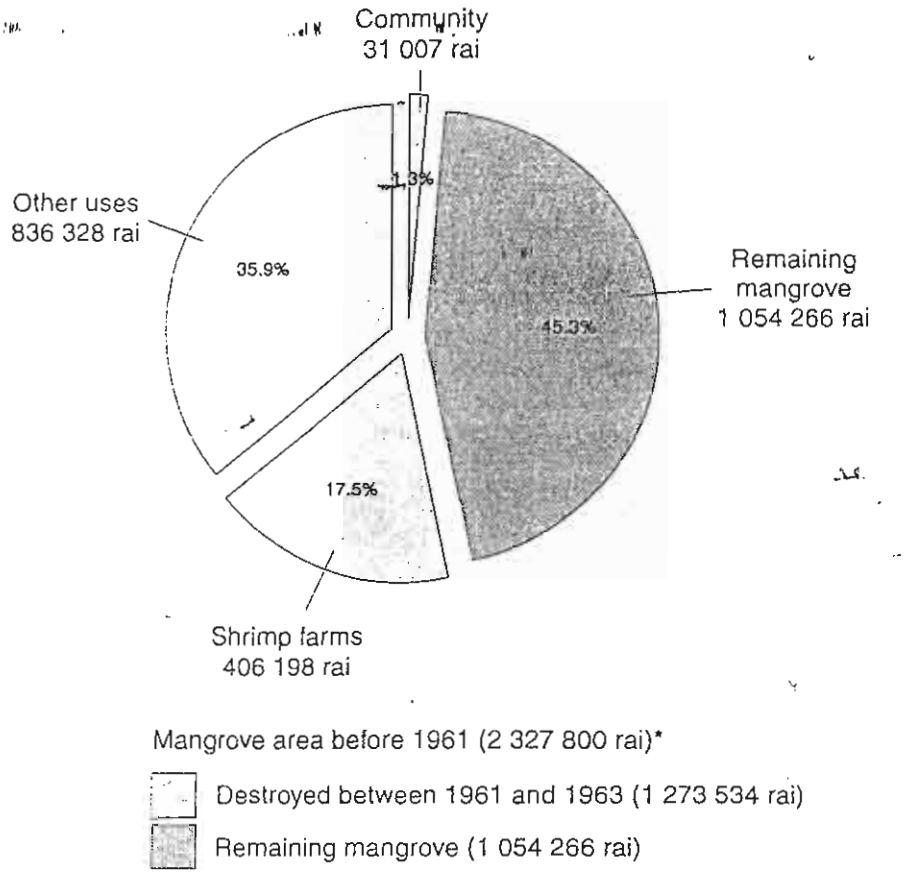
Shrimp culture systems

Shrimp culture in Thailand is conducted at three levels of intensity: extensive, semi-intensive, and intensive. The characteristics of these culture systems are summarized in Table 4.

Extensive systems

Extensive culture systems are characterized by low stocking densities and, therefore, low productivity. This system is characteristic of low income coastal communities. In addition to being a source of charcoal wood and fire wood, the mangrove is also a source of food and income for near-shore communities. Through their long association with the mangrove ecosystem, these communities know that the mangrove is a concentration ground for small aquatic animals such as juvenile fish and crustaceans. The extensive aquaculture system, therefore, is an evolved form of hunting-and-gathering of food for these communities.

Early shrimp culture in Thailand was mostly by extensive culture methods. Shrimp ponds were comparatively large, ranging from 5 to 10 ha each with a peripheral canal around the inner dike.



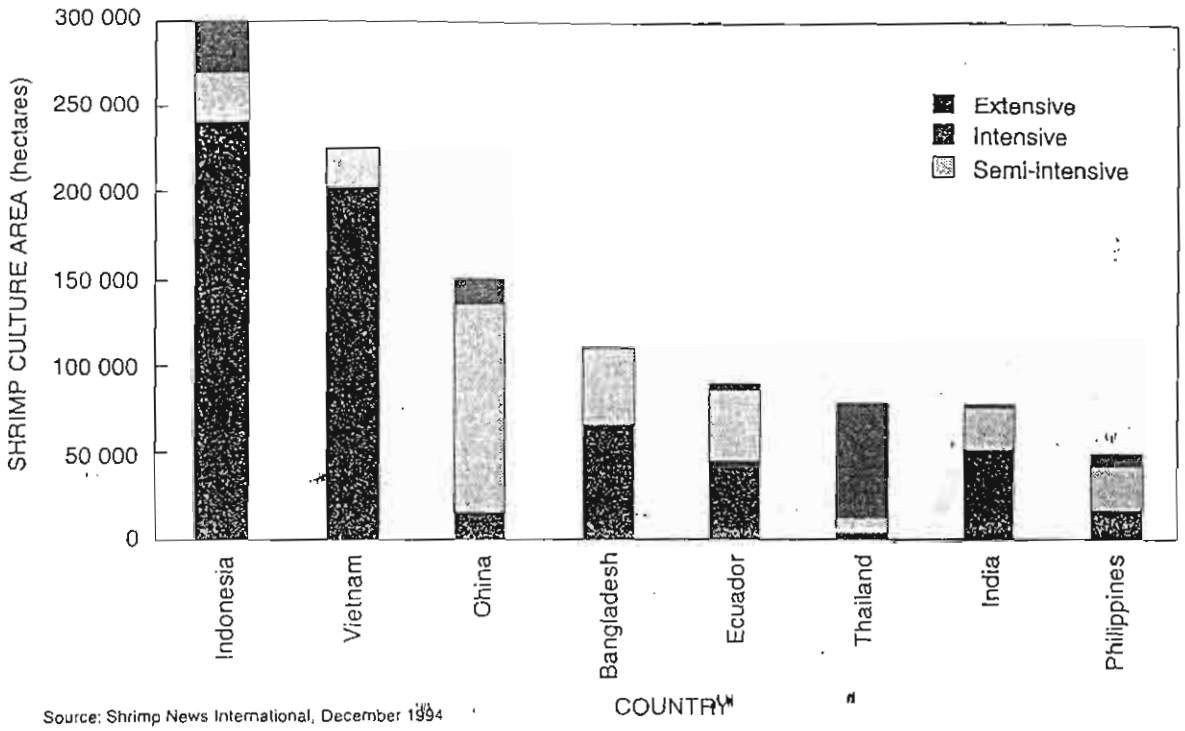
* 6.25 rai = 1 hectare
Sources: Royal Forestry Department, Department of Fisheries, Land Development Department, and National Research Council of Thailand.

Figure 1. Utilization of mangrove area in Thailand in 1993

Table 1. Shrimp production from ocean capture, pond culture and culture area in Thailand

Year	Production by capture (mt)	Production by culture (mt)	Total production (mt)	Shrimp culture area (rai)*
1981	122 706	10 729	133 435	171 619
1982	156 523	10 091	166 614	192 453
1983	127 584	11 550	139 134	222 107
1984	104 394	13 007	117 401	229 949
1985	91 632	15 840	107 472	254 805
1986	102 227	17 886	120 113	283 548
1987	128 100	23 566	151 666	279 812
1988	110 200	55 633	165 833	342 364
1989	110 800	93 495	204 295	444 785
1990	107 400	118 227	225 627	403 787
1991	129 100	162 070	291 170	470 826
1992	116 800	184 884	301 684	454 975
1993	100 000	225 514	325 514	449 292

Source: Royal Thai Department of Fisheries
* 6.25 rai = 1 hectare



Source: Shrimp News International, December 1994

Figure 2. Shrimp culture system and culture area of major producing countries,

Table 2. Destruction of mangrove area between 1961 and 1993

Year	Mangrove area (rai)*	Destroyed area (rai)*	Destroyed (%)	Mean rate destroyed (rai/yr)
Pre-1961	2 327 800			
		28 425	1.23	
1961	2 299 375			
		345 000	14.81	24 643
1975	1 945 375			
		158 700	6.82	39 675
1979	1 795 675			
		567 951	24.38	81 136
1986	1 227 724			
		99 230	4.27	33 076
1989	1 128 494			
		42 113	1.82	21 056
1991	1 086 381			
		32 115	1.39	16 057
1993	1 054 266			
Total/mean		1 273 534	54.72	38 910

Source: Royal Forestry Department
* 6.25 rai = 1 hectare

They are generally called "Wang Koong." Farmers may have used water elevators powered by wind mills for filling the ponds. Shrimp seed entrained by the influent water grew in the ponds for 2 to 3 months before the ponds were drained and the shrimp, usually of small size, were harvested. During the culture period, some fertilizer may have been added to the pond, but feed was not provided. Shrimp production by this extensive methods was rather low, with an average of only 150 kg/ha/year or less. These methods are still used by some Thai shrimp culturists.

Mangrove areas in estuaries have been prime candidates for such pond construction since they typically have gentle slopes, adequate tidal range, abundant wild shrimp seed, and land ownership vested with the government. Such land was usually easy to use and inexpensive.

The tidal pattern, range and wetted area at each stage are particularly important when siting and constructing extensive ponds. For prospective sites in Thailand, which have a maximum tidal range of -0.4 m mean sea level (MSL) to +2.0 m MSL; the most ideal elevation for the ponds is between elevations 0.0 m and 1.4 m MSL. Ponds built within this range can be filled and drained twice monthly since water can only enter the pond during ordinary high tides, and the ponds can also be drained completely

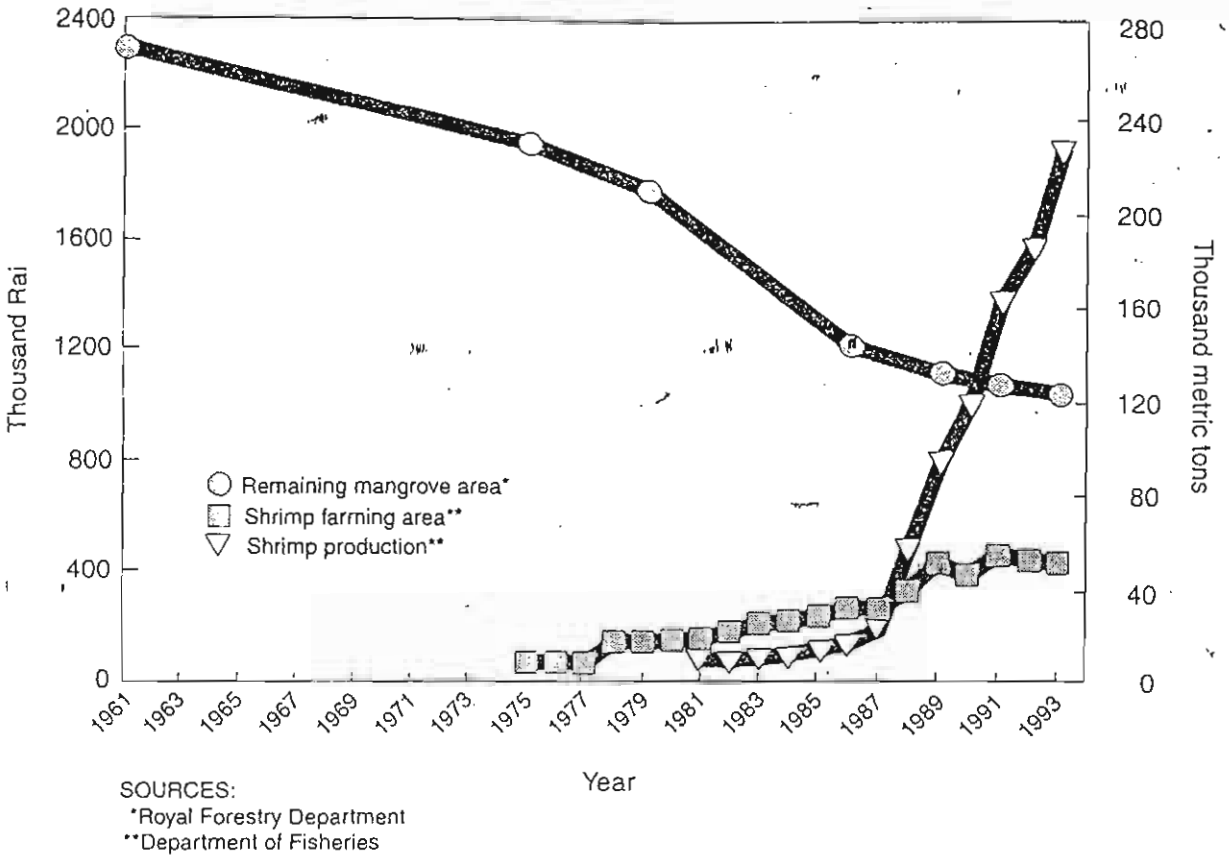


Figure 3. Mangove and shrimp farm area vs shrimp production

only during ordinary low tides. Ponds with bottoms above +1.8 m MSL will normally require mechanical pumping to fill, while those with bottoms below -0.4 m MSL will require pumping to fully drain. Neither situation is desirable if the objective is to rely solely on tidal action for water exchange. Using pumps to drain a pond is highly undesirable in almost all situations for the extensive culture system.

Semi-intensive systems

In the past, as shrimp demand rose and shrimp became a valuable commodity, extensive shrimp farmers started developing techniques in order to increase their yields. Developments towards a monoculture of shrimp included the active harvesting of juvenile shrimp from the wild for stocking into culture ponds.

In 1980, the semi-intensive system was introduced into Thailand through the Aquaculture Development Project, a soft-loan from the Asian Development Bank. Semi-intensive culture ponds may be created through intensification of existing extensive ponds. This may include such things as clearing and leveling the pond bottom, and digging canals in the pond bottom; converting from tidal water exchange to pumped water exchange; control stocking density; greater use of fertilizers; use of fish toxicants to control fish intruders; and provision of feed.[1]

Menasveta and Higuchi[2] described a plan of staged intensification for Thai extensive shrimp farms towards semi-intensive culture. This simulation was conducted as part of an Asian Development Bank planning study. While the original extensive farm consisted of a single 4.8 ha pond with its inlet and outlet on the same canal, the fully intensified case consisted of a sedimentation and shrimp postlarvae collection pond, four nursery ponds and three growout ponds with inlets and outlets on separate canals. Shrimp production was about 150 kg/ha/year with the traditional configuration, but was expected to be 600 to 1800 kg/ha/year or more after intensification due to enhanced use of space, improved intruder control, less down time for sediment removal, and better water quality management.

Intensive systems

About a decade ago, some shrimp culturists began developing advanced technologies for intensive culture systems. The Thai intensive culture system is similar to the Taiwanese method. With these new technologies, shrimp production rate increased to 6.0-10.0 mt/ha/crop, lasting 4 months per crop. The shrimp species used almost exclusively with this type of culture is *Penaeus monodon*, or black tiger shrimp. It should be noted that this type of culture system no longer requires the mangrove zone. Since this culture system requires fast water exchange per day, rapid draining is very important. In this regard, the pond bottom needs to be at a higher elevation, preferably +2.0 MSL. These areas are behind the mangrove zone.

Intensive farms are also liberated from harvesting wild-caught juveniles because large numbers of shrimp fry are

Table 3. Utilization of mangrove area in 1993

Region	Shrimp Farm		Community		Others*		Remaining		Total Area	
	Rai**	%	Rai**	%	Rai**	%	Rai**	%	Rai**	%
Eastern	162 399.00	6.97	15 535.75	0.67	83 298.50	3.58	81 548.00	3.51	342 781.25	14.73
Central	88 481.25	3.80	10 931.75	0.47	285 705.50	12.26	33 519.00	1.45	418 637.50	17.98
Southern (Gulf of Thailand	130 637.50	5.61	3 572.75	0.15	115 942.00	4.98	102 654.00	4.42	352 806.25	15.16
Southern (Andaman Sea)	24 680.75	1.08	966.75	0.06	351 382.50	15.09	836 545.00	35.90	1 213 575.00	52.13
Total	406 198.50	17.46	31 007.00	1.35	836 328.50	35.91	1 054 266.00	45.28	2 327 800.00	100

Source: Joint Working committee of Royal Forestry Department, Department of Fisheries, Land Development Department and National Research Council of Thailand.

* Agriculture, road development, ports, salt farms, mining etc.

** 6.25 rai = 1 hectare

Table 4. Characteristics of shrimp culture at three levels of intensity

Characteristics	Level of Intensity		
	Extensive	Semi-intensive	Intensive
Land elevation (m)	0 to +1.4 MSL*	0 to +1.4 MSL	> +2.0 MSL
Pond size (ha)	>5	1-2	1 or less
Aeration	Natural	Water exchange or mechanical	Continual mechanical and flushing
Stocking rate (PL/m^2/crop)	<5	5 to 15	20 or more
Feed	Natural (no supplement)	Natural + supplement	Formulated
Production level (kg/ha/yr)	100-300	600-1800	>6000

*MSL = Mean Sea Level

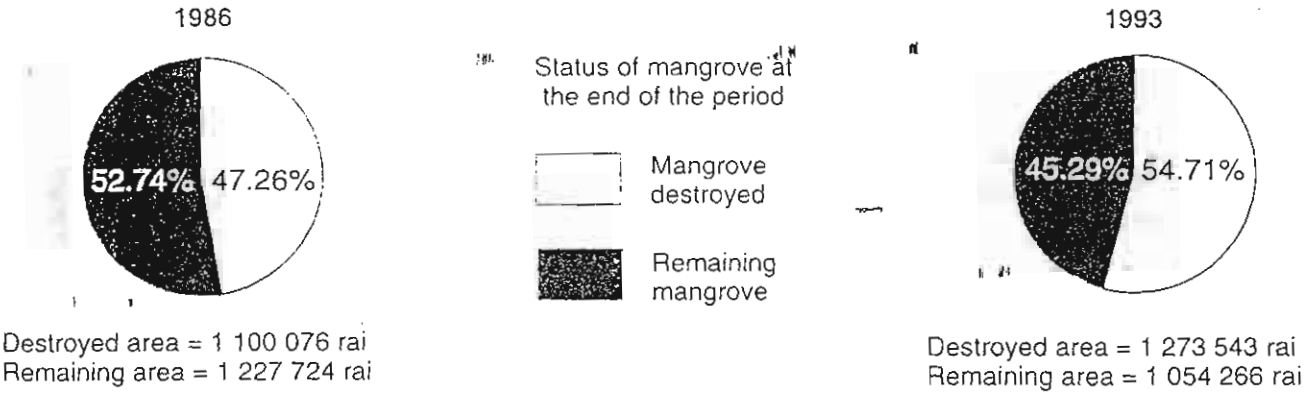
Table 5. Shrimp culture systems of major producers in 1994

Country	Production (mt)	Area (hectares)	Culture System (%)			Productivity (t/ha/yr)
			Extensive	Semi-intensive	Intensive	
THAILAND	250 000	80 000	5	10	85	3.13
ECUADOR	100 000	90 000	50	45	5	1.11
INDONESIA	100 000	300 000	80	10	10	0.33
INDIA	70 000	80 000	65	30	5	0.87
VIETNAM	50 000	225 000	90	10	0	0.22
BANGLADESH	35 000	110 000	60	40	0	0.32
CHINA	35 000	150 000	10	80	10	0.23
PHILIPPINES	30 000	50 000	35	50	15	0.60

Source: Shrimp New s International, December 1994

Table 6. Shrimp culture modernization* and mangrove destruction in Thailand.

Before modernization		After modernization
1961~1986		1987~1993
26 years	Time span	7 years
Extensive & semi-intensive	Shrimp culture system	Intensive
Rather primitive	Culture technology	More advanced
283 548 (1986)	Shrimp culture area (rai)	449 292 (1993)
17 886 (1986)	Annual shrimp production (mt)	225 514 (1993)
63 (1986)	Productivity (kg/rai/year)	502 (1993)
1 100 076	Destroyed mangrove area (rai)	173 458
42 311	Mangrove destruction rate (rai/year)	24 780
47.26	%Mangrove destroyed in period**	7.45



Destroyed area = 1 100 076 rai
Remaining area = 1 227 724 rai

Destroyed area = 1 273 543 rai
Remaining area = 1 054 266 rai

*Modernization of shrimp culture technology started in 1987
**Based on mangrove area of 2 327 800 rai in 1961 as 100%

produced from hatcheries. Furthermore, certain requirements of intensive culture systems make mangrove areas entirely incompatible. Because of the high shrimp densities and the use of supplemental feeding, there is large organic accumulation at the pond bottom after a shrimp crop which requires complete drying to facilitate sanitation and removal. Ponds found in the mangrove are unsuitable because they do not completely dry due to their low elevation. In addition, there is constant seepage from the pond due to mangrove roots which are impossible to remove. Finally, alkaline water conditions are required for optimal shrimp culture, but in mangrove areas the soil is high in organic content (i.e., humus) and therefore acidic and inappropriate for shrimp growth.

The Interaction

Historically, Thailand practiced extensive shrimp culture until the mid-1980s when intensive culture practices were introduced. Nearly all of the extensive farm operators subsequently converted to intensive culture systems. Consequently, intensive culture practices continued to improve, making Thailand the world's leader in shrimp culture technology.

At present, 85% of shrimp culture areas employ the intensive culture system, while the rest use extensive and semi-intensive methods. This corresponds to shrimp productivity of 3.13 mt/ha/yr, significantly higher than in -shrimp farming countries where extensive and semi-intensive culture system are mostly used (Table 5 and Figure 2). While using 80 000 ha, Thailand produced 250 000 mt in 1994. In contrast, to produce only 100 000 mt. Ecuador needed 90 000 ha while Indonesia required 300 000 ha. The ultimate difference in these productivities is the culture systems employed.

In considering the historical development and the physical requirement of the three shrimp culture systems together with the rate of mangrove destruction, it is clear that both extensive

and semi-intensive shrimp culture systems resulted in a higher rate of mangrove destruction (Figure 3). It should be noted that the intensive culture system was introduced in 1986. Since then, shrimp production increased sharply until the present, while mangrove destruction and the total shrimp farming area leveled off (Table 6 and Figure 3).

Any impact from shrimp farming upon mangrove destruction in Thailand is currently minimal and may be accounted for by unscrupulous fly-by-night farm operators out to capitalize on a new industry. The majority of shrimp farmers in Thailand are serious about their livelihood and have made sustainability their goal, and sustainability is incompatible with mangrove utilization.

In the near future, the European Union will impose a Generalized System of Preferences (GSP) for Thailand which will make shrimp farming in Thailand less competitive with other countries. This in turn would encourage more extensive-type culture operations in those countries. For this reason, more mangroves worldwide may be potentially destroyed !

The advent of intensive shrimp farming techniques in 1986 shifted land use away from the unsuitable low lying mangrove areas and toward higher ground. Coupled with technologies such as formulated feed, water quality management, genetics, disease control and modern hatchery techniques, shrimp culture has become more effrcient, capable of producing more shrimp from less culture area. Farmers have come to realize that intensive shrimp culture systems are sustainable systems and that utilization of mangrove areas is not. While shrimp farming was admittedly a factor in destroying mangroves, the distinction must be made that this destruction was the result of the unsophisticated extensive culture systems of ten years ago. Ever since intensive culture came into practice, circa 1986, mangrove destruction by the shrimp industry has been minimal.

Many of Thailand's competitors continue to utilize mangrove areas by virtue of their extensive culture systems. However, these are mostly poor farmers who depend on shrimp farming for their livelihood, and it is important not to take this away from them. As consumer demand for shrimp increases, so will attempts throughout the world to meet this demand. For a majority of these attempts, primitive culture systems — low in efficiency, unsustainable and unfriendly to mangroves— will be practised. If the current global trend continues, the world's mangroves will be seriously endangered. Therefore, the intensive shrimp culture system may be the only means of preventing mangroves from being destroyed by extensive shrimp farming. In Thailand's case, 85% of the shrimp farms practice intensive farming, utilizing a relatively small amount of mangrove land with great efficiency. This efficiency has benefited the mangrove ecosystem of Thailand by eliminating the need for mangrove regions. Intensive shrimp culture technology needs to be promoted by educating and discouraging new shrimp farmers from utilizing the mangroves. This technology should be disseminated globally for the purpose of conserving the remaining mangrove ecosystems around the world.

Even though 17% of the historic mangrove area has been utilized by shrimp farming activities in the past (Figure 1 and Table 3), the shrimp industry is confident that there will be no further destruction from its sector. Presently, Thai technology requires the use of non-mangrove areas for the culture of shrimp. Other shrimp producing countries should study Thailand, the world's largest and most efficient shrimp producer, and adopt the intensive culture technology in order to help preserve the world's mangroves.

Notes and References

1. Fast AW. 1992. Pages 369-381 *in* Marine Shrimp Culture: Principles and Practices (AW Fast, LJ Lester, eds.). Elsevier Science Publishers, Amsterdam.
2. Menasveta P, Higuchi M. 1983. Operation manual for aquaculture development projects in Thailand. Dept. of Marine Science, Chulalongkorn University, Thailand. 141 p. (mimeo).

3. This study is supported by the Thailand Research Fund (TRF) and the National Center for Genetic Engineering and Biotechnology (BIOTEC).

4. One hectare = 6.25 rai.

Piamsak Menasveta is Professor & Director Aquatic Resources Research Institute, Chulalongkorn University, Bangkok, Thailand •

ELSEVIER

Aquaculture 167 (1998) 273–281

Aquaculture

Optimal dietary levels of lecithin and cholesterol for black tiger prawn *Penaeus monodon* larvae and postlarvae

Chalee Paibulkichakul [a], Somkiat Piyatiratitivorakul [a,b,*], Prasat Kittakoop [c], Voranop Viyakarn [a], Arlo W. Fast [d], Piamsak Menasveta [a,b,c]

[a] *Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[b] *Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand*
[c] *Marine Biotechnology Research Unit, National Genetic Engineering and Biotechnology Center, Chulalongkorn University, Bangkok 10330, Thailand*
[d] *Hawaii Institute of Marine Biology, University of Hawaii, Kaneohe, HI 96744, USA*

Accepted 13 July 1998

Abstract

The effect of lecithin and cholesterol on growth and survival of larval and postlarval *Penaeus monodon* was evaluated using semi-purified diets containing four levels of lecithin (0.0, 0.5, 1.0 and 1.5%) and three levels of cholesterol (0.0, 0.5 and 1.0%). Three early stages (zoeal, mysid and postlarval) of *P. monodon* were fed the experimental diets. Growth and survival of shrimp fed diets containing 1.0 and 1.5% lecithin were not significantly different ($P > 0.05$) but these groups had significantly greater growth and survival than those fed 0.0 and 0.5% lecithin diets. Shrimp fed diets containing 1.0% cholesterol had significantly greater ($P < 0.05$) growth and survival than that of shrimp fed diets containing 0.0 and 0.5% cholesterol. There was no interaction between lecithin and cholesterol on growth and survival of *P. monodon*. During a low salinity stress test, PL-15 shrimp fed diets containing 1.0% cholesterol had significantly greater ($P < 0.05$) tolerance to low salinity exposure.

Keywords: Lecithin; Cholesterol; Larval feed; Salinity stress test; *Penaeus monodon*; Black tiger prawn; Marine shrimp

* Corresponding author. Tel.: +66 2 2188161 fax: +66 2 2544259; e-mail: psomkiat@chula.ac.th

0044-8486/98/$19.00
PII: S0044-8486(98)00327-5

1. Introduction

Currently, shrimp larviculture primarily depends on live food organisms such as micro-algae, rotifers, copepods and *Artemia* sp., all of which are often seasonally available. Furthermore, the quality of live feed is not consistent and declines after harvest and storage. A high quality artificial feed that can serve as a replacement for live feeds is needed. An essential step in the development of artificial feed for larval shrimp is defining nutrient requirements. Lecithin and cholesterol are two such nutrients. Phospholipids can be synthesized by shrimp, but the rate of synthesis is too slow to satisfy metabolic requirements of young crustaceans (D'Abramo et al., 1981; Kanazawa, 1983; Kanazawa et al., 1985). All crustaceans, including marine shrimp, are incapable of synthesizing steroid ring compounds (Akiyama et al., 1992). Lecithin plays important roles in lipid and carbohydrate metabolism in the liver of fish, and as an emulsifying agent in biological systems associated with transportation and absorption of fatty acids within the body. Lecithin is an essential component of biomembrane systems in all eukaryotic cells. Cholesterol is an essential precursor of bile acids, steroid hormones, molting hormones, vitamin D_3 and prostaglandins (Steffens, 1989; Tacon, 1990; Akiyama et al., 1992).

Appropriate phospholipid and cholesterol supplementation is known to promote growth and survival of many marine and freshwater crustaceans (Castell et al., 1975; D'Abramo et al., 1981, 1984, 1985; Kanazawa et al., 1985; Briggs et al., 1988; Chen and Jenn, 1991). Most studies of dietary lecithin and cholesterol requirements have focused on juvenile and larger shrimp rather than early life stages of marine shrimp. Therefore, our present study was designed to determine optimal dietary levels of lecithin and cholesterol for growth, survival and low salinity tolerance of postlarval *Penaeus monodon*.

2. Materials and methods

P. monodon eggs were obtained from the Andaman Sea broodstock and were hatched at the Aquaculture Laboratory, Department of Marine Science, Chulalongkorn University in Bangkok, Thailand. After hatching, shrimp larvae were cultured in a static water hatchery system as described by Wilkenfeld et al. (1983). This system had a 50% water exchange every two days. Salinity was maintained at 30‰, temperature at 28 ± 1°C, pH at 7.75 ± 0.25, dissolved oxygen at 6.5–7.7 mg/l, and ammonia concentration at < 0.5 mg/l. Twelve hours of dim light, and 12 h of darkness were provided. Two larval stages (zoeal and mysid) and postlarvae were used to evaluate the effects of dietary lecithin and cholesterol on growth and survival, while 15 day old postlarvae (PL-15) were used in the low salinity stress tests.

The experiment was 4×3 factorials in a completely randomized design with three replicates per dietary treatment combination. Diets included combinations of four levels of lecithin (0.0, 0.5, 1.0 and 1.5%) and three levels of cholesterol (0.0, 0.5 and 1.0%). Diets were semi-purified with mean protein and lipid levels of 50 and 8%, respectively. These diets were produced using methods modified from Kanazawa et al. (1985) and

Table 1
Composition of the test diets before addition of lecithin or cholesterol

Ingredients	Dry matter (%)
Casein	55.0
Dextrin	15.5
Semi-refined fish oil	8.0
Mineral mixture[a]	8.0
Vitamin mixture[b]	4.0
Lecithin[c]	0–1.5
Cholesterol[d]	0–1.0
Carrageenan	5.0
α-cellulose	2.0–4.5

[a]Mineral mixture 100 g contains: K_2HPO_4 2.0 g, $Ca_3(PO_4)_2$ 2.720 g, $MgSO_4.7H_2O$ 3.041g, $NaH_2PO_4.2H_2O$ 0.790 g.
[b]Vitamin mixture 100 g contains: p-aminobenzoic acid, 10.0 mg; biotin, 0.40 mg; inositol, 400.0 mg; nicotinic acid, 40.0 mg; Ca-pantothenate, 60.0 mg; pyridoxine-HCl, 12.0 mg; riboflavin, 8.0 mg; thiamin-HCl, 4.0 mg; menadione, 4.0 mg; α-tocopherol, 20.0 mg; cyanocobalamine, 0.08 mg; calciferol, 1.20 mg; folic acid, 0.80 mg; choline chloride, 120.0 mg; ascorbic acid, 20 mg; astaxanthin, 10 mg.
[c]Soy lecithin, feed grade.
[d]Ninety five percent cholesterol, laboratory grade, Sigma.

ingredient composition is presented in Table 1. All diets were ground and sieve sorted into three sizes; < 53 μ for the zoeal diet, 53–125 μ for the mysid diet, and > 125–500 μ for the postlarval diet. All diets were prepared at a temperature < 40°C and stored in dark containers at -20°C until used.

In preparation for the zoeal trials, stage VI nauplii were separated into 4-l, cylindrical rearing units at a density of 100 nauplii/l. The trial was terminated after larvae reached mysis stage I. For the mysid and postlarval trials, mysis stage I and PL-1 were prepared the same as in the zoeal trial, but densities were 30 and 10 individuals/l, respectively.

During each trial, shrimp were fed four times daily (0800, 1200, 1600 and 2000 h). Each morning (0900–1000 h) 10 larvae from each replicate of the zoeal and mysid trials were randomly sampled for growth determination and then returned. Growth index was calculated using the method of Kanazawa et al. (1985). The total length of postlarvae was determined 15 days after metamorphosis (at PL-15).

During the low salinity stress tests, ten PL-15 from each dietary feed group were placed in 500 ml of dilute seawater (2‰). Shrimp mortalities were observed every 5 min for 2 h. Data were analyzed using a probit analysis to determine median tolerance time for each treatment group.

3. Results

There was no significant ($P > 0.05$) interaction between dietary lecithin and cholesterol on growth and survival of larval and postlarval *P. monodon*. Therefore, the effects of lecithin and cholesterol on growth and survival are discussed separately.

Mean zoeal growth rate was not significantly different ($P > 0.05$) for the four levels of dietary lecithin (Fig. 1). For the mysid stage, the growth rate of shrimp fed the diet

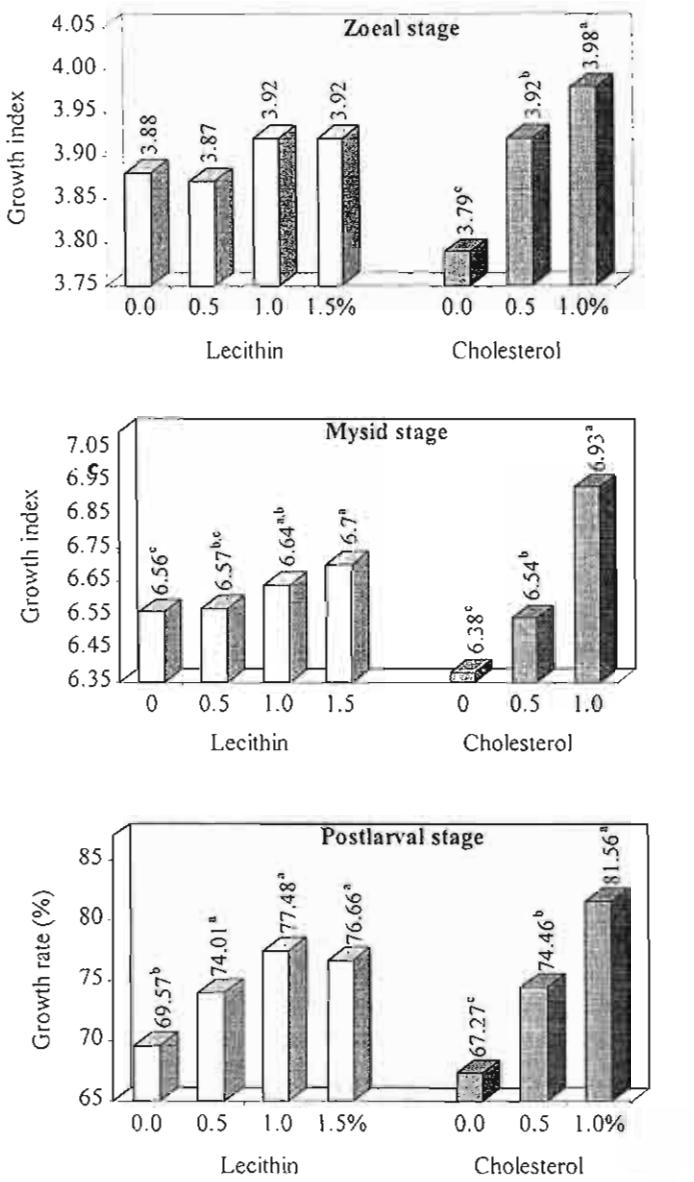


Fig. 1. Effects of lecithin and cholesterol on growth of *P. monodon* zoeal, mysid and postlarval stages. Mean values are shown at the top of each bar. Means with the same superscript are not significantly different.

containing 1.5% lecithin was significantly greater ($P < 0.05$) than that of shrimp fed diets containing 0.0 and 0.5% lecithin. Postlarval shrimp fed diets containing 0.5, 1.0 and 1.5% lecithin had a growth rate that was significantly greater ($P < 0.05$) than that of postlarvae fed a diet containing no lecithin.

The growth rates of larval and postlarval *P. monodon* fed diets containing each level of cholesterol were significantly different ($P < 0.05$) (Fig. 1). In all cases, the highest dietary cholesterol content (1.0%) always resulted in the greatest growth rate.

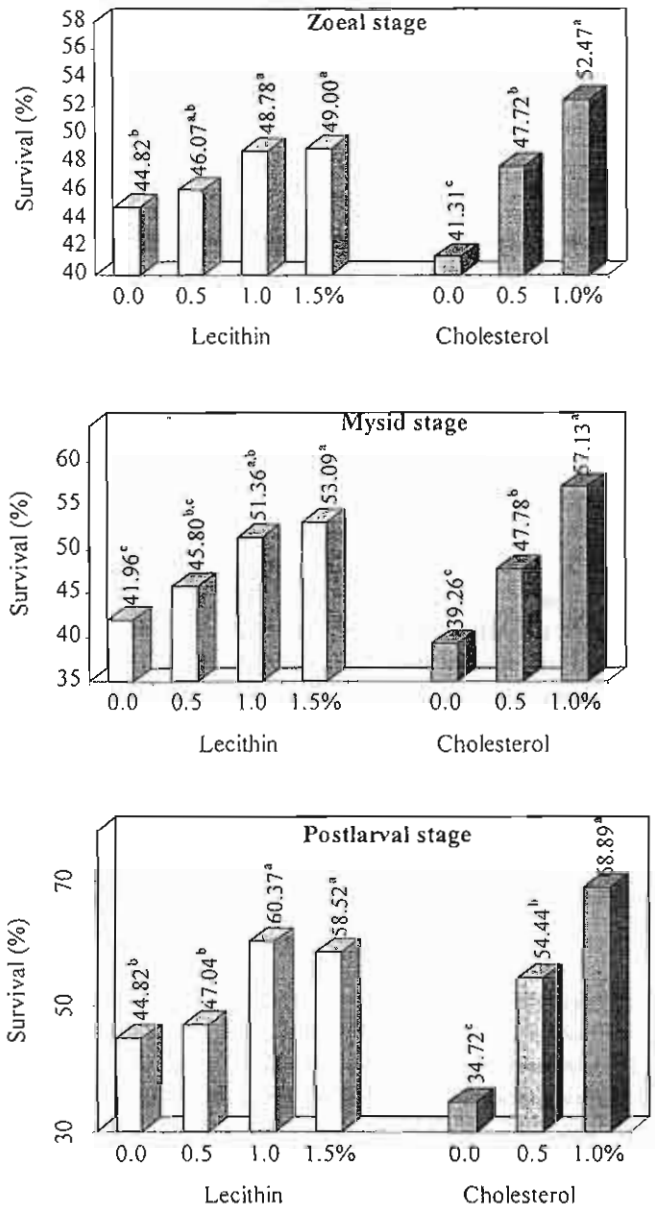


Fig. 2. Effects of lecithin and cholesterol on survival of *P. monodon* zoeal, mysid and postlarval stages. Mean values are shown at the top of each bar. Means with the same superscript are not significantly different.

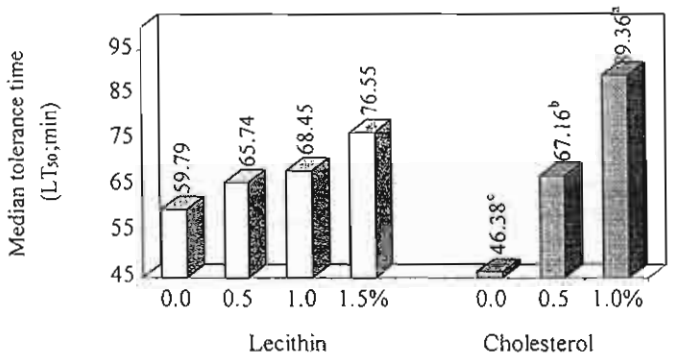


Fig. 3. Effects of lecithin and cholesterol on survival of *P. monodon* PL-15 exposed to low salinity (2‰) stress. Median tolerance times (TL_{50}) in minutes are shown at the top of each bar. Values with the same superscript are not significantly different.

The effects of lecithin and cholesterol on survival of larval and postlarval *P. monodon* followed essentially the same patterns as those for growth rate (Fig. 2). Each larval stage fed diets containing 1.0 and 1.5% lecithin had significantly greater ($P < 0.05$) survival than that for 0.0 and 0.5% lecithin containing diets. Shrimp fed diets containing 1.0% cholesterol had the greatest survival, significantly greater than that of shrimp fed a diet containing no cholesterol.

As found in survival and growth responses, there was no significant ($P > 0.05$) interaction between lecithin and cholesterol content of diets on PL-15 tolerance of *P. monodon* to low salinity stress tests (Fig. 3). Survival of *P. monodon* fed diets containing different levels of lecithin was not significantly different ($P > 0.05$). However, regression analysis indicates that shrimp fed diets containing higher levels of lecithin survived significantly ($r = 0.96$; $P < 0.05$) longer than shrimp fed low concentrations of lecithin when exposed to low salinity.

Survival of PL-15 shrimp fed diets containing 1.0% of cholesterol was significantly greater ($P < 0.05$) than that PL-15 shrimp fed other diets (Fig. 3). Regression analysis revealed a significant correlation ($r = 0.99$; $P < 0.05$) between dietary levels of cholesterol and median tolerance time of shrimp exposed to low salinity.

4. Discussion

Our findings of no interactions between dietary lecithin and cholesterol relative to survival, growth and salinity tolerance of larval and postlarval *P. monodon* agree with published results for other crustaceans, including; *Homarus americanus* (Kean et al., 1985), *Macrobrachium rosenbergii* (Briggs et al., 1988), *Penaeus penicillatus* (Chen and Jenn, 1991) and juvenile *P. monodon* (Chen, 1993). Although lecithin and cholesterol are essential nutrients, the nutritional functions of these compounds are independent.

The dietary phospholipid requirement for *P. penicillatus* was reported as 1.25% of the total diet (Chen and Jenn, 1991), and 0.4–2.0% of the total diet for other marine

shrimp (Akiyama et al., 1992). Reported dietary cholesterol requirements for *Penaeus japonicus* vary from 0.5% (Kanazawa et al., 1971) to 2.0% (Deshimaru and Kuroki, 1974), and 0.5% or higher for *P. penicillatus* (Chen and Jenn, 1991). In our present study, *P. monodon* larvae and postlarvae fed a diet containing either 1.0 or 1.5% of lecithin had significantly better growth and survival, whereas 1.0% cholesterol added to the diet significantly increased growth and survival of *P. monodon*. Greater tolerance to low salinity stress was also associated with higher survival and increased growth of shrimp.

Mammals synthesize phospholipids from 1,2-diglycerides with the aid of CDP-choline (Imai and Sakagami, 1966, cited by Kanazawa et al., 1985). Larvae of *P. japonicus*, however, are believed to have a limited ability to biosynthesize phospholipid because of the inefficacy of CDP-choline (Kanazawa et al., 1985). Our study indicates that *P. monodon* larvae are similar to *P. japonicus* larvae in their ability to biosynthesize phospholipids, based on our observed growth and survival of *P. monodon*. This indicates that dietary phospholipids such as phosphatidylcholine and phosphatidylinositol improved growth and survival of *P. monodon* larvae and PL by effects other than enhancement of dietary lipid emulsification in the digestive tract. Previous work has shown that the hemolymph of juveniles of *P. japonicus* contains high-density lipoproteins rich in phospholipids as the major lipoproteins (Teshima and Kanazawa, 1980a), and also that the high-density lipoproteins were involved in the transport of lipids through the hemolymph (Teshima and Kanazawa, 1980b). We, therefore, suspect that phosphatidylcholine and phosphatidylinositol containing high levels of n-3 and n-6 fatty acids serve as the lipid moieties of high-density lipoproteins in *P. japonicus* larvae.

Conklin et al. (1980) and D'Abramo et al. (1981) demonstrated a positive effect of phospholipid on survival of juvenile *Homarus americanus*. Kanazawa et al. (1985) demonstrated the relative efficacy of different sources of phospholipids in improving growth and survival of *P. japonicus*. The relative nutritional value of phospholipids on growth and survival of crustaceans seems to vary with the quality of esterified fatty acids, in addition to the kinds of compounds esterified with phosphoric acid at the C-3 position.

Studies of the effects of lecithin and cholesterol on juvenile, *Macrobrachium rosenbergii* (Briggs et al., 1988) and lobster *H. americanus* (Kean et al., 1985) have found that there is no interaction between lecithin and cholesterol in influencing growth or survival. They used semi-purified diets such as crab protein rather than purified diet such as casein as the primary protein source. D'Abramo et al. (1982) indicated that the absence of soy lecithin in purified diets fed to juvenile lobsters caused a significant decrease in the concentration of total cholesterol and phospholipid in the serum, possibly owing to insufficient levels of certain dietary amino acids when casein was the main source of protein.

Kanazawa et al. (1971) demonstrated that diets containing 0.5% cholesterol significantly improved the growth and survival of *P. japonicus*. Teshima et al. (1983) reported that diets containing 1.0% cholesterol improved growth and survival of *P. japonicus* larvae. At these cholesterol levels, larval shrimp grew as well as those fed natural diets. Sheen et al. (1994) reported that diets containing 0.2–0.8% cholesterol improved growth and survival of shrimp.

During our trials, we observed greater mortality of zoea during the first three days of this stage, compared with that for the last three days. This higher mortality was associated with incomplete ecdysis and feed residue in the digestive tract. This mortality syndrome has been previously observed and may be related to problems with sterol metabolism (Conklin et al., 1980; Bowser and Rosemark, 1981). Additional amounts of dietary cholesterol may overcome this problem. In our study survival, growth and salinity tolerance continued to increase even at the highest cholesterol levels used (1.0%) (Figs. 1–3); therefore optimal dietary cholesterol levels may well exceed 1.0%.

Acknowledgements

Financial support for this research was provided by the Thailand Research Fund (TRF).

References

Akiyama, D.M., Dominy, W.G., Lawrence, A.L., 1992. Penaeid shrimp nutrition. In: Fast, A.W., Lester, L.J. (Eds.), Marine Shrimp Culture: Principles and Practices. Elsevier Science Publishing, New York, pp. 535–568.

Bowser, P.R., Rosemark, P., 1981. Mortalities of cultured lobsters, *Homarus*, associated with a molt–death syndrome. Aquaculture 23, 11–18.

Briggs, M.R.P., Jauncey, K., Brown, J.H., 1988. The cholesterol and lecithin requirements of juvenile prawn *(Macrobrachium rosenbergii)* fed semi-purified diets. Aquaculture 70, 121–129.

Castell, J.D., Mason, E.C., Covey, J.F., 1975. Cholesterol requirements of juvenile American lobsters *(Homarus americanus)*. J. Fish. Res. Board Can. 38, 1431–1435.

Chen, H.Y., 1993. Requirements of marine shrimp, *Penaeus monodon*, juvenile for phosphatidylcholine and cholesterol. Aquaculture 109, 165–176.

Chen, H.Y., Jenn, J.S., 1991. Combined effects of dietary phosphatidylcholine and cholesterol on the growth, survival and body lipid composition of marine shrimp, *Penaeus penicilliatus*. Aquaculture 96, 167–178.

Conklin, D.E., D'Abramo, L.R., Bordner, C.E., Baum, N.A., 1980. A successful purified diet for the culture of juvenile lobsters: the effect of lecithin. Aquaculture 21, 243–249.

D'Abramo, L.R., Bordner, C.E., Conklin, D.E., Baum, N.A., 1981. Essentiality of dietary phosphatidylcholine for the survival of juvenile lobsters. J. Nutr. 111, 425–431.

D'Abramo, L.R., Bordner, C.E., Conklin, D.E., 1982. Relationship between dietary phosphatidylcholine and serum cholesterol in the lobster *Homarus* sp. Mar. Biol. 67, 231–235.

D'Abramo, L.R., Bordner, C.E., Conklin, D.E., Baum, N.A., 1984. Sterol requirement of juvenile lobsters, *Homarus* sp. Aquaculture 42, 13–25.

D'Abramo, L.R., Wright, J.S., Wright, K.H., Bordner, C.E., Conklin, D.E., 1985. Sterol requirement of cultured juvenile crayfish, *Pacifastacus leniusculus*. Aquaculture 49, 245–255.

Deshimaru, O., Kuroki, K., 1974. Studies on a purified diet for prawn-II, optimum contents of cholesterol and glycosamine in the diet. Bull. Jpn. Soc. Sci. Fish. 40, 421–424.

Imai, Y., Sakagami, T., 1966. Metabolism of compound lipids. In: Biochemistry of lipids, Tokyo: Asakura-shoten, Cited by Kanazawa, A., Teshima, S., Sakamoto, M., 1985. Effects of dietary lipids, fatty acids, and phospholipids on growth and survival of prawn *(Penaeus japonicus)* larvae. Aquaculture Vol. 50, pp. 39–49.

Kanazawa, A., 1983. Effects of dietary phospholipids on growth of the larval red sea bream and knife jaw. Mem. Fac. Fish. Kagoshima Univ. 32, 109–114.

Kanazawa, A., Tanaka, N., Teshima, S., Kashiwada, K., 1971. Nutritional requirement of prawn-II, requirement of sterols. Bull. Jpn. Soc. Sci. Fish. 37, 211–215.

Kanazawa, A., Teshima, S., Sakamoto, M., 1985. Effects of dietary lipids, fatty acids, and phospholipids on growth and survival of prawn *(Penaeus japonicus)* larvae. Aquaculture 50, 39–49.

Kean, J.C., Castell, J.D., Boghen, A.G., D'Abramo, L.R., Conklin, D.E., 1985. A re-evaluation of the lecithin and cholesterol requirements of juvenile lobster *(Homarus americanus)* using crab protein-based diets. Aquaculture 47, 1431–1449.

Sheen, S.S., Liu, P.C., Chen, S.N., Chen, J.C., 1994. Cholesterol requirement of juvenile tiger shrimp (*Penaeus monodon*). Aquaculture 125, 131–137.

Steffens, W., 1989. Principles of Fish Nutrition. Ellis Horwood, West Sussex.

Tacon, A.G.J., 1990. Standard Methods for the Nutrition and Feeding of Farmed Fish and Shrimp. Vol. 1: The Essential Nutrients. Argent Laboratories Press, Washington.

Teshima, S., Kanazawa, A., 1980a. Lipid constituents of serum lipoproteins in the prawn. Bull. Jpn. Soc. Sci. Fish. 46, 57–62.

Teshima, S., Kanazawa, A., 1980b. Transport of dietary lipids and role of serum lipoproteins in the prawn. Bull. Jpn. Soc. Sci. Fish. 46, 51–55.

Teshima et al., 1983.

Wilkenfeld, J.W., Lawrence, A.L., Kuban, F.D., 1983. Rearing penaeid shrimp in a small scale system for experimental purposes. In: Rogers, G.L., Day, R., Lim, A. (Eds.), Proceedings of the First International Conference on Warm Water Aquaculture Crustacea. Brigham Young University Hawaii Campus, Office of Continuing Education, Laie, HI, USA, pp. 72–81.

Optimal lipid:carbohydrate and protein:energy ratios in semi-purified diets for juvenile black tiger shrimp *Penaeus monodon*

Benjamas Chuntapa[1], Somkiat Piyatiratitivorakul[1,2], Charoen Nitithamyong[1], Voranop Viyakarn[1] & Piamsak Menasveta[1,2]

[1]Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[2]Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand

Correspondence: Somkiat Piyatiratitivorakul, Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand, e-mail address psomkiat@chula.ac.th, Fax 66-2-2544259

Abstract

This study was aimed at determining the optimal lipid:carbohydrate and protein:energy ratios for growth and survival of juvenile *Penaeus monodon*. Two experiments were performed using completely randomized designs in semi-closed recirculating water systems. Juveniles of 0.4-0.8 g in weight and 4.0-5.5 cm in length stocked at a density of 80 individuals/m^2 were fed semi-purified diets. The first experiment determined optimal lipid:carbohydrate using isonitrogenous (35%) and isocaloric (330 kcal/100g) diets with five lipid:carbohydrate ratios; 4:39, 7:32, 9:25, 14:18 and 16:12 (% W/W): The lipid:carbohydrate ratio of 7:32 gave the highest growth rate ($P<0.05$), while survival rates of shrimp in all other diet groups were similar but less. Thus optimal lipid to carbohydrate ratio for the juvenile black tiger shrimp was approximately 1:4.6. In the second experiment, optimal protein:energy (*P:E*) ratio was studied using 5 protein levels (25, 30, 35, 40 and 45%) with a fixed lipid:carbohydrate ratio of 1:4.6. Nine diets containing energy content (203-459 kcal/100g) with protein:energy ratio (63-171 mg protein/kcal) were formulated. Shrimp fed the diet containing 33-44% protein and energy content of 223-371 kcal/100g had a significantly higher growth rate than the other diets ($P<0.05$). A regression analysis indicated that an optimal *P:E* ratio for the optimal growth and survival of juvenile black tiger shrimp was 150 and 146 mg protein/kcal, respectively, which diet containing 33-44% protein and optimal energy was 263-331 kcal/100g.

Introduction

Because of the black tiger shrimp's (*Penaeus monodon* Fabricius) high growth rate, adaptability to various culture systems (Alava & Lim 1983), high market value, and excellent response to compounded feed (Lee 1971; Forster 1972; AQUACOP 1977), this species become very popular for commercial farming in Asia. This has led to the development of more intensive culture systems, which in-turn created the need for more nutritionally complete and well balanced prepared feeds. Currently, feed costs in intensive shrimp culture are 50-60% of total production costs; protein is the single most expensive component in shrimp feed.

Lower feed costs can be achieved by optimizing the rations between expensive protein and lower cost energy sources in feed. If protein is in excess over energy, protein will be used for metabolic energy maintenance rather than growth, thus reducing growth (Phillips 1972). If the energy component of feed is in excess relative to protein, growth will also be reduced due to decreased feed consumption consistent with the protein limitation (Maynard & Loosli 1969; Page & Andrews 1973; Takeda *et al.* 1975).

Protein and energy relationships in shrimp nutrition are not well documented (Sedgwick 1979; Cuzon & Guillaume 1997). AQUACOP (1977) estimated that a total dietary energy content of 330 kcal/100g was required for optimal *P. monodon* growth. In their review, Cuzon & Guillaume (1997) found that energy levels in crustacean diets generally ranged from 310 to 410 kcal/100 g. Both Bautista (1986) and Shiau & Peng (1991) concluded that a protein:energy ratio of 125 mg protein/kcal is optimal for *P. monodon* growth. Since protein and energy relationships in shrimp feed are so poorly understood, but important, these need better definition.

In addition to the relationship between feed protein and energy, the source of energy is also very important. As lipids and carbohydrates are the two energy sources, we first established optimal ratios of lipid:carbohydrate for *P. monodon* juveniles, followed by a determination of an optimal ratio between protein:energy, and lipid:carbohydrate.

Materials and methods

Two experiments were conducted using a completely randomized design with three replications per treatment. Semi-closed recirculating water systems were used in both experiments with 50% water exchange every three days. Prior to each experiment, shrimp were acclimated in the systems for two weeks. Experiments I and II lasted 30 and 45 days respectively.

Diet formulations in both experiments were modified from those of Kanazawa *et al.* (1977) and Shiau & Chou (1991), and are shown in Tables 1 and 2 respectively. Diets were pelletized with 1-2 mm diameters, while proximate composition was determined according to AOAC (1984). Gross energy values of diets were determined by ballistic bomb calorimeter. Energy levels were adjusted by varying ratios of lipid, carbohydrate and protein in diets. Standard physiological values used for calculating energy levels were; 4 kcal/g protein, 4 kcal/g carbohydrate, and 9 kcal/g lipid (Halver 1976). In both experiments, shrimp were fed four times daily (0800, 1200, 1600 and 2000 hr) at 5% of body weight in glass aquaria (0.3x0.6x0.3 m) at 15 shrimp/aquarium (80 shrimp/m^2). All diets were conducted with 3 replicates. Water quality was monitored weekly during both experiments for; temperature, salinity, pH, and dissolved oxygen. The photoperiod was 12:12 hr light:dark cycle.

In experiment I, five iso-nitrogenous (35% protein; Colvin 1976; New 1976; Colvin & Brand 1977) and iso-caloric (330 kcal/100g; AQUACOP 1977; Bautista 1986) diets were formulated with five lipid to carbohydrate ratios by % w/w (4:39, 7:32, 9:25, 14:18 and 16:12; Sick & Andrews 1973; Deshimaru & Kuroki 1974; Tacon 1990; Akiyama *et al.* 1992; Cuzon *et al.* 1994; Table 1). The effects of these diets on shrimp survival and growth were evaluated. Two hundred and twenty five juvenile *P. monodon* with initial sizes of 0.4-0.5 g and 4-5 cm were used in this experiment.

In experiment II, nine diets with constant lipid to carbohydrate ratio (1:4.6), with five protein levels (25, 30, 35, 40 and 45%), and with nine energy levels (203-459 kcal/100g), and with corresponding protein:energy (*P:E*) ratios (63-171 mg

protein/kcal) were formulated to determine optimal protein:energy ratio for juvenile shrimp (Table 2). Four hundred and five juvenile *P. monodon* with initial size of 0.4-0.8 and 4.0-5.5 cm were used to evaluate effects of diets on shrimp survival and growth.

Shrimp survival and weight in each tank were recorded every 15 days in both experiments. Shrimp growth and survival were evaluated using ANOVA and Duncan's New Multiple Range Test (Ott 1977). Optimal *P:E* ratio for juvenile shrimp weight gained and survival was evaluated by regression analysis in experiment II.

Results

Water quality in both experiments was quite similar in all experimental units, and within acceptable ranges for aquatic life with; water temperature 26.5 to 28.5 °C, salinity 23 to 25 ppt, pH 7.5 to 8.0, and dissolved oxygen 6.4 to 7.8 mg/l.

Experiment I: Optimal lipid and carbohydrate level for weight gained and survival.

There was a significant difference ($P<0.05$) in growth rate of juvenile shrimp with different ratios of lipid:carbohydrate, with iso-nitrogenous (35% protein) and iso-caloric (330 kcal/100g) prepared diets. The greatest weight gained occurred in shrimp fed diet with a 7:32 (% by weight) lipid:carbohydrate ratio (Fig. 1). There was no significant growth difference in shrimp fed other diets. Optimal shrimp growth therefore occurred with a lipid:carbohydrate ratio of 1:4.6, and when 39% of the diet consisted of lipid and carbohydrate (i.e. 7% lipid, 32% carbohydrate). Mean survival of shrimp ranged from 78% to 84% (Fig. 2) with no significant differences ($P>0.05$).

Experiment II: Optimal protein energy ratio for weight gained and survival.

Mean growth rates of shrimp fed diets containing 25-45% protein with 203-459 kcal/100g energy levels were non-uniform and could be separated into two groups. First, shrimp fed diets < 33% protein with energy contents of 399, 291 and 203 kcal/100g diet had reduced growth rates. Other shrimp fed diets containing greater protein (33-44%) and energy content (223, 263, 331, 371, 439, and 459 kcal/100g) had greater growth rates. Shrimp fed diets containing 23.5% and 26.1% protein with 291 and 399 kcal/100g energy, and 29.1% protein with 203 kcal/100g had growth rates which were not significantly different ($P>0.05$). Shrimp fed a diet containing 34.1% protein and 223 kcal/100g showed a growth rate similar to shrimp fed a diet containing 34.0% protein and 331 kcal/100g, but for shrimp fed a diet containing 33.1% protein with energy increased to 439 kcal/100g the growth rate tended to decrease. Shrimp fed diet containing 36.1% protein and 459 kcal/100g showed a growth rate similar to shrimp fed a diet containing 33-34% protein at all energy levels. Shrimp fed diet containing 44.0% protein and 263-371 kcal/100g exhibited a growth rate similar to shrimp fed the diet containing protein in range of 34.0-36.1%. Thus the diets containing 35-45% protein, all supported similar growth in the shrimp. The optimal protein:energy ratio for growth was determined for shrimp fed 33-44% protein diets by regression analysis. The following equation provided best fit (Fig. 3).

$$Y_W = -9.054 + 1.436X - 0.005X^2$$
$$(r^2=0.73)$$

Where: Y_W = growth rate (% weight gained), and X = *P:E* ratio in diet (mg protein/kcal). The equation indicated that the optimal *P:E* ratio of diet containing 33-44% protein for shrimp growth was 150 mg protein/kcal.

Using survival rate and protein:energy ratio data, the regression analysis (Fig. 4) indicated the best fit for the relationship between survival rate and *P:E* ratio as,

$$Y_S = 10.016 + 0.993X - 0.003X^2$$
$$(r^2=0.53)$$

Where: Y_S = survival rate (%), and X = *P:E* ratio in diet (mg protein/kcal). The equation determined that the optimal *P:E* ratio for the best survival of shrimp juvenile was 146 mg protein/kcal

Discussion

Experiment I. In our lipid:carbohydrate trials we used corn starch, a relatively pure carbohydrate as available data indicated that shrimp are best able to use starch carbohydrates (polysaccharides) rather than dextrin or glucose (Andrews *et al.* 1972). Forster & Gabbott (1971) found that corn starch permitted a better performance than did many other carbohydrate sources. Our results demonstrated that shrimp fed a diet containing lipid:carbohydrate ratio of 1:4.6 (or 7:32% by weight) gave the best growth rate. Andrews *et al.* (1972) and Akiyama, Dominy & Lawrence (1992) reported that marine shrimp required optimal dietary lipids in the range of 6-7.5%, while Sick & Andrews (1973) and Cuzon, Guillaume & Cahu (1994) reported optimal dietary carbohydrate in the range of 20-40% for most aquatic animals.

In our studies, shrimp fed diet containing high lipid and low carbohydrate (9:25%, 14:18% and 16:12% by weight) had low growth rates compared with other diets. Excess dietary lipids are known to adversely affect normal metabolic functions, decrease growth or/and survival of shrimp. Shrimp consuming excess lipids reduce their feed consumption because of excessive food energy. Excess dietary lipids also affect nutritional balance. Shrimp fed diet containing low lipid and high carbohydrate (4:39% by weight) had a lower growth rate than shrimp fed a diet containing more lipid and less carbohydrate, 7:32% by weight. Excess dietary carbohydrates are known to cause degenerative change of the digestive gland resulting in poor growth (Pascual *et al.* 1983). Mean survival of shrimp with no significant differences in this study, indicated that all diets had adequate energy for life maintenance. AQUACOP (1977) estimated that a total dietary energy content of 330 kcal/100g was required for optimal growth of *P. monodon.*

Experiment II. Our results demonstrated that shrimp fed diets with protein ranging from 23.5-29.1% at all energy levels showed a lower growth rate compared with shrimp fed higher protein levels; protein levels below 30% appear insufficient for optimal growth. Bautista (1986) reported that *P. monodon* (0.60 to 0.80 g) fed diets with 30% protein and energy ranging from 205-335 kcal/100g had lower growth rates compared with shrimp fed diets containing 35-45% protein at all energy levels.

Shrimp fed 36.1% protein and 459 kcal/100g diet showed a similar growth rate compared with shrimp receiving 33.1-34.1% and 44.0% protein in all energy levels. Bautista (1986) observed that shrimp growth rates declined when energy level reached 400 kcal/100g. In our case, shrimp fed diets with protein in the range of 33-44% exhibited better growth rates when the dietary energy was close to 263-331 kcal/100g.

Our optimal protein:energy ratios (146-150 mg protein/kcal) differ from those reported in earlier studies. Bautista (1986) found that *P. monodon* fed a 40% protein diet required a *P:E* ratio of 121 mg protein/kcal for optimal growth. Sedgwick (1979) found that *P. merguiensis* fed diets with a 34-42% protein range needed a *P:E* ratio of 72.7 mg protein/kcal for optimal growth. In their review, Cuzon & Guillaume (1997) found *P:E* requirements for *P. monodon* growth ranged from 83-209 mg protein/kcal. Lee & Lawrence (1997) demonstrated that P:E ratios in crustaceans ranged from 142 to 167 mg protein/kcal. These differences may be explained in the past by nutrient quality difference in protein, lipid and carbohydrate (Cuzon & Guillaume 1997), shrimp species, size, and feeding habit (Deshimaru & Yone 1978; Teshima & Kanazawa 1984), protein quality and quantity (Bautista 1986; Alava & Pascual 1987; Shiau & Chou 1991), salinity (Hajra *et al.* 1988) and perhaps other differences in feed ingredients.

Acknowledgments

This research was funded by the Thailand Research Fund (TRF) and in part of the Graduate School of Chulalongkorn University. The authors thank Dr. Arlo W. Fast for English and technical editorial works.

References

Akiyama D.M., Dominy W.G. & Lawrence A.L. (1992) Penaeid shrimp nutrition. In: *Marine shrimp culture: Principles and practices* (ed. by Fast A.W. & Lester L.J.), pp. 535-568. Elsevier, Amsterdam, The Netherlands.

Alava V.R. & Lim C. (1983) The quantitative dietary protein requirements of *Penaeus monodon* juveniles in a controlled environment. *Aquaculture* **30**, 53-61.

Alava V.R. & Pascual F.P. (1987) Carbohydrate requirements of *Penaeus monodon* (Fabricius) juveniles. *Aquaculture* **61**, 211-217.

Andrews J.W., Sick L.V. & Baptist G.J. (1972) The influence of dietary protein and energy level on growth and survival of penaeid shrimp. *Aquaculture* **1**, 341-347.

AOAC. (1984) *Official Methods of Analysis of the Association of Official Analytical Chemists*, 14th edn. AOAC, Arlington, VA, USA.

AQUACOP. (1977) Reproduction in captivity and growth of *Penaeus monodon* Fabricus in Polynesia. In: *Proceeding of the 8th Annual Workshop of the World Mariculture Society* (ed. By Avault J.W.), pp. 927-945. Louisiana State University Press, Baton Rouge, LA, USA.

Bautista M.N. (1986) The response of *Penaeus monodon* juveniles to varying protein/energy ratios in test diets. *Aquaculture* **53**, 229-242.

Colvin P.M. (1976) Nutritional studies on penaeid prawns: protein requirement in compounded dits for juvenile *Penaeus indicus* (Milne Edwards). *Aquaculture* **7**, 315-326.

Colvin L.B. & Brand C.W. (1977) The protein requirement of penaeid shrimp at various life-cycle stages in controlled environment systems. *Proceedings of World Mariculture Society* **8**, 821-840.

Cuzon G. & Guillaume J. (1997) Energy and protein: energy ratio. In: *Crustacean nutrition, Advances in World Aquaculture, Volume 6* (ed. by D'Abramo L.R., Conklin D.E. & Akiyama D.M.), pp. 51-70. The World Aquaculture Society, Baton Rouge, Louisiana, USA.

Cuzon G., Guillaume J. & Cahu C. (1994) Review: composition, preparation and utilization of feeds for Crustacea. *Aquaculture* **124**, 253-267.

Deshimaru O. & Kuroki K. (1974) Studies on purified diet for prawn. I. Basal composition of diet. *Bulletin of the Japanese Society of Scientific Fisheries* **40**, 413-419.

Deshimaru O. & Yone Y. (1978) Optimum level of dietary protein for prawn. *Bulletin of the Japanese Society of Scientific Fisheries* **44**, 1395-1397.

Forster J.R.M. (1972) Studies on compounded diets for prawn. *Proceeding of World Mariculture Society* **3**, 389-402

Forster J.R.M. & Gabbott P.A. (1971) The assimilation of nutrients from compounded diets by prawns *Palaemon serratus* and *Pandalus platyceros*. *Journal of the Marine Biological Association of the United Kingdom* **51**, 943-961.

Hajra A., Ghosh A. & Mandal S.K. (1988) Biochemical studies on the determination of optimum dietary protein to energy ratio for tiger prawn *Penaeus monodon* Fabricius juveniles. *Aquaculture* **71**, 71-79.

Halver J.E. (1976) *The nutritional requirements of cultivated warmwater and coldwater fish species.* Paper No. 31, 9 pp. 26 May-2 June, 1976. FAO Technical Conference on Aquaculture, Kyoto, Japan.

Kanazawa A., Teshima S. & Tokiwa S. (1977) Nutritional requirements of prawn. VII. Effect of dietary lipids on growth. *Bulletin of the Japanese Society of Scientific Fisheries* **43**, 849-856.

Lee D.L. (1971) Studies on the protein utilization related to growth in *Penaeus monodon. Aquaculture* **1**, 1-13.

Lee P.G. & Lawrence A.L. (1997) Feed digestibility in crustaceans. In: *Book of abstracts from World Aquaculture '97.* (ed. by World Aquacuture Society) p. 278. 19-23 February 1997. Washington State Convention Center, Seattle, Washington, U.S.A.

Maynard L.A. & Loosli J.K. (1969) *Animal nutrition.* McGraw-Hill Book company, New Delhi, India. 613 pp.

New M.B. (1976) A review of dietary study with shrimp and prawn. *Aquaculture* **9**, 101-144.

Page J.W. & Andrews J.W. (1973) Interactions of dietary levels of protein and energy on channel catfish (*Ictalurus punctatus*). *Journal of Nutrition* **103**, 1139-1346.

Pascual F.P., Coloso R.M. & Tamse C.T. (1983) Survival and some histological changes in *Penaeus monodon* Fabricius juveniles fed various carbohydrates. *Aquaculture* 31, 169-180.

Phillips A.M.Jr. (1972) Caloric and energy requirements. In: *Fish nutrition* (ed. by Halver J.E.), pp. 1-28. Academic Press, New York, NY, USA.

Ott L. (1977) *An introduction to statistical methods and data analysis.* PWS Publishers, Boston, USA. 730 pp.

Sedgwick R.W. (1979) Influence of dietary protein and energy on growth, food consumption and food conversion efficiency in *Penaeus merguiensis* de Man. *Aquaculture* **16**, 7-30.

Shiau S.Y. & Chou B.S. (1991) Effects of dietary protein and energy on growth performance of tiger shrimp *Penaeus monodon* reared in seawater. *Nippon Suisan Gakkaishi* **57**, 2271-2276.

Shiau S.Y. & Peng C.Y. (1991) Utilization of different carbohydrates at different dietary protein levels in gross prawn *Penaeus monodon* reared in seawater. *Aquaculture* **101**, 240-250.

Sick L.V. & Andrews J.W. (1973) The effect of selected dietary lipids, carbohydrates and proteins on the growth, survival and body composition of *Penaeus duorarum*. *Proceeding of World Mariculture Society* **4**, 263-276.

Tacon A.G.J. (1990) *Standard method for the nutrition and feeding of farmed fish and shrimp: The essential nutrients. Vol.1.* Argent Laboratory Press, Washington D.C., USA.

Takeda M., Shimeno S, Hosokawa H., Kajiyama H. & Kaisyo T. (1975) The effect of dietary caloric to protein ratio on the growth, feed conversion and body composition of young yellowtail. *Bulletin of the Japanese Society of Scientific Fisheries* **41**, 443-447.

Teshima S. & Kanazawa A. (1984) Effects of protein, lipid and carbohydrate levels in purified diets on growth and survival rates of the prawn larvae. *Bulletin of the Japanese Society of Scientific Fisheries* **50**, 1709-1715.

Table 1 Prepared diets compositions in experiment I. (values are in % dry weight).

L:C Ratio	1:9.8	1:4.6	1:2.8	1:1.3	1:0.8
L:C (% weight)	4:39	7:32	9:25	14:18	16:12
			Percent dry weight		
Ingredients					
Casein	35.0	35.0	35.0	35.0	35.0
corn starch	38.5	31.8	25.0	18.3	11.6
Semi-refined fish oil	4.0	7.0	10.0	13.0	16.0
Cellulose	16.2	19.9	23.7	27.4	31.1
Others*	6.3	6.3	6.3	6.3	6.3
Nutrients					
Moisture	2.5	2.5	2.4	3.9	3.3
Crude protein	34.0	34.1	35.2	35.3	34.6
Crude lipid	3.5	7.1	9.2	13.7	16.3
Calorie+ (kcal $100g^{-1}$)	330.7	330.5	330.1	330.2	330.1

* others : vitamin c 0.02%, astaxanthin 0.02%, lecithin 0.5%, cholesterol 0.5%, aquabind 1.5%, mineral mixture 2% (mineral mixture 100 g containing K_2HPO_4 2.0 g; $Ca_3(PO_4)_2$ 2.720 g; $MgSO_4.7H_2O$ 3.041g; $NaH_2PO_4.2H_2O$ 0.790 g); and vitamin mixture 1.76% (vitamin mixture 100 g containing ρ-Aminobenzoic acid 10.0 mg; Biotin 0.40 mg; Inositol 400.0 mg; Nicotinic acid 40.0 mg; Ca-Pantothenate 60.0 mg; Pyridoxine-HCl 12.0 mg; Riboflavin 8.0 mg; Thiamine-HCl 4.0 mg; Menadione 4.0 mg; α-Tocopherol 20.0 mg; Cyanocobalamine 0.08 mg; Calciferol 1.20 mg; Folic acid 0.80 mg; Choline chloride 120.0 mg)

+ Total calorie (gross energy) was measured by ballistic bomb calorimeter.

Table 2 Prepared diets compositions in experiment II.

Protein:Energy Ratio (mg of protein $kcal^{-1}$)	148	157	171	86	108	121	63	80	87
					Percent dry weight				
Ingredients									
Casein	30.0	35.0	45.0	25.0	35.0	45.0	25.0	33.9	36.9
Corn starch	13.6	13.6	13.6	31.7	31.7	31.7	49.9	48.3	46.0
Semi-refined fish oil	3.0	3.0	3.0	7.0	7.0	7.0	11.0	10.6	10.1
Cellulose	47.1	42.1	32.1	30.0	20.0	10.0	7.8	0.9	0.7
Others*	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Nutrients									
Moisture	3.7	3.7	3.5	2.9	3.5	3.8	3.4	3.1	3.0
Crude protein	29.1	34.1	44.0	23.5	34.0	44.1	26.1	33.1	36.1
Crude lipid	3.2	3.5	4.1	7.4	7.0	8.2	11.5	11.0	11.2
Calorie (kcal $100g^{-1}$)	203	223	263	291	331	371	399	439	459

* As in experiment I (Table 1).

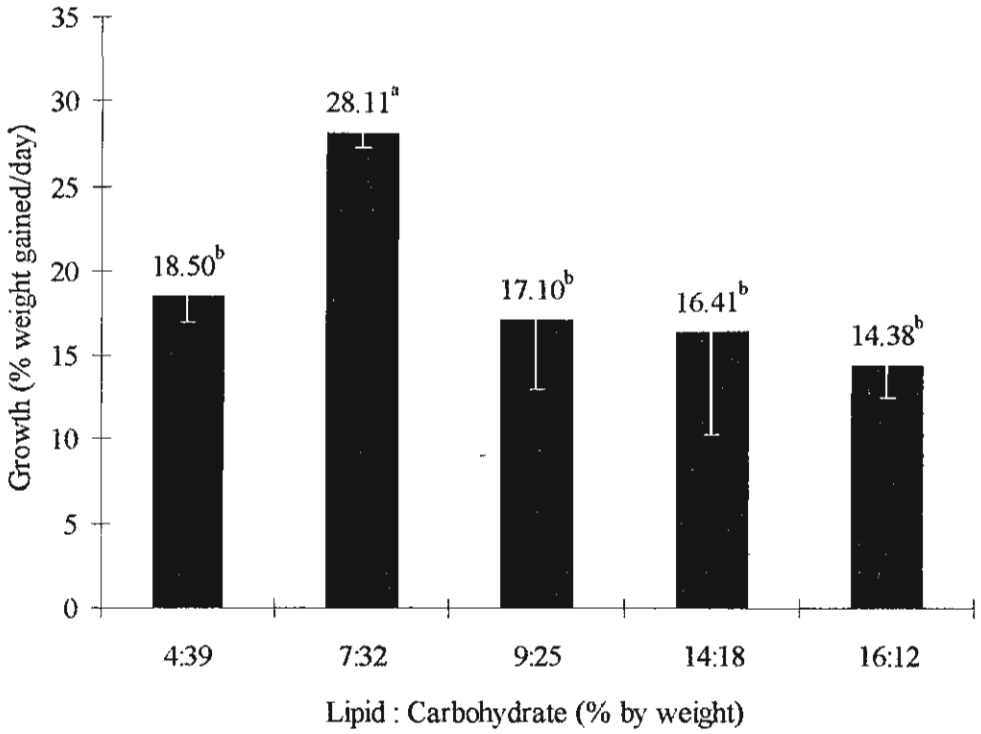


Figure 1 Mean growth rates ± S.D. of juvenile *Penaeus monodon* fed diets with different lipid:carbohydrate ratios for 30 days in experiment I (based upon 15 animals per aquarium; three aquaria per diet). Mean growth values with the same superscripts were not significantly different ($P>0.05$).

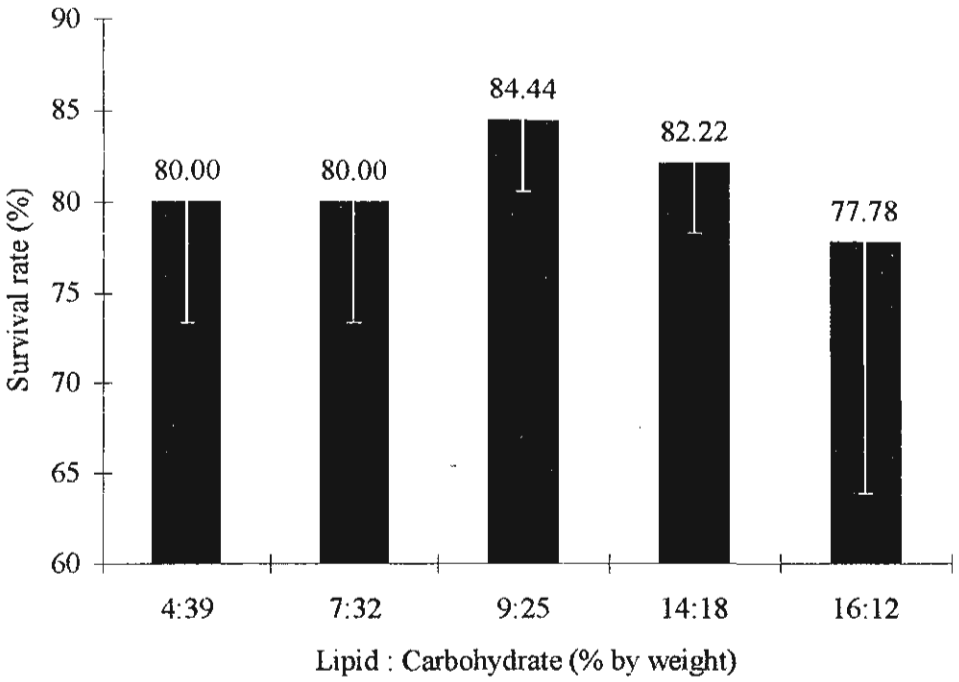


Figure 2 Mean survival rates ± SD of juvenile *Penaeus monodon* fed diets with different lipid:carbohydrate ratios for 30 days in experiment I.

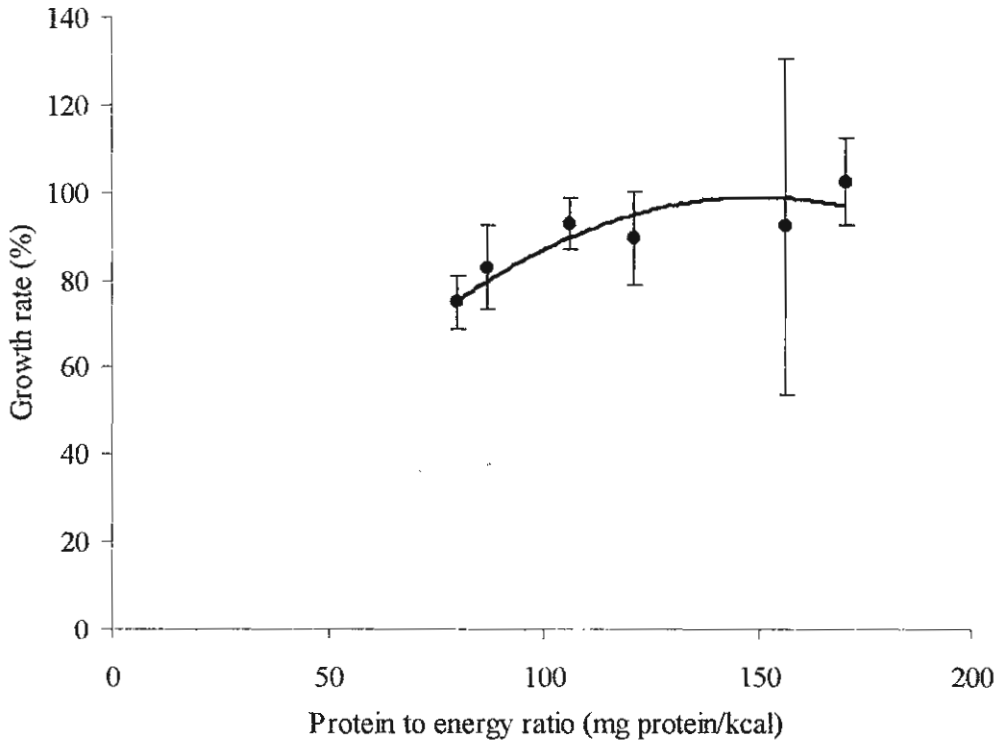


Figure 3 Growth rate ± SD of juvenile *Penaeus monodon* fed diets with different *P:E* ratios in experiment II (based on 15 animals per aquarium; three aquaria per diet).

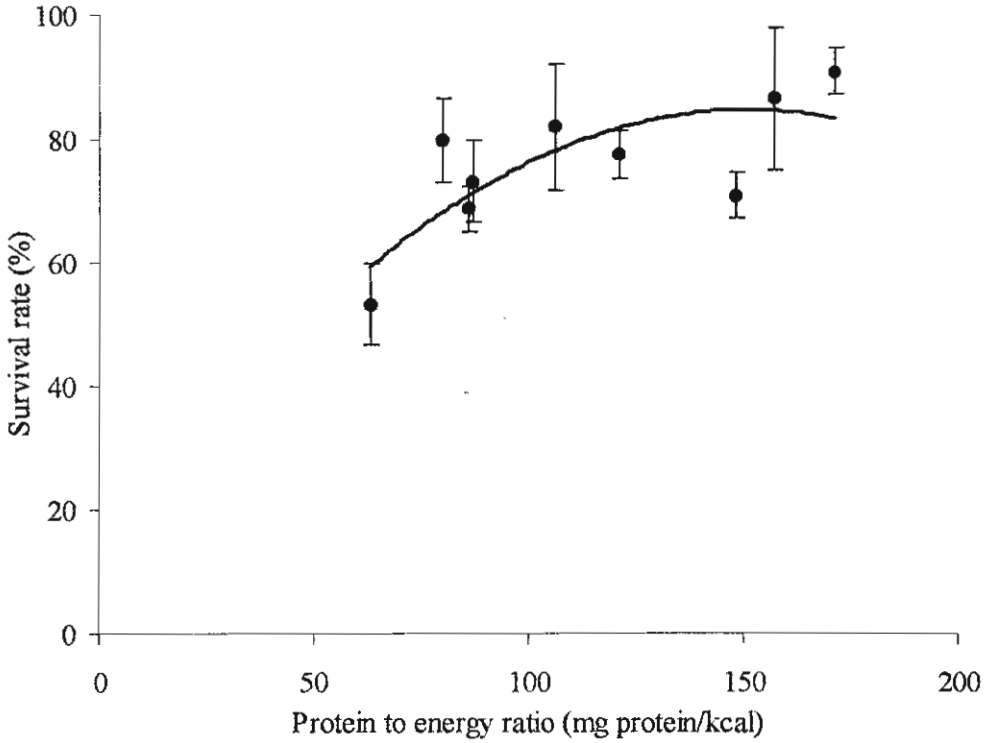


Figure 4 Survival rates ± SD of juvenile *Penaeus monodon* fed diets with different *P:E* ratios in experiment II.

ELSEVIER

Aquaculture 167 (1998) 301–313

Aquaculture

Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth

Sirirat Rengpipat [a,*], Wannipa Phianphak [b], Somkiat Piyatiratitivorakul [c], Piamsak Menasveta [c]

[a] *Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[b] *Thai Irradiation Center, Office of Atomic Energy for Peace, Ministry of Science, Technology and Environment, Klong 5, Klonglaung, Pathumtani 12120, Thailand*
[c] *Department of Marine Science, Faculty of Science, and Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand*

Accepted 8 June 1998

Abstract

Bacillus S11 bacterium isolated from black tiger shrimp habitats was added to shrimp feed as a probiotic in three forms: fresh cells, fresh cells in normal saline solution, and a lyophilized form. After a 100-day feeding trial with probiotic supplemented and non-supplemented (control) feeds, *Penaeus monodon* (from PL30) exhibited no significant difference ($p > 0.05$) in growth, survival nor external appearance between all three probiotic treatments, but significant differences ($p < 0.05$) occurred between probiotic and control groups. After challenging shrimps with a shrimp pathogen, *Vibrio harveyi*, by immersion for 10 days, all probiotic treatment groups had 100% survival; whereas the control group had only 26% survival. In addition, the control group had an unhealthy external appearance, and deformed texture of the hepatopancreas and intestine, while treatment group shrimp appeared healthy and normal.

Keywords: Probiotics; *Penaeus monodon*; Black tiger shrimp; *Bacillus* sp.; Immersion; *Vibrio harveyi*; Shrimp health

1. Introduction

During the past 50 years, numerous trials were conducted with microorganisms known as probiotics in efforts to improve culturability of food species, and to improve

* Corresponding author. Tel.: +66 2 2185089; fax: +66 2 2530337; e-mail: sirirat@mail.sc.chula.ac.th

0044-8486/98/$19.00
PII: S0044-8486(98)00305-6

human health and welfare. Appropriate probiotic applications were shown to improve intestinal microbial balance, thus leading to improved food absorption (Parker, 1974; Fuller, 1989), and reduced pathogenic problems in the gastrointestinal tract (Lloyd et al., 1977; Snoeyenbos et al., 1978; Pivnick et al., 1981; Cole and Fuller, 1984; Goren et al., 1984). Several probiotic species were used, including *Lactobacillus* spp. (Muralidhara et al., 1977; Pollman et al., 1980; Jonsson, 1986), *Saccharomyces* sp. (Burnett and Neil, 1977; Surawicz et al., 1989), *Bacillus* spp. (Ozawa et al., 1981; Ogle and Inborr, 1987; Spriet et al., 1987), and mixed cultures (Pollman et al., 1980; Lessard and Brisson, 1987). With some trials, growth promotion was clearly demonstrated in poultry (Alder and Damassa, 1980) and pigs (Pollman et al., 1980) compared with control groups. Those results were most promising and gave confidence that further improvements in probiotic applications were possible (Fuller, 1992).

Austin et al. (1995) recently found that the probiont *Vibrio alginolyticus* applied to salmon could reduce diseases caused by *Aeromonas salmonicids*, *V. anguillarum* and *V. ordalii*. Other published reference to probiotic application have been scarce, however, especially for shrimp culture application (Douillet and Langdon, 1994; Sugita et al., 1996).

Black tiger shrimp (*Penaeus monodon*) culture has considerable economic importance in Thailand. During 1992–1995, *P. monodon* exports from Thailand were valued at US$1.5–2.0 billion per year (Thai Department of Business Economics, 1996). Unfortunately, yields declined beginning in 1996 due to epidermic outbreaks of infectious diseases, including; yellow-head baculovirus, systemic ectodermal and mesodermal baculovirus and luminous bacterium (*V. harveyi*, Wendy and Kevan, 1992). Probiotics were considered a possible solution to some of these disease problems, but no information existed on such applications. Therefore, the aim of our current research was to evaluate probiotic potentials of selected bacterial strains on growth and survival of *P. monodon*.

2. Materials and methods

2.1. Bacteria strain

Several samples of mud, water and black tiger shrimp from shrimp ponds along the Gulf of Thailand were collected and homogenized, serially diluted with sterilized normal saline solution (NSS, NaCl 0.85% w/v), then plated on Tryptic soy agar (Difco) with 1% (w/v) NaCl. Serial dilution ranged from 10^{-1} to 10^{-8} with triplicate plate counts. Isolated colonies were selected and checked for antimicrobial properties against *V. harveyi* D311 and *V. parahaemolyticus* using an agar diffusion technique described in detail by Naclerio et al. (1993). Of 377 isolates, one strain produced the greatest antimicrobial performance. This strain was identified as *Bacillus* sp. and designated *Bacillus* S11, a Gram-positive, rod shape, catalase positive and spore former (Phianphak, 1996). *Bacillus* S11 was cultured in Tryptic soy broth (Difco) with 1% (w/v) NaCl and stocked on Tryptic soy agar (included 1% (w/v) NaCl). Culture conditions were at 37°C in 2-l flasks for 24 h, afterward *Bacillus* S11 cells were centrifuged and washed in

sterile normal saline solution three times, then stored at −20°C until further use. We routinely checked its purity during this investigation. We included three forms of *Bacillus* S11 in three shrimp feed formulations, including *Bacillus* S11 as; fresh cells, fresh cells in normal saline solution, and with lyophilized cells. Fresh cells aged 24 h ($\sim 10^{13}$ colony forming units (CFU) per 1 g of cells wet weight) were harvested and maintained at −20°C prior to use. Cell aliquots were freeze-dried using a Lyophilizer FD-1 as prescribed by the manufacturer (Eyela, Tokyo Rikakikai) and kept in a sterilized container (1 l) at −20°C before use. *Bacillus* S11 cells brought to room temperature from −20°C were used as fresh cells (3.0×10^{13} CFU g^{-1}). Some of these cell aliquots were suspended in NSS (1 g of cell wet weight suspended in 100 ml of NSS). They were designated fresh cells in NSS (3.4×10^{10} CFU ml^{-1}). Lyophilized cells were used at 5×10^{12} CFU g^{-1}.

Formulated shrimp feed consisted of 32% fish meal, 25% soy bean, 10% shrimp head meal, 1% lecithin, 20% wheat flour, 5% wheat gluten, 2% vitamin complex, 3% mineral complex, and 5% fish oil by weight. These ingredients were mixed and extruded, heated at 110°C for 10 min, then kept at 80°C overnight. After returning to room temperature, this diet was kept at −20°C until used.

Total bacterial count in unaltered diet was 2×10^2 CFU g^{-1}, of which ~100% was *Bacillus* spp. The ratio of formulated shrimp feed to *Bacillus* S11 form was 3:1 (except the control). After mixing, feed was kept at −20°C until used. Final *Bacillus* S11 concentrations in treatment feeds was $\sim 10^{10}$ CFU g^{-1}.

2.2. Preparation of experimental animals and experimental design

Healthy, black tiger shrimp aged PL-15 were acclimatised in 12 concrete test tanks (each measuring $80 \times 74 \times 87$ cm) until PL-30 (0.7–0.8 g), and were fed unaltered, formulated feed three times daily. The PL-15 were obtained from a hatchery located near Chachoengsao, Thailand, using broodstock captured from the Andaman sea. A closed, recirculating water system was used. This system and culture procedures are described by Menasveta et al. (1991).

The experiment was conducted as a completely randomized design with four treatments: (a) shrimp fed unaltered diet (control); (b) shrimp fed diet mixed with fresh cells of *Bacillus* S11; (c) shrimp fed diet mixed with *Bacillus* S11 cell suspension in NSS; and (d) shrimp fed diet mixed with lyophilized *Bacillus* S11 cells. Each treatment had three replicates of 40 shrimp each. Shrimp were fed three times daily at 800, 1200 and 1600 h. Daily feeding rate was ~10% of total body weight.

Lengths and weights of 10 randomly collected shrimp from each pond were determined every 3 weeks. At the same time, shrimp survival was also determined in each tank.

Weekly water samples of 120 ml were collected from the center of each tank, along with shrimp feces (~200 mg), and one live shrimp once every 3 weeks for bacterial determination starting from the first day of the feeding trials. Water quality was monitored weekly, including; pH, dissolved oxygen, temperature, salinity, ammonium, nitrate, nitrite and phosphate as described by Strickland and Parsons (1972). Shrimp were dissected using sterilized surgical scissors where the hepatopancreas and intestines

were removed for microbial enumeration and identification. Bacterial determinations were made using serial dilution in NSS, followed by plating on Nutrient agar (with 1% w/v NaCl), Tryptic soy agar (with 1% w/v NaCl), and Thiosulphate citrate bile salt agar (TCBS, Difco). After 24–48 h of incubation at 37°C, plate colonies were counted and recorded. Microbial strains were re-examined using Gram staining, spore staining and selected biochemical tests as described by Sneath (1986). The effects of *Bacillus* S11 on shrimp growth and survival, and on *V. harveyi* D331 resistance were evaluated using analysis of variance and Duncan's multiple range tests (Statistical Analysis System, 1983).

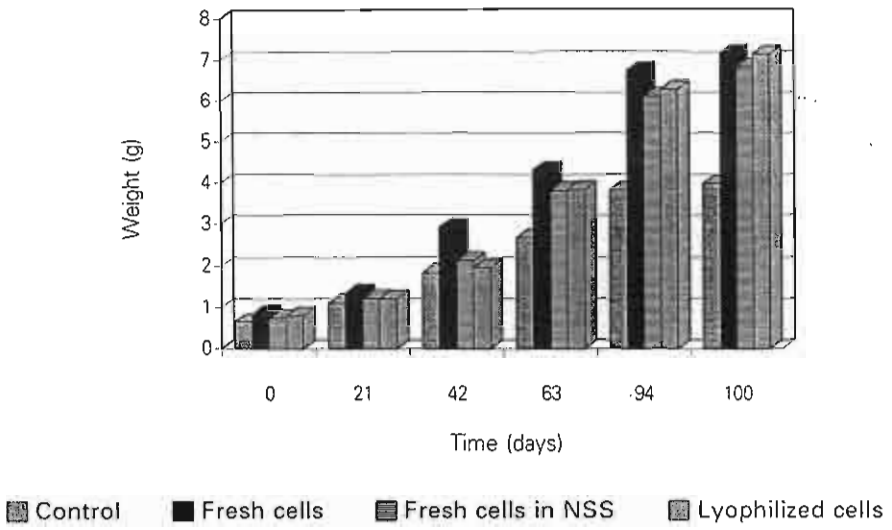
2.3. Pathogen challenge test

After feeding for 100 days, shrimp in each treatment were challenged with the luminous bacteria *V. harveyi* D331 which had been cultured and maintained using TCBS broth and agar. Shrimp in all replicated immersed in a suspension of *V. harveyi* D331 at $\sim 10^5$ CFU ml^{-1} according to Austin et al. (1995). This was followed by a second immersion of $\sim 10^7$ CFU ml^{-1} after 7 days. During the immersion test, water and shrimp were collected and checked every 2 days from each tank for microbial examination and shrimp survival. After 10 days of the challenge test, shrimp in each treatment group were dissected by sterile surgical scissors and examined microscopically as described above. Shrimp survival was determined for each replicate. *V. harveyi* culture isolated from shrimp hepatopancreas and intestines were purified and identified using Gram staining, oxidase test, and motility test, and were compared with the original *V. harveyi* D331 cultures. *V. harveyi* D331 and *V. parahaemolyticus* cultures were kindly provided by the Shrimp Culture Research Center, Charoen Pokphand Feedmill, Samutsakorn, Thailand. We reconfirmed the identity of *V. harveyi* and *V. parahaemolyticus* by following procedures described by Holt et al. (1986).

3. Results and discussion

There were no obvious effects of *Bacillus* S11 on the water quality in the three feed treatments. Dissolved oxygen (5–6 mg l^{-1}), pH (7.9–8.2), temperature (26–27°C) and salinity (20 mg l^{-1}) values were stable and within acceptable ranges. Likewise, ammonium (0–0.5 mg l^{-1}), nitrate (0–1.2 mg l^{-1}), nitrite (0–2.5 mg l^{-1}), and phosphate (2–3 mg l^{-1}) were unremarkable and considered safe for shrimp culture (Menasveta et al., 1989; Boyd, 1990; Menasveta et al., 1991). During the first week, however, nitrite increased to 2.5 mg l^{-1} but later decreased to near zero.

After 100 days, mean shrimp weights of all probiont treatment groups averaged 7.06 ± 0.48 g with no significant difference ($p > 0.05$) between these groups. Probiont treatment group mean weights were significantly greater ($p < 0.05$) than control shrimp mean weight (3.99 ± 0.38 g; Fig. 1). Shrimp survival after 100 days was significantly greater ($p < 0.05$) in the treatment groups ($33.3 \pm 4.4\%$) compared with the control group ($15.8 \pm 5.2\%$; Fig. 1). Cannibalism associated with clear water and concrete tanks apparently caused low survival rate. Treatment group *P. monodon* appeared healthier



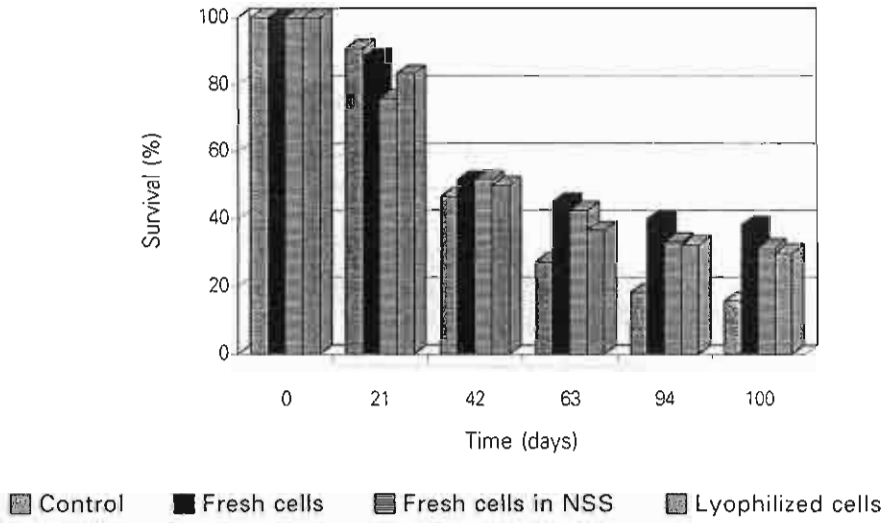
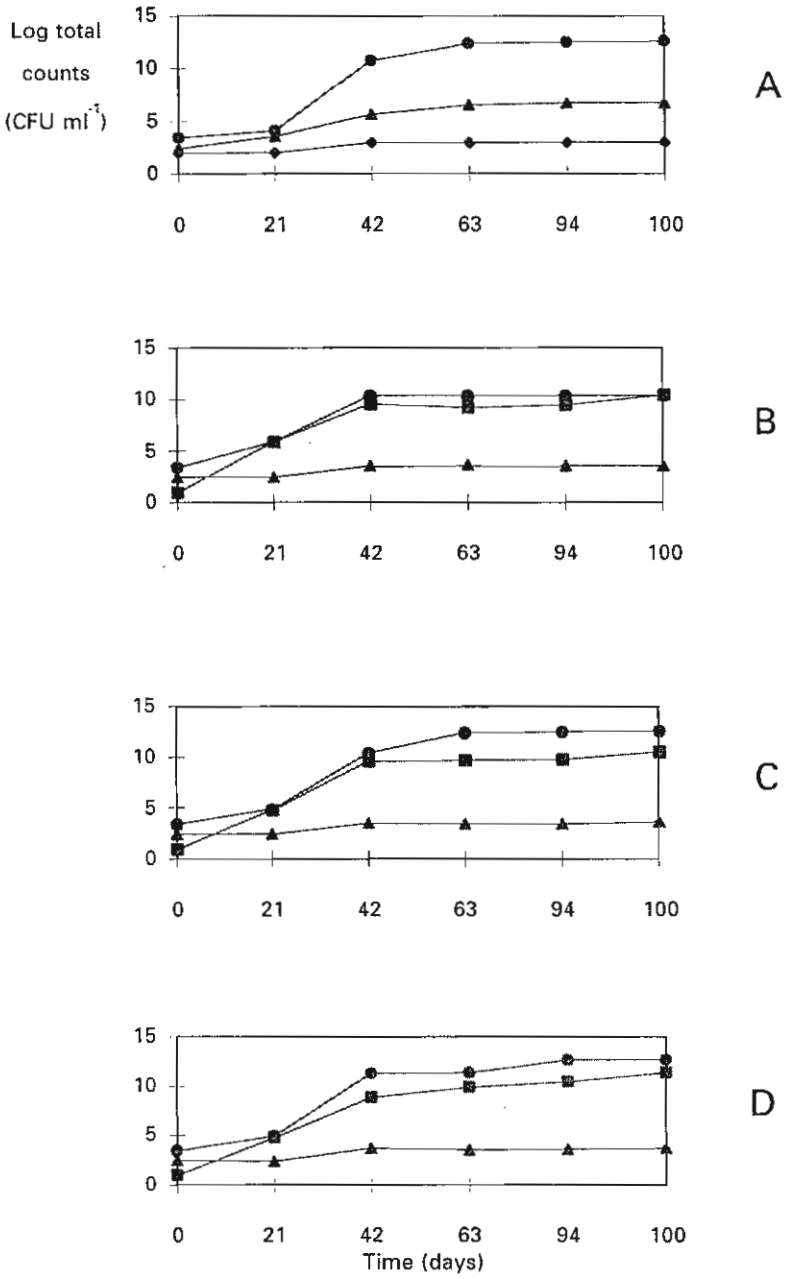


Fig. 1. Live weights (g) and survival (%) of *P. monodon* cultured for 100 days in four feed treatments.

and were active than those of the control group. Clearly, probiotic treatment with *Bacillus* S11 appeared to enhance both growth and survival of *P. monodon*. *Bacillus* S11 preparations with fresh cells, fresh cells in NSS and lyophilized forms gave similar results without any significant differences between *Bacillus* S11 forms.



Total bacteria (–●–), *Bacillus* spp. (–✦–), *Bacillus* S11 (–■–), and *Vibrio* spp. (–▲–)

Fig. 2. Bacterial counts in rearing tank water during 100 days of feeding with: (A) Control; (B) Fresh cells; (C) Fresh cells in NSS; and (D) Lyophilized cells. All values are means of three replicates per treatment.

After 42 days culture, total bacterial counts of water from each culture tank was $\sim 10^{10}$ CFU ml^{-1} regardless of treatment (Fig. 2). These concentrations were generally greater than those normally found in earthen ponds used for commercial shrimp culture (e.g., 10^7 to 10^8 CFU ml^{-1}; Colorni, 1985). Higher values in our culture system was probably due to low algal density and lack of grazer organisms. Our tanks were turbid, grey coloured water. *Vibrio* spp. concentrations in control tanks (Fig. 2A) were greater than those of treatment groups (Fig. 2B,C and D). *Bacillus* S11 concentrations increased

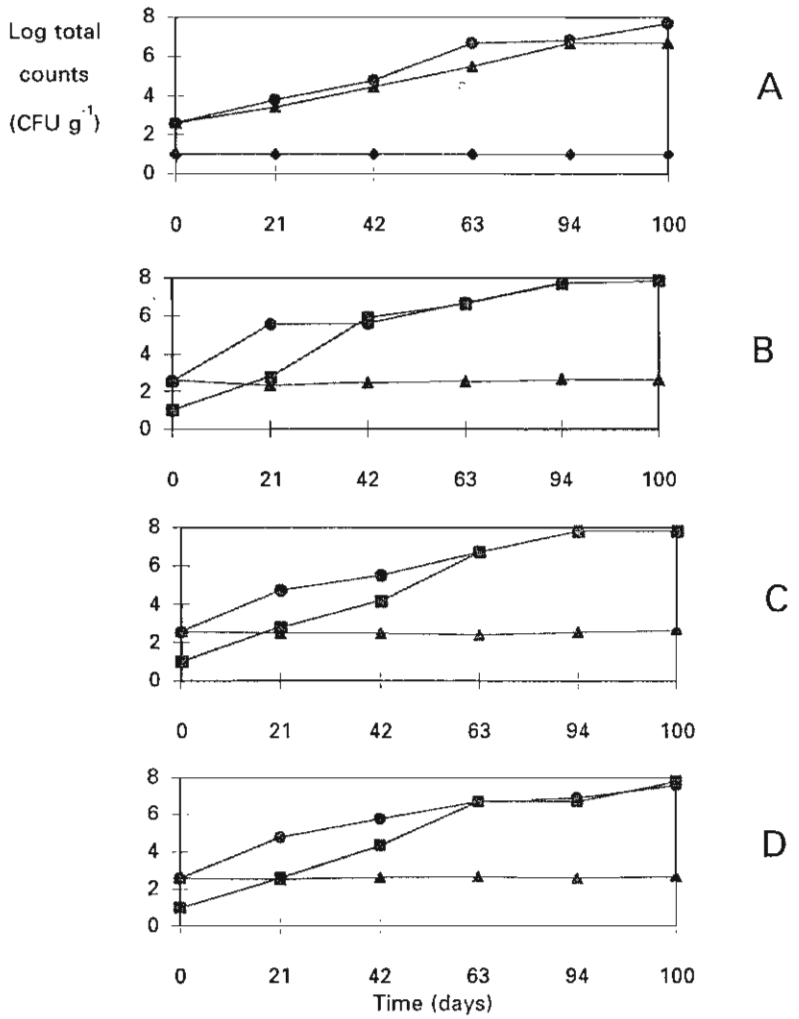
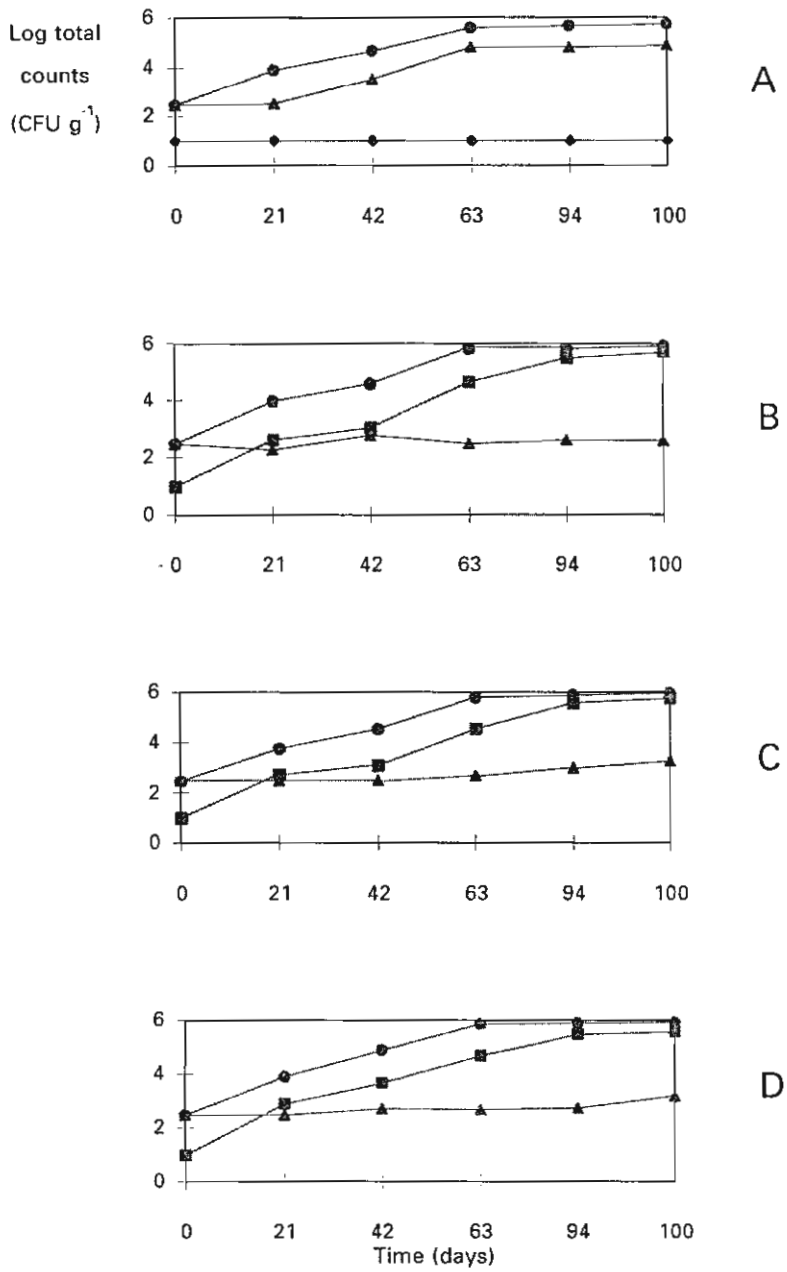


Fig. 3. Bacterial counts in shrimp guts (hepatopancreas and intestine) during 100 days of feeding with: (A) Control; (B) Fresh cells; (C) Fresh cells in NSS; and (D) Lyophilized cells. All values are means of three replicates per treatment.



Total bacteria (–●–), *Bacillus* spp. (–✦–), *Bacillus* S11 (–■–), and *Vibrio* spp. (–▲–)

Fig. 4. Bacterial counts in shrimp feces during 100 days of feeding with: (A) Control; (B) Fresh cells; (C) Fresh cells in NSS; and (D) Lyophilized cells. All values are means of three replicates per treatment.

to 10^8–10^{10} CFU ml^{-1} in all treatment tanks through the first 42 days, but were not found in the control tanks. *Bacillus* spp. concentrations were $\sim 2 \times 10^2$ CFU ml^{-1} in the control tanks, but were nearly absent in the treatment tanks. *Bacillus* spp. presence may have been due to non-competitive, saprophytic growth (Yasuda and Kitao, 1980). These results indicate that the probiont *Bacillus* S11 replaced both *Vibrio* spp. and other bacterial in the culture water.

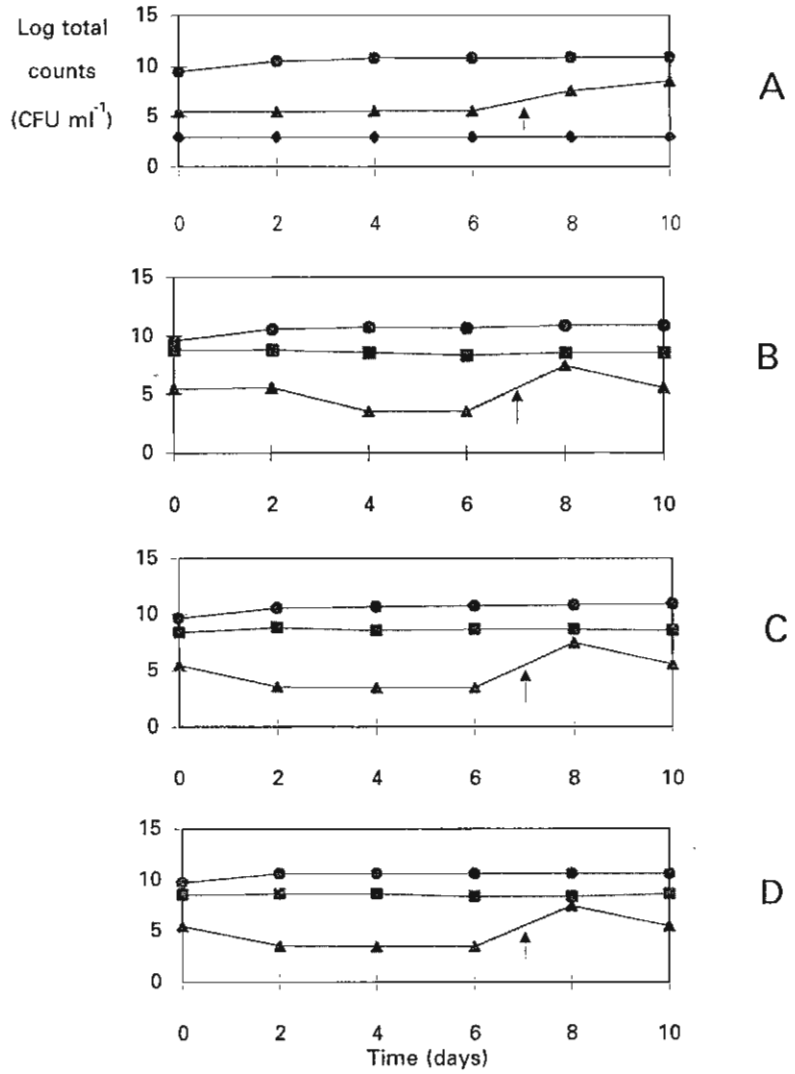


Fig. 5. Bacterial counts in tank water of probiotic treatments and control during 10 days challenge with *V. harveyi* D331 where: (A) Control; (B) Fresh cells; (C) Fresh cells in NSS; and (D) Lyophilized cells. (↑ second booster of *V. harveyi* D331 10^7 CFU ml^{-1} by immersion.) All values are means of three replicates per treatment.

Total bacterial flora, both Gram-positive and Gram-negative rods, found in shrimp hepatopancreas and intestines (mid- to hind gut) in all treatments and controls on day 100 were in a range of 10^7–10^8 CFU g^{-1} (Fig. 3); the same values as reported by Dempsey et al. (1989). The main flora in control group shrimp guts were *Vibrio* spp. (10^6 CFU g^{-1}) and a few *Bacillus* spp. (< 10 CFU g^{-1}), while those in all the

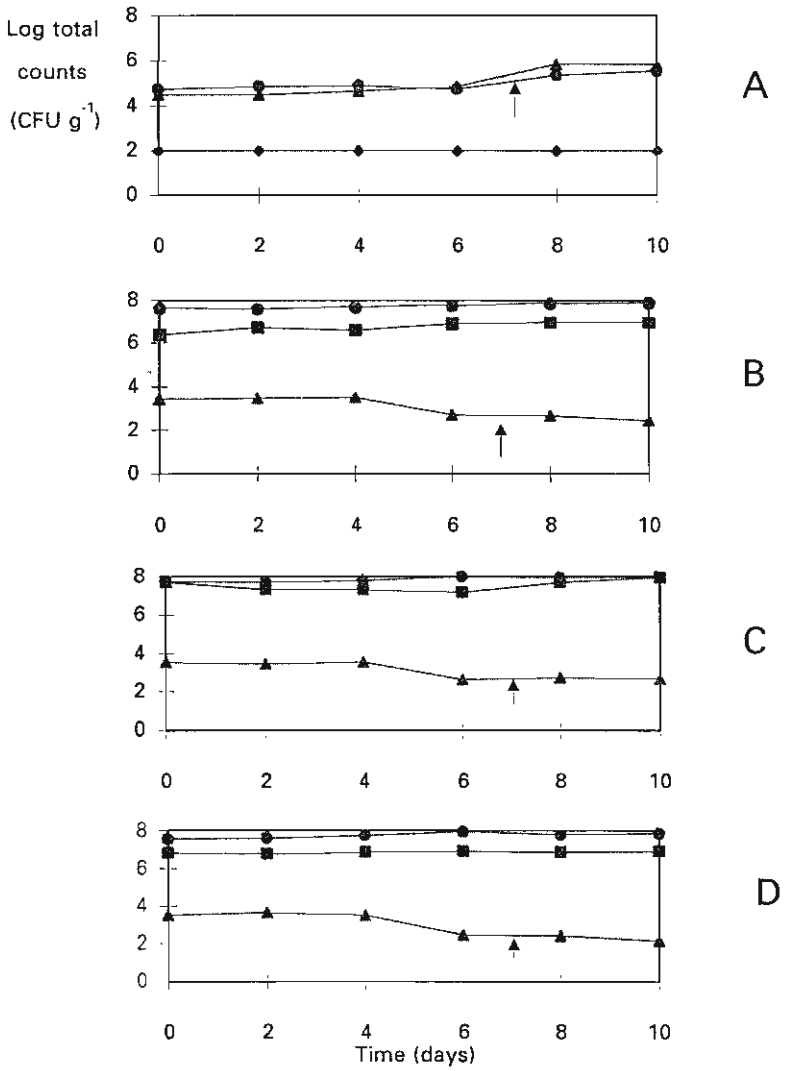


Fig. 6. Bacterial counts in shrimp guts (hepatopancreas and intestine) of probiotic treatments and control during 10 days challenge with *V. harveyi* D331 where: (A) Control; (B) Fresh cells; (C) Fresh cells in NSS; and (D) Lyophilized cells. (↑ second booster of *V. harveyi* D331 10^7 CFU ml^{-1} by immersion.) All values are means of three replicates per treatment.

treatment groups were mostly *Bacillus* S11 (10^6–10^7 CFU ml^{-1}) and a few *Vibrio* spp. ($\sim 10^2$ CFU g^{-1}). Clearly, *Vibrio* spp. in treatment shrimp guts was replaced by *Bacillus* S11. This same pattern of bacterial replacement occurred in shrimp feces of both control and treatment groups (Fig. 4), where *Vibrio* spp. was replaced by *Bacillus* S11. These results re-confirm the agreement in bacterial type and concentration between shrimp guts and feces (Yasuda and Kitao, 1980). More importantly, these results are an indirect indication that the probiont *Bacillus* S11 viably colonize shrimp guts and proliferate in a manner that benefited the host.

During the challenge test, *Vibrio* spp. concentrations in both tank water and shrimp guts of the control group increased from 10^5 to 10^7 CFU ml^{-1} or g^{-1}, while only 10^2 CFU of *Bacillus* spp. were detected (Figs. 5 and 6). Conversely, with all treatment groups, the probiont *Bacillus* S11 was the principal bacteria (10^7–10^8 CFU ml^{-1} or g^{-1}), with lower concentration of *Virio* spp. in water (10^5 CFU ml^{-1}) and in the guts (10^2 CFU g^{-1}). The main *Vibrio* spp. found in all challenge tanks was identified as *V. harveyi* D331. After 10 days challenge with *V. harveyi* D331, survival of probiotic treatment groups (100%) was significantly greater ($p < 0.05$) than with the control group (26%; Fig. 7).

Even after the second high dose of *V. harveyi* D331, healthy shrimps in the treatment survived well. Moreover, no external pathology was observed in treatment groups. They looked normal in size, colour and texture. On the contrary, guts from the control group had pale colour and deformed texture, and survival continued to decrease following the second challenge (Fig. 7). Although treatment groups were also immersed in *V. harveyi* D331, they resisted both external and internal infection by *V. harveyi* D311. *Bacillus* S11 colonization of shrimp guts apparently acted as an interferer or competitor against *V. harveyi* D311 infection. *Bacillus* S11 may produce some anti-microbial substances, or some unknown by-products negatively effected *V. harveyi* D331 (Hastings and Nealson, 1981).

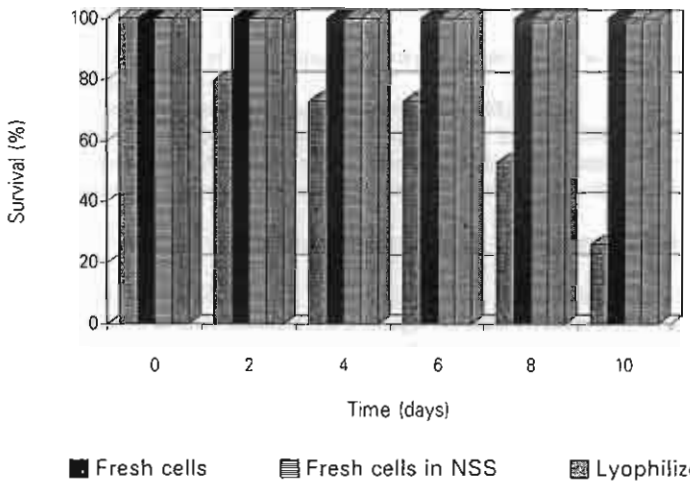


Fig. 7. *P. monodon* survival in three probiotic treatment groups and control with *V. harveyi* D331 for 10 days.

Our findings still leave open questions regarding the effects of probionts on shrimp immune response. Our data suggests competitive exclusion by non-pathogenic *Bacillus* S11 was the main source of benefits in our case, but we did not measure possible immunities. We can conclude, however, that use of *Bacillus* S11 in shrimp feeds can reduce *P. monodon* mortalities during culture. Our findings should be confirmed in outdoor, earthen pond trials before they are applied commercially.

Acknowledgements

The authors are grateful to Dr. Arlo W. Fast for revising the manuscript and providing many valuable suggestions and comments. This research was supported by the Thailand Research Fund, (TRF) and The Royal Thai Government Budget for Research.

References

Alder, H.E., Damassa, A.J., 1980. Effects of ingested *Lactobacilli* on *Salmonella infantis* and *Escherichia coli* and on intestinal flora, pasted vent and chick growth. Avian Diseases 24, 868–878.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, J., Griffith, D.R.W., 1995. A probiotic reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J. Fish. Dis. 18, 93–96.

Boyd, C.E., 1990. Water quality in ponds for aquaculture. Alabama Agricultural Experimental Station, Auburn University, Auburn, AL, 482 pp.

Burnett, G.S., Neil, E.L., 1977. A note on the effect of probioticum feed additive on the live-weight gain, feed conversion and carcass quality of bacon pig. Anim. Prod. 25, 95–98.

Cole, C.B., Fuller, R., 1984. A note on the effect of host specific fermented milk on the coliform population of the neonatal rat gut. J. Appl. Bacteriol. 56, 495–498.

Colorni, A., 1985. A study on the bacteria flora of giant prawn, *Macrobrachrium rosanbergii*. Larva fed with *Artemia salina naplii*. Aquaculture 49, 1–10.

Dempsey, A.C., Kitting, C.L., Rosson, R.A., 1989. Bacterial variability among individual penaeid shrimp digestive tracts. Crustaceana 56, 267–276.

Douillet, P.A., Langdon, C.J., 1994. Use of a probiotic for culture of larvae of the Pacific oyster (*Crassostrea gigas* Thuberg). Aquaculture 119, 25–40.

Fuller, R., 1989. Probiotic in man and animals. J. Appl. Bacteriol. 66, 365–378.

Fuller, R., 1992. Probiotic the scientific basis. 1st edn. Chapman & Hall, London, 398 pp.

Goren, E., De Jong, W.A., Doornenbal, P., Koopman, J.P., Kennis, H.M., 1984. Protection of chicks against *Salmonella* infection induced by spray application of intestinal microflora in the hatchery. Veterinary Quarterly 6, 73–79.

Hastings, J.W., Nealson, K.H., 1981. The symbiotic luminous bacteria. In: The Prokaryotes II. Springer-Verlag, New York, 1960 pp.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., 1986. Facultatively anaerobic Gram-negative rods. In: Bergey's Manual of Determinative Bacteriology, 9th edn. Williams and Wilkins, Baltimore, pp. 175–289.

Jonsson, E., 1986. Persistence of *Lactobacillus* strain in the gut of sucking piglets and its influence on performance and health. Swed. J. Agric. Res. 16, 43–60.

Lessard, M., Brisson, G.J., 1987. Effect of a *Lactobacillus* fermentation product on growth, immune response and fecal enzyme activity in weaned pigs. Can. J. Anim. Sci. 67, 509–516.

Lloyd, A.B., Comming, R.B., Kent, R.D., 1977. Prevention of *Salmonella typhimurium* infection in poultry by pretreatment of chickens and poultry with intestinal extracts. Australian Veterinary Journal 53, 82–87.

Menasveta, P., Aranyakanonda, P., Rungsupa, S., Moree, N., 1989. Maturation and larviculture of penaeid prawns in closed recirculation seawater system. Aquacultural Engineering 8, 357–368.

Menasveta, P., Fast, A.W., Piyatiratitivorakul, S., Rungsupa, S., 1991. An improved closed seawater recirculation maturation system for giant tiger prawn (*Penaeus monodon* Fabricius). Aquacultural Engineering 10, 173–181.

Muralidhara, K.S., Sheggeby, G.G., Elliker, P.R., 1977. Effect of feeding *Lactobacilli* on the coliform and *Lactobacillus* flora of intestinal tissue and feces from piglets. J. Food Protect. 40, 288–295.

Naclerio, G., Ricca, E., Sacco, M., Felice, M.D., 1993. Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. Appl. Environ. Microbiol. 59, 4313–4316.

Ogle, R.B., Inborr, J., 1987. Alternatives to low dose antibiotics in piglet feeds in Sweden. 38th Meeting of the European Association for Animal Production, Portugal, Commission on Pig Production, Session 4, pp. 1–6.

Ozawa, K., Yokota, H., Kimura, M., Mitsuoka, T., 1981. Effect of administration of *Bacillus subtilis* strain BN on intestinal flora of weanling piglets. Jpn. J. Vet. Sci. 43, 771–775.

Parker, R.B., 1974. Probiotics, the other half of the antibiotics story. Anim. Nutr. Health 29, 4–8.

Phianphak, W., 1996. Use of bacteria as probiotics supplement in shrimp feed. MS Thesis, Chulalongkorn University, Thailand, 123 pp.

Pivnick, H., Blanchfield, B., D'Anst, J.-Y., 1981. Prevention of *Salmonella* infection in chicks by treatment with fecal cultures from mature chickens (Nurmicultures). J. Food Protect. 44, 909–916.

Pollman, D.S., Danielson, D.M., Peo, E.R. Jr., 1980. Effect of microbial feed additive on performance of starter and growing–finishing pigs. J. Anim. 51, 577–581.

Sneath, P.H.A., 1986. Endospore-forming Gram-positive rods and cocci. In: Sneath, P.H.A., Mair, N.S., Sharp, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins, Baltimore, pp. 1104–1105.

Snoeyenbos, G.H., Weinack, O.M., Smyser, C.F., 1978. Protecting chicks and poultry from *Salmonellae* by oral administration of normal gut microflora. Avian Diseases 22, 273–287.

Spriet, S.M., Decuypere, J.A., Hendericky, H.K., 1987. Effect of *Bacillus toyoi* (Toyocerin) on the digestibility of the nutrient and the small intestinal mean retention time in pig. Med. Fac. Landbouw. Rijksuriv. Gent. 52, 1673–1683.

Statistical Analysis System, 1983. SAS Introductory Guide. SAS Institute, Cary, USA, 93 pp.

Strickland, J.D.H., Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis, 2nd edn. Fisheries Research Board of Canada Bull., 167. Alger Press, 310 pp.

Sugita, H., Mutsuo, N., Shibuya, K., Deguchi, Y., 1996. Production of antibacterial substances by intestinal bacteria isolated from coastal crab and fish species. J. Mar. Biotechnol. 4, 220–223.

Surawicz, C.M., Elmer, G.W., Speelman, P., 1989. Prevention of antibiotic-associated diarrhea by *Saccharomyces boulardii*: a prospective study. Gastroenterology 84, 1285–1287.

Thai Department of Business Economics, 1996. Export value of Thai frozen shrimp 1986–1996. Shrimp Culture Newsletter, 96, pp. 1–4.

Wendy, F., Kevan, L.M., 1992. Diseases of cultured penaeid shrimp in Asia and the United States. The Oceanic Institute, 382 pp.

Yasuda, K., Kitao, T., 1980. Bacterial flora in the digestive tract of prawn, *Penaeus japonicus* Bate. Aquaculture 19, 229–234.

Probiotic Use of *Lactobacillus* spp. for Black Tiger Shrimp, *Penaeus monodon*

Wannipa Phianphak[1], Sirirat Rengpipat[2], Somkiat Piyatiratitivorakul[3], and Piamsak Menasveta[3]

A mixture of *Lactobacillus* species and strains (designated L-P) was isolated from chicken gastrointestinal tracts and mixed with a formulated shrimp diet. Growth and survival of *Penaeus monodon* juveniles fed the L-P diet for 100 days was significantly greater ($p<0.05$) than those shrimp fed a control diet without L-P added. A 10-day immersion challenge test with *Vibrio harveyi* D331 resulted in 74% mortality in the control group, while none of the treatment group shrimp died.

Key words: Probiotics, shrimp health, *Lactobacillus* spp., *Penaeus monodon*, *Vibrio harveyi* D331.

[1] *Thai Irradiation Center, Office of Atomic Energy for Peace, Ministry of Science, Technology and Environment, Klong 5, Klonglaung, Pathumtani 12120, Thailand.*

[2] *Corresponding author*
Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

[3] *Department of Marine Science and Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand.*

การใช้ *Lactobacillus* spp. เป็นโพรไบโอติกในการเลี้ยงกุ้งกุลาดำ *Penaeus monodon*

วรรนิภา เพี้ยนภักตร์, ศิริรัตน์ เร่งพิพัฒน์, สมเกียรติ ปิยะธีรธิติวรกุล
และ เปี่ยมศักดิ์ เมนะเศวต (2542)

วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **24 (1)**

Lactobacillus spp. หลายสายพันธุ์ (L-P) แยกได้จากทางเดินอาหารไก่ นำมาผสมกับอาหารกุ้ง ในการเลี้ยงกุ้งกุลาดำ *Penaeus monodon* ด้วยอาหารปกติ (กลุ่มควบคุม) และอาหารผสม L-P พบว่า การเจริญเติบโต และการรอดชีวิตของกุ้งกุลาดำที่เลี้ยงด้วยอาหารผสม L-P เป็นเวลา 100 วัน มีค่ามากกว่า ในกลุ่มกุ้งกุลาดำที่เลี้ยงด้วยอาหารปกติอย่างมีนัยสำคัญ ($p<0.05$) เมื่อนำกุ้งทั้งสองกลุ่มมาเหนี่ยวนำให้เกิดโรคด้วย *Vibrio harveyi* D331 โดยวิธีการแช่เป็นเวลา 10 วัน กุ้งในกลุ่มควบคุมมีการตาย 74 % ในขณะที่กลุ่มได้รับอาหารผสม L-P รอดชีวิต 100 %

คำสำคัญ โพรไบโอติก, สุขภาพกุ้ง, *Lactobacillus* spp., *Penaeus monodon*, *Vibrio harveyi* D311

INTRODUCTION

Metchnikoff first used the term "probiotics" in 1907, where it was defined as a microorganism feed supplement which is non-pathogenic but which propagates in the host animals, gastrointestinal (GI) tract thus serving as a growth promotor and/or providing resistance to infectious disease.[1] Probiotic bacteria and other microbes include *Lactobacillus* spp., *Bifidobacterium* sp., *Clostridium* sp., *Enterococcus* sp., *Escherichia coli*, *Bacillus* sp., *Streptococcus* sp., yeast, and mixed cultures. Probiotics are widely used commercially as feed supplements for terrestial animals such as pigs[2-5] and chickens.[6-7] Probiotic growth promotion, health enhancement, and infectious disease inhibition greatly reduce investment risks and costs. At the same time, probiotics have not yet been used widely in aquaculture, although a substantial market currently exists in the agro-industry for these products.[8] However, some successful probiotics trials have been reported with *Vibrio alginolyticus* in salmon.[9] Recently, Sugita *et al.* (1996)[10] isolated bacterial flora from the GI tract of marine crabs and fish which have potential uses as probiotics. Futhermore, Rengpipat et al. (1998)[11] clearly demonstrated that *Bacillus* S11 could be used as a probiotic for *Penaeus monodon*.

Lactobacillus, a group of lactic acid bacteria, has long been used as starter culture for several milk products,[12,13] and other fermented foods.[14] The end product of this fermentation is lactic acid which is a safe food preservative and flavor enhancer.[15] Several *Lactobacillus* spp. strains are used as probiotics for terrestial animals, including, *Lactobacillus lactis*, *L. bulgaricus*, *L. fermentum*, *L. acidophilus* and *L. reuteri*.[2,4,5,16] These *Lactobacillus* spp. reduced *Escherichia coli* in feces,[4] guts[5] and stomachs[2] of small pigs, and they suppressed and neutralized *E. coli* toxin in calves.[16] *Lactobacillus* spp. feed additives also benefited host metabolism, including reduced serum cholesterol[17] and amine[3] in pigs, and they produced hydrolytic enzymes which improved digestion in rats,[18] chicks[6,7] and pigs.[19] The main purpose of our study was to evaluate potential benefits of *Lactobacillus* spp. mixed cultures with black tiger shrimp, *Penaeus monodon*.

MATERIALS AND METHODS

Five strains of *Lactobacillus* spp., including *Lactobacillus acidophilus* TISTR 1338, *L. bulgaricus* TISTR 1339, *L. casei* TISTR 1340, *L. casei* subsp. *tolerans* TISTR 1341, and *L. jensenii* TISTR 1342 were used in this study. They were provided by Bangkok MIRCEN, Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand. They were originally isolated from intestines of healthy chickens obtained from local markets in Bangkok. Prior studies by Thanaruttikannont (1996)[20] confirmed that they possessed good probiotic properties for rearing chickens. Each *Lactobacillus* spp. was grown in MRS broth (DIFCO) at 37°C for 24 h and then harvested by centrifuging at 5,000 rpm for 30 min. Cells were immediately washed twice with sterile normal saline solution. Cells of each strain were mixed in equal amounts to achieve final concentration of ~10^{10} cells g^{-1} wet weight. This *Lactobacillus* spp. mixture was designated L-P. *Lactobacillus* spp. confirmations were performed on MRS agar by picking isolated colonies for examination, using Gram staining, a catalase test, and a biochemical test. Confirmation was by standard techniques.[21]

The shrimp diet was prepared consisting of 32% fish meal, 25% soybean, 10% shrimp head meal, 1% lecithin, 20% wheat flour, 5% wheat gluten, 1% vitamin complex, 1% mineral complex, and 5% fish oil, all by weight. These ingredients were mixed, extruded, heated at 100°C for 10 min, and then dried at 80°C overnight. After cooling to room temperature, feed was kept at -20°C until used. Total bacterial count revealed mostly *Bacillus* spp. (10^2 CFU g^{-1}). Prior to use, L-P was added to feed in a 1:3 ratio, thoroughly mixed, and kept at -20°C until use. L-P in this aliquot was at approximately 10^{10} CFU g^{-1}. Feed without any addition (non-treated feed) was used as a control diet.

Healthy black tiger shrimp aged PL-15 were acclimatized in six concrete test tanks (each measuring 80x74x87 cm) until PL-30

(0.7-0.8 g), and were fed non-treated feed three times daily. The shrimp culture procedure was as described by Menasveta *et al.* (1991),[22] including a closed, recirculating water system. The experiment was conducted in a completely randomized design with two treatments: a treatment group (shrimp fed L-P diet); and a control group (shrimp fed non-treated diet). Three replications with 40 shrimps per replicate were used in each treatment. Feeding was conducted three times daily at 0800, 1200 and 1600 h. Total daily feed was ~10% of total body weight. Total shrimp weights and the number of shrimp in each tank were measured every three weeks.

Water samples (120 ml) were collected weekly from the center of each tank, along with shrimp feces (~200 mg), and one live shrimp once every three weeks for bacterial determination starting from the first day of the feeding trials. Water quality was monitored weekly, including pH, dissolved oxygen, temperature, salinity, ammonium, nitrate, nitrite and phosphate as described by Strickland and Parsons (1972).[23] Shrimp were dissected using sterile surgical scissors to remove the hepatopancreas and intestines for microbial enumeration and identification. All samples for bacterial determination were serially diluted in NSS and plated on nutrient agar, tryptic soy agar, MRS agar, and thiosulfate citrate bile salt agar (TCBS). All agar media included NaCl at 1% w/v. Reagents were from DIFCO Laboratories, USA. Colonies formed on plates after 24-48 h of incubation at 37°C were counted and recorded. Confirmation of each strain was microbiologically re-examined by Gram staining, spore staining, and selected biochemical tests using standard techniques.[21]

After feeding for 100 days, shrimp in each treatment were challenged with *Vibrio harveyi* D331, kindly provided by the Shrimp Culture Research Center, Charoen Pokphand Feedmill Co. Ltd., Thailand. *Vibrio harveyi* D331 is a pathogenic bacterium causing luminescent disease in *P. monodon*. Both TCBS broth and agar were used as culturing and maintaining media for *V. harveyi* D331. Immersion techniques[9] (Austin *et al.*, 1995) were used with *V. harveyi* D331 suspension and final concentrations of ~10^5 CFU ml^{-1} in tank waters at Day 0, with a second booster of 10^7 CFU ml^{-1} on Day 7. Water and shrimp samples were taken every two days from each tank for bacterial determination as described above, along with measurements of shrimp survival. After 10 days of the challenge test, shrimp in each treatment group were dissected and their internal organs were observed microscopically. Final survival was measured.

V. harveyi isolated from shrimp guts was purified and identified by examining their microbiological and biochemical characteristics, including. Gram staining, on oxidase test, and a motility test. These results were compared with the results from tests with *V. harveyi* D331. The identity of *V. harveyi* and *Vibrio* spp. were reconfirmed by following procedures described by Holt et al. (1986).[24]

The effects of L-P on shrimp growth and survival and *V. harveyi* D331 resistance, were analysed using analysis of variance and Duncan's multiple range tests.[25]

RESULTS AND DISCUSSION

All water quality values were within acceptable and safe ranges for shrimp culture (Table 1).[22] After 100 days, shrimp survival was quite low, averaging 34% in the treatment group and 16% in the control group (Fig. 1). Percent of shrimp survival calculated from the average of survival at each time point without subtacting the number of shrimp removed for microbiological study, in terms of percent. Cannibalism in clear water may have caused low survival. Furthermore, from our observations survival of shrimp in cement tank is generally quite low. No infection was found and shrimp were very healthy. Average shrimp weights of the the treatment group were significantly greater than those of the controls after 100 days (Fig. 2, $P<0.05$). It appears that L-P contributed to both increased yields and growth. This finding supports our earlier finding with other probiotics for the same purposes.[11,27]

The most useful probiotic micro-organisms for shrimp are those that grow and propagate well in the shrimp gastrointestinal

tract, producing useful end products for their host is benefit. Until now, *Lactobacillus* spp. was not known to inhabit shrimp GI tracts.[26,27] We have demonstrated, however, that *Lactobacillus* spp. (L-P) isolated from chicken intestines can colonize *P. monodon* GI tracts and function as probiotics to increase survival and growth of *P. monodon* (Fig. 1). Interestingly, only *L. casei* subsp. *tolerans* was the predominant strain in the GI tract, while other species disappeared (data not shown). *Lactobacillus* spp. were found in both tank water and shrimp guts and tank water in our control group (Figs. 3 and 4). This might be due to cross-contamination from using the same utensil for handling the diet on the first day. However, *Lactobacillus* spp. concentrations in the control group were insignificant compared with those of the treated groups. It is possible that *L. casei* subsp. *tolerans* can adapt and survive once introduced into a culture system.

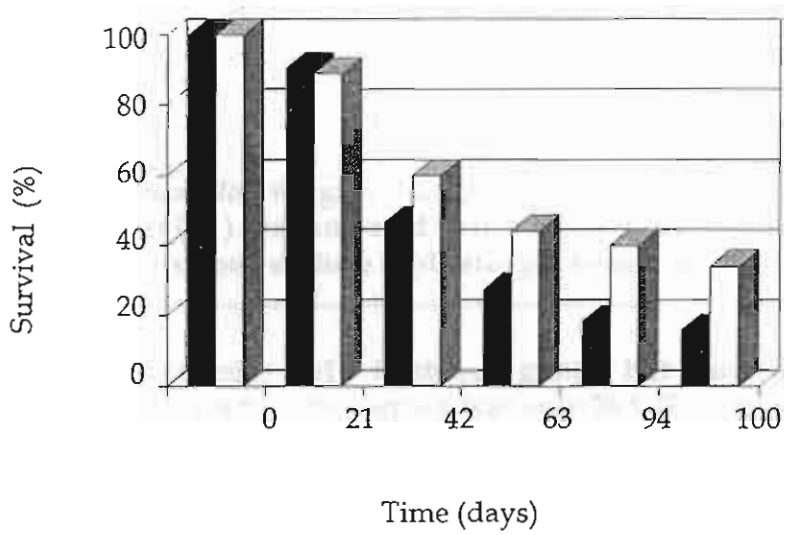


Figure 1. Average *Penaeus monodon* survival during 100 days of feeding trial. Shrimp fed non-treated diet (■) are compared with a treatment group fed a L-P diet (□). All values are means of three replicates per treatment.

Table 1. Range of water quality values in shrimp rearing tanks during 100 days of L-P diet applications.

pH	7.9-8.2
Dissolved oxygen	5-6 mg L^{-1}
Temperature	26-27 °C
Salinity	20 ppt
Ammonium	0-0.05 mg L^{-1}
Nitrite	0-2.5 mg L^{-1}
Nitrate	0-1.2 mg L^{-1}
Phosphate	2-3 mg L^{-1}

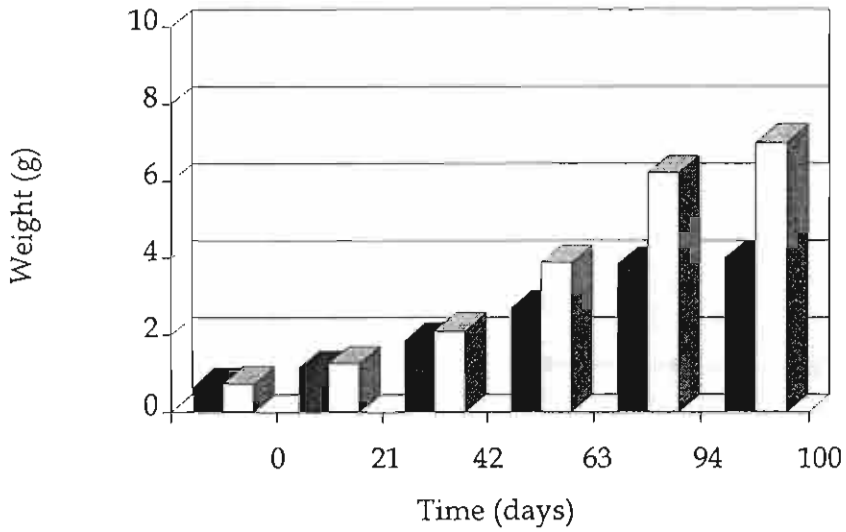


Figure 2. Average *Penaeus monodon* weights during 100 days of feeding trial. Shrimp fed non-treated diet (■) are compared with a treatment group fed a L-P diet (□). All values are means of three replicates per treatment.

It is possible that L-P could colonize and adhere to shrimp GI tract surface, leading to nearly compete exclusion of other microbes. Evidence for this conclusion includes the presence of *Vibrio harveyi* D331 (majority among *Vibrio* detection) in water and guts of the control group in much greater concentration than those of the treated group during challenge with *V. harveyi* D331 in the last 10 days (Figs. 3B and 4B). Futhermore, L-P was detected in both the guts (Fig. 3A) and feces (Fig. 6) of treatment group shrimp after feeding with the L-P diet. In addition, their end products of lactic acid (Gilliland, 1985), or some other anti-microbial substances[28-30] could tentatively interfere with or suppress the growth of *V. harveyi* D331 and lead to the indirect eradication of *V. harveyi* D331 infection. That could explain the lower numbers of *V. harveyi* D331 in guts and feces of treated shrimp compared with the controls. Obviously, *V. harveyi* D331 of 10^2 CFU g^{-1} in shrimp guts did not result in detectable disease symptoms.

During the 10 day challenge test with *V. harveyi* D331, no mortality occurred in the treatment group, but survival in the control group was only 26% (Fig. 5). Surviving shrimp in the control group looked unhealthy. These survival rates were significantly different ($p< 0.05$).

We still do not have a clear idea about exactly how probiotics protect shrimp and improve their survival and growth. We have demonstrated these benefits here and in our other findings, but we are uncertain whether the protective mechanism involves competitive exclusion of harmful microbes, or immune response by the shrimp.

We also need to evaluate *Lactobacillus* spp. applications during the entire culture cycle in ponds. Application times and dosages, shelf life of the probiotics, and other factors an in open cycle system need to be evaluated before large scale commercial applications are made. However, our work clearly demonstrates potential probiotic benefits of *Lactobacillus* spp. for control of certain serious diseases in *P. monodon*.

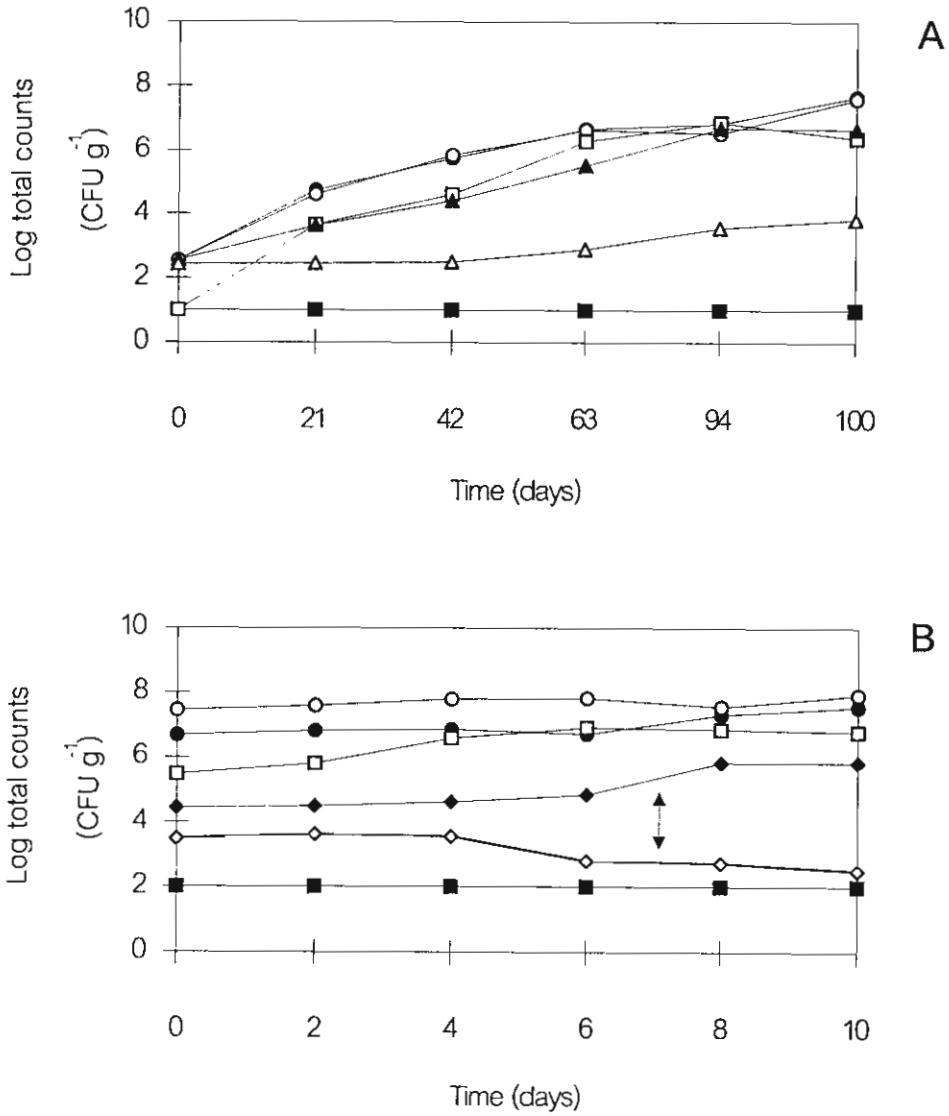


Figure 3. Average bacterial counts in shrimp guts (hepatopancreas and intestine) for: (A) shrimp cultured for the first 100 days on feeding trial and (B) shrimp cultured for the last 10 days during challenge with *V. harveyi* D331 (↕ 2nd booster with *V. harveyi* D331). Total bacteria (o), *Lactobacillus* spp. (□), *Vibrio* spp. (Δ), and *Vibrio harveyi* D331 (◊). Closed symbols represent mean values for controls whereas open symbols represent mean values for the treatment group. Each value is the mean of three replicates per treatment.

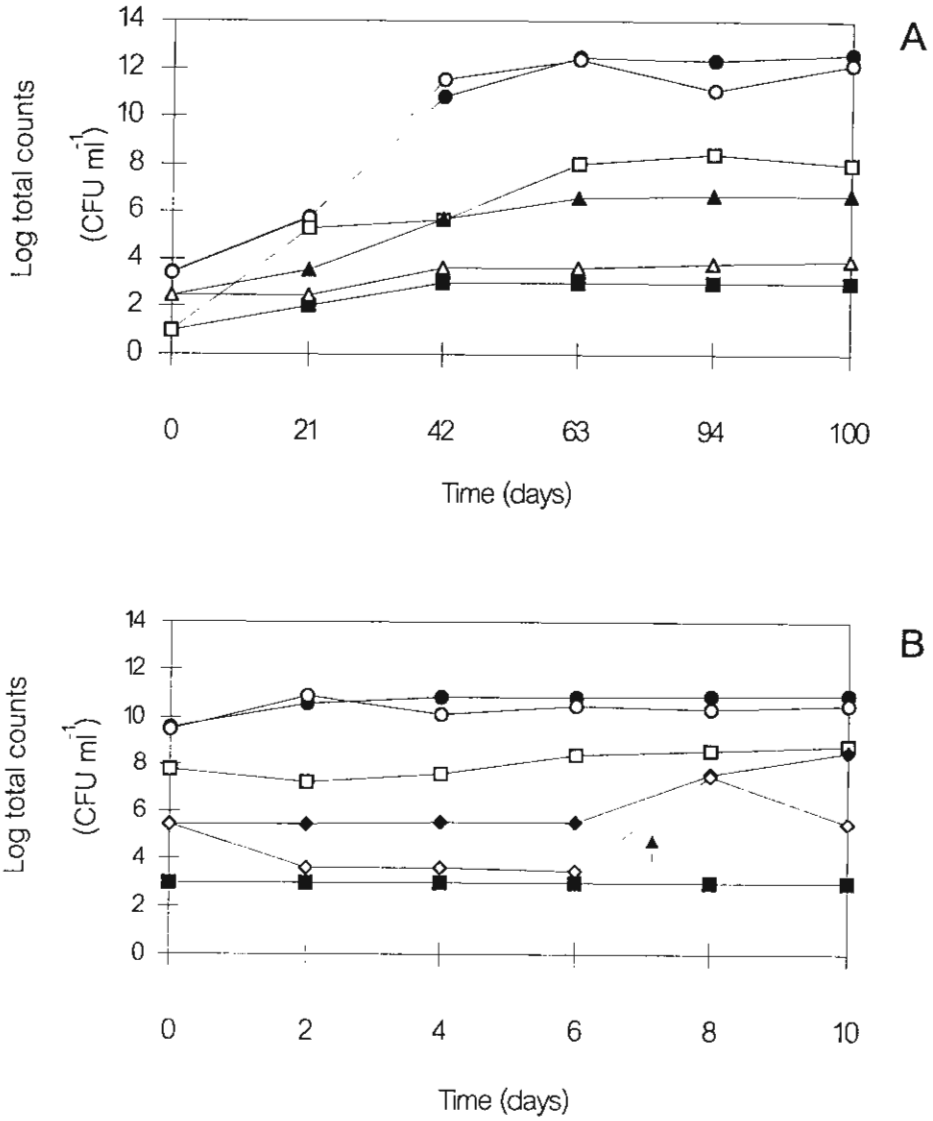


Figure 4. Bacterial counts in rearing tank water for: (A) shrimp cultured for the first 100 days on feeding trial; and (B) shrimp cultured for last 10 days during challenge with *V. harveyi* D331 (↑ 2nd booster with *V. harveyi* D331). Total bacteria (o) , *Lactobacillus* spp. (□) , *Vibrio* spp. (Δ) and *Vibrio harveyi* D331 (◊). Closed symbols represent mean values for controls whereas open symbols represent mean values for the treatment group. Each value is the mean of three replicates per treatment.

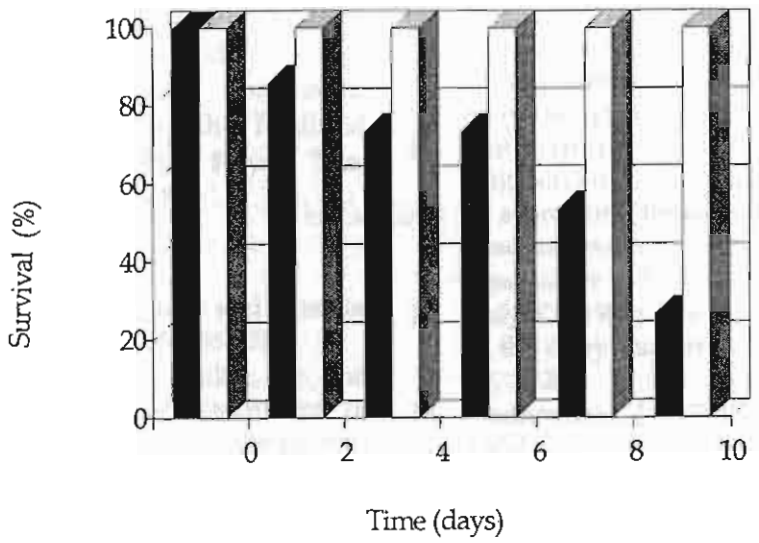


Figure 5. Average *Penaeus monodon* survival during 10-day challenge with *Vibrio harveyi* D331. Shrimp fed non-treated diet (■) are compared with a treatment group fed a L-P diet (□). All values are means of three replicates per treatment.

CONCLUSIONS

Lactobacillus spp. and especially *L. casei* ubsp. *torelans* can be used as probiotics for feeding black tiger shrimp, *Penaeus monodon*, leading to higher growth and survival. *Lactobacillus* spp. demonstrates potential of control *Vibrio harveyi* in the shrimp GI tract and provides highly healthy shrimp protected against such diseases.

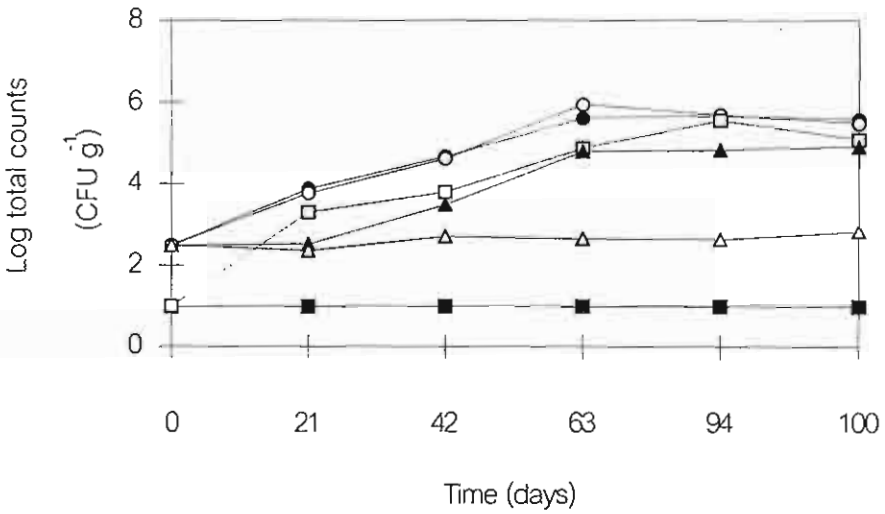


Figure 6. Bacterial counts in shrimp feces during 100 days of feeding trial. Total bacteria (o), *Lactobacillus* spp. (□), and *Vibrio* spp. (Δ). Closed symbols represent mean values for controls whereas open symbols represent mean values for the treatment group. Each value is the mean of three replicates per treatment.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Arlo W. Fast for revising the manuscript and providing many valuable suggestions and comments. This research was supported by the Thailand Research Fund (TRF) and The Royal Thai Government Budget for Research.

REFERENCES

1. Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology* **66**, 365-378.
2. Barrow, P.A., Brooker, B.E., Fuller, R. and Newport, M.J. (1980) The attachment of bacteria to the gastric epithelium of the pig and its importance in the microecology of the intestine. *Journal of Applied Bacteriology* **48**, 147-154.
3. Hill, J.R., Kenworthy, R. and Porter, P. (1970) Studies of the effect of dietary lactobacilli on intestinal and urinary amines in pigs in relation to weaning and post-weaning diarrhoea. *Research in Veterinary Science* **11**, 320-326.
4. Muralidhara, K.S., Sheggeby, G.G., Elliker, P.R., England, D.C. and Sandine, W.E. (1977) Effect of feeding lactobacilli on the coliform and *Lactobacillus* flora of intestinal tissue and feces from piglets. *Journal of Food Protection* **40**, 288-295.
5. Ratcliffe, B., Cole, C.B., Fuller, R. and Newport, M.J. (1986) The effect of yoghurt and milk fermented with a porcine intestinal strain of *Lactobacillus reuteri* on the performance and gastrointestinal flora of pigs weaned at two days of age. *Food Microbiology* **3**, 203-211.
6. Barrow, P.A., Simpson, J.M. and Lovell, M.A. (1988) Intestinal colonization on the chicken by food-poisoning *Salmonella* serotypes. Microbial characteristics associated with faecal excretion. *Avian Pathology* **17**, 571-588.
7. Fuller, R., Houghton, S.B. and Brooker, B.E. (1981) Attachment of *Streptococcus faecium* to the duodenal epithelium of the chicken and its importance in colonization of the small intestine. *Applied and Environmental Microbiology* **41**, 1433-1441.
8. Thai Department of Business Economics (1996) Export value of Thai frozen shrimp 1986-1996. *Shrimp Culture Newsletter* **96**, 1-4.
9. Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I. and Griffith, D.R.W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Diseases* **18**, 93-96.
10. Sugita, H., Matsuo, N., Shibuya, K. and Deguchi, Y. (1996) Production of antibacterial substances by intestinal bacteria isolated from coastal crab and fish species. *Journal of Marine Biotechnology* **4**, 220-223.
11. Rengpipat, S., Phianphak,W., Piyatiratitivorakul, S. and Menasveta, P. (1998) Effect of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* **167**, 301-313.
12. Daly, C. (1983) The use of mesophillic cultures in the dairy industry. *Antonie van Leeuwenhoek* **49**, p. 297.
13. Stadhouders, J. (1974) Dairy starter cultures. *Milchwissenschaft* **29**(6), p. 329.
14. Jay J.M. (1992) Fermented foods and related products of fermentation. In: *Modern Food Microbiology*, Fouth edition, (ed. J. L. Jay) Van Nostrand Reinhold, New York. pp. 371-409.
15. Gilliland, S.E. (1985) Role of starter culture bacteria in food preservation. In: *Bacterial Starter Cultures for Food* (ed. S. E. Gilliland), CRC Press, Boca Raton, FL. p.175.
16. Gilliland, S.E., Bruce, B.B., Bush, L.J. and Stanley, T.E. (1980) Comparison of two strains of *Lactobacillus acidophilus* as dietary adjuncts for young calves. *Journal of Dairy Science* **63**, 964-972.
17. Gilliland, S.E., Nelson, C.R. and Maxwell, C. (1985) Assimilation of cholesterol by *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* **49**, 377-381.
18. Garvie, E.I., Cole, C.B., Fuller, R. and Hewitt, D. (1984) The effect of yoghurt on some components of the gut microflora and the metabolism of lactose in the rat. *Journal of Applied Bacteriology* **56**, 237-245.
19. Jonsson, E. and Henningsson, S. (1991) Establishment in the piglet gut of lactobacilli capable of degrading mixed-linked β-D-glucans. *Journal of Applied Bacteriology* **70**, 512-516.
20. Thanaruttikannont, T. (1996) Use of lactic acid bacteria as probiotic supplement in chicken feed. M.S.Thesis, Chulalongkorn University, Thailand, 133 pp.
21. Kandler, O. and Weiss, N. (1986) Regular, nonsporing gram-positive rods. In: *Bergey's Manual of Systematic Bacteriology* Volume 2 (eds. P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt) Williams & Wilkins, Baltimore, USA. pp. 1208-1260.
22. Menasveta, P., Fast, A.W., Piyatiratitivorakul, S. and Rungsupa, S. (1991) An improved closed seawater recirculation maturation system for

giant tiger prawn (*Penaeus monodon* Fabricius). *Aquacultural Engineering* **10**, 173-181.

23. Strickland, J.D.H. and Parsons T.R. (1972) *A Practical Handbook of Seawater Analysis*, Second edition. Fisheries Research Board of Canada Bulletin No.167, Alger Press, 310 pp.
24. Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., 1986. Facultatively anaerobic gram-negative rods. In: *Bergey's Manual of Determinative Bacteriology*, Ninth edition. Williams and Wilkins, Baltimore, pp. 175-289.
25. SAS Institute (1983) *The Statistics Analysis System*. SAS Institute Inc., Gary, Indiana, USA, 93 pp.
26. Dempsey, A.C. and Kitting, C.L. (1987) Characteristics of bacteria isolated from penaeid shrimp. *Crustaceana* **52**, 90-93.
27. Phianphak, W. (1996) Use of bacteria as probiotic supplement in shrimp feed M.S. Thesis, Chulalongkorn University, Thailand. 123 pp.
28. Gilliland, S.E. and Speck, M.L. (1977) Antagonistic action of *Lactobacillus acidophilus* towards intestinal and food borne pathogens in associative culture. *Journal Food Protection* **40**, 820-823.
29. Toba, T., Yoshioka, E. and Itoh, T. (1991a) Lacticin, a bacteriocin produced by *Lactobacillus delbrueckii* subsp. *lactis*. *Letters in Applied Microbiology* **12**, 43-45.
30. Toba, T., Yoshioka, E. and Itoh, T. (1991b) Acidophilucin A, a new heat-labile bacteriocin produced by *Lactobacillus acidophilus* LAPT 1060. *Letters of Applied Microbiology* **12**, 106-108.

Received: January 19, 1999
Accepted: April 21, 1999

Probiotics in Aquaculture: A Case Study of Probiotics for Larvae of the Black Tiger Shrimp (*Penaeus monodon*)

*[1]Sirirat Rengpipat, [1]Sombat Rukpratanporn
[2]Somkiat Piyatiratitivorakul, [2]Piamsak Menasveta

[1]Department of Microbiology and [2]Department of Marine Science, Faculty of Science
Chulalongkorn University, Bangkok 10330, Thailand
[2]Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: The trend of using probiotics in aquaculture is increasing due to research results indicating their ability to increase production and prevent disease in farm animals. The development of suitable probiotics for biocontrol in aquaculture would result in less reliance on chemicals and antibiotics and result in a better environment. In this investigation, a Thai *Bacillus* isolate (strain S11) was used as a probiotic bacterium by passage through *Artemia* sp. fed to the black tiger shrimp (*Penaeus monodon*). It was found that black tiger shrimp larvae reared using the *Bacillus*-fortified *Artemia* probiotic as a feed had significantly shorter development times and fewer disease problems than larvae reared without the probiotic.

Key words: *Penaeus monodon*, black tiger shrimp larvae, probiotics

INTRODUCTION

Microbes and aquaculture

Microbes play both direct and indirect roles in aquaculture. They are important causes of diseases which may readily spread through water to aquatic animal hosts. As flora in soil and water, they also influence the aquatic environment by involvement in C, N, S and P cycles important for ecological balances. Other microbes live in and on aquatic plants and animals. These may be specific for individual organisms and important to their health. An inbalance in the microbial flora in the water or in these organisms often leads to pathogenesis. For example, an inbalance in *Vibrio* species in the rearing water or in the GI tracts of shrimp and fish can lead to pathogenesis (Rengpipat 1996).

Marine culture of shrimp, crabs, fish, oysters and mussels in Thailand provides good income for producers and products that are popular because of their good taste and reasonable price. The diminishing seafood from capture fisheries has paved the way to industrial scale aquaculture. In Thailand, particularly for the black tiger shrimp (*Penaeus monodon*), most farmers build large ponds and raise shrimp intensively. Even though the production has been good for the past 10 years (Thai Department of Business Economics 1996), the trend for the last 2-3 years has been diminished production due to viral diseases such as yellow head virus and white spot syndrome virus (WSSV) (also called systemic ectodermal and mesodermal baculovirus or SEMBV) and to luminescent bacterial disease (Rengpipat 1996). These agents can cause sudden death on a massive scale.

Probiotic microbes in aquaculture

For more than 50 years, beneficial microbes defined as probiotics (Fuller, 1989, 1992 and 1997) have been used successfully for raising healthy and disease-tolerant farm animals like swine (Baird 1977; Pollman et al. 1980) and chickens (Dilworth & Day 1978; Miles et al.1981). These probiotics are now widely used for enhancing production of land animals and they have gained acceptance as being better, cheaper and more effective in promoting animal health than administration of antibiotics or chemical substances.

More recently (within the past 10 years) researchers have sought beneficial microbes for aquaculture by attempting to isolate from seawater, sediments and GI tracts those capable of producing antibiotics and/or antimicrobial substances that can inhibit pathogens *in vitro* (Table 1). Bacteria and unicellular algae capable of inhibiting pathogenic bacteria have been found (Munro et al. 1995; Austin and Day 1990). In setting criteria for the most suitable probiotics in aquaculture, one must be concerned with indirect effects on ecosystem cycles and food chains.

Douillet and Langdon (1994) used a commercial probiotic bacterium (CA2) as a larval feed supplement to increase production in oysters. Other researchers have found that probiotics prevent diseases in salmon (Austin et al. 1992 and 1995), larvae of scallop (Requelme et al. 1997) and black tiger shrimp (Rengpipat et al. 1998 and unpublished data). Austin et al. (1992) stressed that probiotics control diseases by prophylaxis, and that they are not meant to be used as

Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S, Menasveta P (1998) Probiotics in Aquaculture: A Case Study of Probiotics for Larvae of the Black Tiger Shrimp (*Penaeus monodon*). *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: sirirat@mail.sc.chula.ac.th

Table 1. Reduction of pathogens by microorganisms that possess probiotic properties at *in vitro*.

Aquatic animal	Microorganisms	Pathogens	References
Fish	Antibiotic-producing marine bacteria (Obligate halophilic bacteria)	*Aeromonas hydrophila* B-32 *A. salmonicida* ATCC 14174	Dopazo et al. (1988)
Prawns	*Tetraselmis suecica*(microalgae)	*Vibrio alginolyticus* *V. anguillarum* *V. parahaemolyticus* *V. vulnificus*	Austin and Day (1990)
Fish	*Planococcus*	*Serratia liquefaciens*	Austin and Billaud (1990)
Fish	Gram -ve rod Gram +ve rod	*V. anguillarum* HI 11345 *A. salmonicida* *A. hydrophila*	Westerdahl et al. (1991)
Fish (Turbot larvae)	*Flavobacterium* sp. *V. fluvialis* *V. natvigens* *Vibrio* spp.	*Pavlova lutheri* (unicellular algae)	Munro et al. (1995)

Table 2. Probiotics and feed supplements used in aquaculture.

Aquatic animal	Probiotic strain	Challenge test with	Results	References
Salmon	*Tetraselmis suecica*	*A. salmonicida* *A. hydrophila* *Lactobacillus* spp. *S. liquefaciens* *V. anguillarum* *V. salmonicida* *Yersinia ruckeri* type I	-good control of diseases by Prophylaxis	Austin et al. (1992)
Oyster (larval culture)	CA2	N.D.	-better yield	Douillet and Langdon (1994)
Salmon	*V. alginolyticus*	*A. salmonicida* *V. anguillarum* *V. ordalii*	-good control of disease	Austin et al. (1995)
Salmon	*L. plantarum* (lyophilized form) could inhibit *V. anguillarum*	*A. salmonicida*	-*Lactobacillus* colonized intestinal wall -could not control disease	Gildberg et al. (1995)
Scallop (larval stage)	*Vibrio* spp. *Pseudomonas* sp.	*V. anguillarum* related (VAR)	- good control of disease	Riquelme et al. (1997)
Black tiger shrimp	*Bacillus* strain S11	*V. harveyi*	- better yield - good control of disease	Rengpipat et al. (1998)
Black tiger shrimp	*Lactobacillus* spp.	*V. harveyi*	- better yield - good control of disease	Rengpipat et al. (unpublished data)

therapeutics. For example, *Lactobacillus plantarum* inhibitory to the salmon pathogen *Vibrio anguillarum* could not be used to treat fish infected with the lethal pathogen, *A. salmonicida*. However, lyophilized *Lactobacillus plantarum* fed to salmon was shown to be able to survive in the GI tract (Gildberg et al, 1995).

Probiotics and black tiger shrimp culture

Rengpipat et al. (1998) isolated *Bacillus* strain S11 from the GI tract of *Penaeus monodon* broodstock caught in the Gulf of Thailand. It inhibited the luminescent disease bacterium, *Vibrio harveyi*, 100% and could promote better yields of black tiger shrimp. Mixed *Lactobacillus* species isolated

from the GI tract of local Thai chickens was also used as a feed supplement to black tiger shrimp (Rengpipat et al., unpublished data) and also resulted in higher shrimp production (Table 2).

Probiotics can be freshly prepared and mixed with the shrimp diet as described by Rengpipat et al. (1998). However, this study was carried out to determine the effectiveness of feeding the *Artemia* encapsulated probiotic *Bacillus* strain S11 for enhancing growth and survival of shrimp larvae.

MATERIALS AND METHODS

The present study was conducted at the aquaculture laboratory, Department of Marine Science and at the Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

Bacterial culture

Bacillus strain S11 (Rengpipat et al. 1998) was stocked on tryptic soy agar (TSA) (Difco) and cultured in tryptic soy broth (TSB) (Difco). Culture conditions were at 37°C in 2-L flasks for 24 h, after which the cells were centrifuged and washed in sterile normal saline solution (NSS) three times immediately before use.

Preparation of encapsulated probiotic *Artemia* sp.

Artemia cysts (Great Lake Artemia, Salt Lake City, Utah, U.S.A.) were hatched (1 g of cysts per liter gently aerated 30 ppt seawater) and harvested at 24 h. During harvesting the cysts and nauplii were seperated. The nauplii were then fed directly to the shrimp postlarvae or kept at 4°C for further use. For probiotic encapsulation, freshly prepared cell cultures of *Bacillus* strain S11 were added to *Artemia* cultures at the begining of the hatching process at a concentration of 10^4 cells/ml. The *Artemia* were subsequently harvested at 24 h and fed immediately to the shrimp postlarvae.

Experimental design

Penaeus monodon postlarvae-10 were bought from a backyard hatchery in Chonburi Province, Thailand. The postlarvae were from a single parent. After acclimatization at the laboratory for 5 days, uniform size postlarvae were selected for probiotic testing.

Experimental units for postlarvae rearing comprised 10-L cylindrical fiber glass tanks containing 7.5-L of 25 ppt seawater and an inititial stocking of 50 postlarvae. Three replicates were used for a total of 150 postlarvae per treatment. Each rearing unit was a closed recirculating system with self-contained filtering unit consisting of sand and oyster shells. Water was continuously pumped through the filtering unit by an air lift system. A small amount of dechlorinated tap water was added every two days to compensate for evaporation and maintain a constant salinity.

Statistical comparisons of the control group (fed *Artemia* only) and the treated group (fed probiotic *Artemia*) were carried out using a student t-test. The experiment was monitored for two weeks. Shrimp were fed three times daily at 9:00, 13:00 and 18:00 h with an excess of *Artemia* nauplii. All materials used for each experimental unit were separated to avoid any cross contamination.

Lengths and weights of 15 randomly selected shrimp from each tank were recorded weekly. Shrimp survival was also determined in each tank at the end of the first and second weeks. Weekly water samples of 100 ml were collected from the center of each tank for two weeks. Water quality was monitored weekly and included the parameters of temperature, pH, salinity, dissolved oxygen, ammonium ion and phosphate ion measured using techniques described by Strickland and Parsons (1972).

Pathogen challenge test

After feeding for two weeks, shrimp were challenged with the luminous bacterium *V. harveyi* D331 which had been cultured and maintained using thiosulphate citrate bile salt TCBS broth and agar (Difco). Shrimp in the control and treated groups (85 shrimp per treatment) were immersed in a suspension of *V. harveyi* D 331 at ~ 10^7 CFUml^{-1} according to Austin et al. (1995). Shrimp survival was determined after 4 days of challenge. At the same time, three shrimp from each treatment were randomly sampled. Each whole shrimp was cut into small pieces using sterile surgical sciccors and transferred to a sterile tube. Bacterial determinations were then made using serial dilutions in NSS, followed by plating on TSA and TCBS agar. After 24-48 h of incubation at 37°C, colonies were counted and recorded. Microbial strains from TSA were re-examined using Gram staining, spore staining and selected biochemical tests as described by Sneath (1986). *V. harveyi* cultures isolated from shrimp were purified and identified using Gram staining, an oxidase test and motility test and they were compared with the original *V. harveyi* D331 culture. *V. harveyi* D331 culture was kindly provided by the Shrimp Culture Research Center, Charoen Pokphand Feedmill Co. Ltd., Samutsakorn, Thailand. We reconfirmed the identity of *V. harveyi* by following procedures described by Holt et al. (1986). Shrimp survival was determined for each treatment after 4 days of the challenge test.

RESULTS AND DISCUSSION

Bacillus strain S11 showed no inhibitory effect on *Artemia* hatching when compared to *Artemia* alone or *Artemia* fed with *Saccharomyces cerevisiae*. *Artemia* nauplii at ~1.84×10^5 g^{-1} were counted after hatching for 24 h. *Bacillus* strain S11 on *Artemia* nauplii were found to be ~2×10^2, ~6.4×10^4 and ~1×10^2 CFU g^{-1} (wet weight) at 0, 4 and 8 h, respectively, after hatching.

The raising of black tiger shrimp postlarvae using *Artemia* encapsulated *Bacillus* strain S11 showed an increase in body weight and length (Table 3). No obvious effects of *Bacillus* strain S11 on water quality were found (Table 4). During the first week, however, ammonium increased to 1.67 mgL^{-1} in one control group, but later decreased to near zero. At two weeks, *Penaeus monodon* survival was significantly different between the control group (85%) and the treated group (89%)(Figure 1).

Table 3. Average live weight and length of *Penaeus monodon* cultured for 2 weeks in two feed treatments

Parameters	Control	Probiotics
Weight (mg)	26.0*	43.8*
Length (cm)	$1.71^{b} \pm 0.20$	$1.83^{a} \pm 0.31$

Control: shrimp with artemia; Probiotics: shrimp with *Bacillus* strain S11- fed artemia; *Total weight divided by a number of shrimp (85 shrimp); [b,a] Different superscripts in the same row significantly different

Table 4. Range of water quality values in shrimp culture water during 2 weeks of probiotic trial.

	Range of water quality values	
Parameter	Control	Probiotics
Temperature (°C)	29.5	29.5
pH	7.79 - 8.23	7.78 - 8.22
Salinity (ppt)	25	25
Dissolved oxygen (mg L^{-1})	7.9 - 8.1	8.0 - 8.1
Ammonium (mg L^{-1})	0 - 1.67	0 - 0.5
Phosphate (mg L^{-1})	3	3

Control : shrimp with artemia
Probiotics : shrimp with *Bacillus* strain S11- fed artemia

When challenged with the luminous disease bacterium *Vibrio harveyi*, the shrimp treated with probiotics showed a higher survival (13%) when compared to the control group (4%) (Figure 2). *Vibrio harveyi* was more virulent to younger stages of shrimp. High numbers of *Vibrio harveyi* were present in both the rearing water and the shrimp themselves on the fourth day of this experiment (Table 5). However, *Bacillus* strain S11 was also detected in significant numbers (10^5-10^6 CFU ml^{-1} or g^{-1}) in the rearing water and the shrimp, clearly showing that *Artemia* was an effective probiotic carrier.

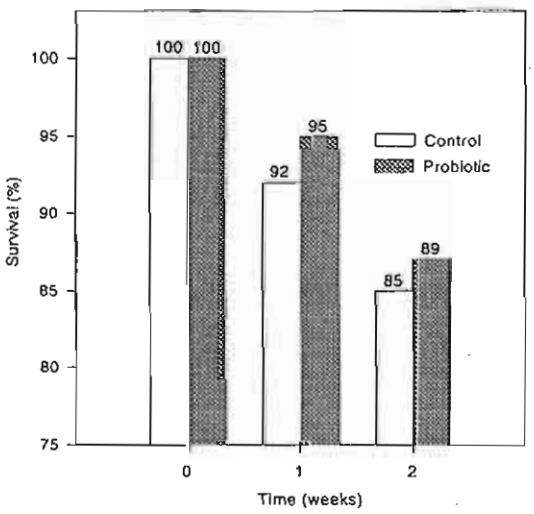


Figure 1. *Penaeus monodon* survival after culture for 2 weeks on control and probiotic feeds.

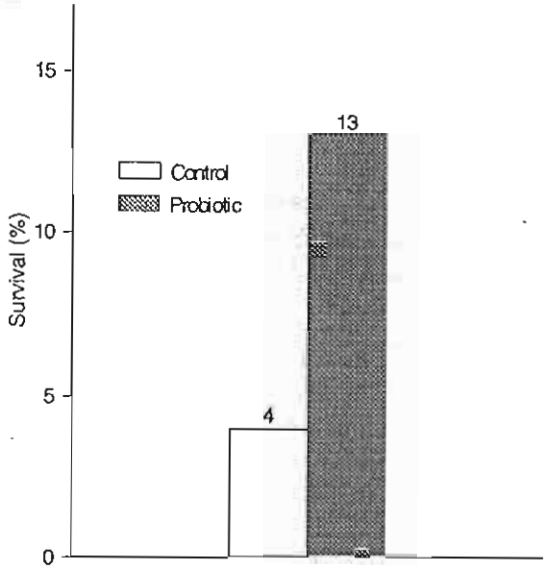


Figure 2. Percentage survival of *Penaeus monodon* after challenge with *Vibrio harveyi* D331.

Bacillus strain S11 is considered a saprophytic strain which is environmental-friendly and has been proven before as a probiotic bacterium for black tiger shrimp (Rengpipat et al., 1998) when mixed with shrimp feed. Therefore, this investigation supported the previous work and also showed that probiotics could be passed through *Artemia* which are routinely used to feed shrimp larvae. Our method may prove beneficial as an enhancement for hatchery postlarvae or for improvement of young shrimp survival at the initial stages of earthen pond culture.

Mechanisms of probiotic action in the host are not fully understood. However, a user may select strains that are suitable or specific for a particular host and environmentally safe. The purpose of their use in aquaculture is to reduce the dependence on antibiotics and chemicals, thus improving environmental safety. Use of local isolates is recommended for biosafety reasons and to avoid sudden changes in the microbial flora of the ecosystem.

ACKNOWLEDGEMENTS: The authors are grateful to Dr. Hunsa Punnapayak for proof reading. This research was supported by the Thailand Reseach Fund (TRF) and The Royal Thai Government Budget for Research.

LITERATURE CITED

Austin B, Billaud AC (1990) Inhibition of the fish pathogen, *Serratia liquefaciens*, by an antibiotic-producing isolate of *Planococcus* recovered from sea water. J Fish Dis 13:553-556

Austin B, Day JG (1990) Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture 90:389-392

Austin B, Baudet E, Stobie M (1992) Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. J Fish Dis 15:55-61

Austin B, Stuckey LF, Robertson PAW, Effendi I, Griffith DRW (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J Fish Dis 18:93-96

Table 5. Average bacterial counts in water and whole shrimp during challenged with *V. harveyi* D331 by immersion

		V. harveyi (CFU ml^{-1} or g^{-1})		*Bacillus* strain S11 (CFU ml^{-1} or g^{-1})	
		Control	Probiotics	Control	Probiotics
Water	- day 1	$1.28x10^7$	$1.31x10^7$	0	75
	- day 4	$1.07x10^6$	$3.64x10^5$	0	50
Shrimp	- day 1	$6.36x10^6$	$6.09x10^6$	0	$1.20x10^3$
	- day 4	TNTC	TNTC	0	$2.76x10^2$

Control : shrimp with artemia; Probiotics : shrimp with *Bacillus* strain S11- fed artemia
TNTC : Too numerous to count

Baird DM (1977) Probiotics help boost feed efficiency. Feedstuffs 49:11-12

Dilworth BC, Day EJ (1978) Lactobacillus cultures in brooder diets. Poultry Science 57:1101

Dopazo CP, Lemos ML, Lodeiros C, Bolinches J, Barja JL, Toranzo AE (1988) Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J Appl Bacteriol 65:97-101

Douillet PA, Langdon CJ (1994) Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). Aquaculture 119:25-40

Fuller R (1989) Probiotic in man and animals. J Appl Bacteriol 66: 365-378

Fuller R (1992) Probiotics: The scientific basis. 1st ed. London: Chapman & Hall 398 pp.

Fuller R (1997) Probiotics 2 Applications and practical aspects. 1st ed. London: Chapman & Hall 211 pp.

Gildberg A, Johansen A, Bogwald J (1995) Growth and survival of Atlantic salmon (*Salmo salar*) fry given diets supplemented with fish protein hydrolysate and lactic acid bacteria during a challenge trial with *Aeromonas salmonicida*. Aquaculture 138:23

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1986) Facultatively anaerobic Gram-negative rods. In: Bergey's manual of determinative bacteriology. ninth edition, William&Wilkins, Baltimore, pp 175-289.

Miles RD, Arafa AS, Harms RH Carlson CW Reid BL, Crawford JS (1981) Effects of a living non-freeze dried *Lactobacillus acidophilus* culture on performance, egg quality and gut microflora in commercial layers. Poultry Science 60;993-1004

Munro PD, McLean HA, Barbour A, Birkbeck TH (1995) Stimulation or inhibition of growth of the unicellular alga *Pavlova lutheri* by bacteria isolated from larval turbot culture systems. J Appl Bacteriol 79:519-524

Pollman DS, Danielson DM, Peo ER, (1980) Effect of microbial feed additives on performance of starter and growing-finishing pigs. J Anim Sci 51:577-581

Rengpipat S, (1996) Microbes and black tiger shrimp culture. Thai J Aquat Sci 3:42-51

Rengpipat S, Phianpak W, Piyatiratitivorakul S, Menasveta P (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture (in press)

Rengpipat S, Phianpak W, Piyatiratitivorakul S, Menasveta P Utilization of *Lactobacillus* spp. as probiotics for feeding *Penaeus monodon*, black tiger shrimp. (unpublished data)

Riquelme C, Araya R, Vergara N, Rojas A, Guaita M, Candia M (1997) Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). Aquaculture 154:17-26

Sneath, PHA (1986) Endospore-forming Gram-positive rods and cocci. In: PHA Sneath, NS Mair, ME Sharp and JG Holt (Editors), Bergey's manual of systematic bacteriology. Vol 2 Baltimore:Williams&Wilkins, pp.1104-1105

Strickland JDH , Parsons TR (1972) A practical handbook of seawater analysis. 2nd edition. Fisheries Research Board of Canada Bull., 167. Alger Press, 310pp.

Thai Department of Business Economics (1996) Export value of Thai frozen shrimp 1986-1996. Shrimp Culture Newsletter 96:1-4

Westerdahl A, Olsson JC, Kjelleberg S, Conway PL (1991) Isolation and characterisation of Turbot (*Scophtalmus maximus*)-associated bacteria with inhibitory effects agianst *Vibrio anguillarum*. Appl Environ Microbiol 57:2223-2228

A Genetic Marker Application with Giant Tiger Shrimp (*Penaeus monodon*) for Closed Life-Cycle Culture

Wannalux Wudthijinda[1], Padermsak Jarayabhand[2,3,4*] and Piamsak Menasveta[2,4]

[1]Department of Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand

[2]Aquatic Resources Research Institute, Chulalongkorn University, Bangkok, 10400, Thailand

[3]Angsila Marine Biological Research Station, Department of Marine Science, Faculty of Science, Chulalongkorn University, Chon Buri, 20210, Thailand

[4]Marine Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Bangkok, 10400, Thailand

Key words: structure, *Penaeus monodon*, DNA markers, RAPD-PCR, Selective breeding program

*corresponding author; at address: Aquatic Resources Research Institute, Chulalongkorn University, Bangkok, 10330, Thailand; Tel: +66-2-2185400; Fax: +66-2-2547680; e-mail: jpaderms@chula.ac.th

Running title: Genetic population structure in *Penaeus monodon*

Abstract

Genetic diversity of giant tiger shrimp (*Penaeus monodon*) was examined by randomly amplified polymorphic DNA (RAPD) analysis in shrimp collected from five areas: Chumphon and Trat (Gulf of Thailand); and Phangnga, Satun and Trang (Andaman Sea). A total of 53 polymorphic fragments from three selected primers (UBC 268, UBC 273, and UBC 299) were scored across all shrimp samples. Twenty-six, thirty and thirty-two genotypes were generated from the respective primers, while average number of scorable bands was 13.7 to 15.3 per primer. Percentage of polymorphic fragments within a sample ranged from 46.7% (Trang) to 61.4% (Trat). A 260 bp RAPD fragment generated by the primer UBC 268 was observed in 92.3% and 5.0% of Trat and Chumphon *P. monodon*, respectively. Population differentiation of *P. monodon* between the Andaman Sea and Gulf of Thailand was clearly demonstrated ($P < 0.0001$). Based on our genetic analyses, *P. monodon* from our five sampling locations are from three different stocks: A (Trat); B (Chumphon); and C (Phangnga, Satun and Trang).

Introduction

Giant tiger shrimp (*Penaeus monodon*) is one of the world's most economically important, cultured crustaceans. Annual production of farmed *P. monodon* in Thailand alone equaled or exceeded 200,000 metric tons since 1993 (Asian Shrimp Culture Council, 1996). Still, *P. monodon* farming relies almost entirely on ocean caught females for farm seed supply. This open reproductive cycle and reliance on wild stocks of *P. monodon* results in heavy exploitation of female broodstock from wild populations (Klinbunga, 1996). Perhaps as importantly, at least for the shrimp culture industry, selective breeding for improved culture performance, disease resistance, and other desirable traits cannot be achieved until this species is domesticated and seed are mass produced using closed life-cycle, captive populations (Jarayabhand et al., 1998). This will require use of high quality, pond reared broodstock rather than ocean caught broodstock (Benzie et al., 1994).

Throughout Thailand, almost all female *P. monodon* broodstock used in hatcheries are taken from the Andaman Sea, especially from Phuket, Satun and Trang provinces. This situation resulted from farmer demands, since farmers believe that progeny of Andaman Sea *P. monodon* exhibit greater survival and possibly greater growth rates than do progeny from broodstock shrimp caught elsewhere in Thailand (Sodsuk, 1996). As a result, broodstock prices for female *P. monodon* from the Andaman Sea are 3 to 5 times greater than for those from the Gulf of Thailand (Klinbunga, 1996).

There could be sound genetic bases for farmer preference of seed stocks. Klinbunga et al. (1999) measured genetic diversity in *P. monodon* from Satun (Andaman Sea), and from Surat and Trat (Gulf of Thailand) using mtDNA-RFLP analysis. They found large genetic differences between paired samples from each coast (Satun-Surat and Satun-Trat). Geographic heterogeneity analysis clearly illustrated isolated, genetic population structures between *P. monodon* from the Andaman Sea and from the Gulf of Thailand ($P < 0.0001$). However, comparative culture performance evaluations of these different stocks has not yet been conducted.

Identification of different shrimp stocks, followed by comparative growout performance evaluations of these stocks in commercial settings are important first steps in selecting shrimp stocks for domestication and selective breeding. Ideally, these comparisons should include co-culture of different stocks in the same ponds. These comparisons require identification and use of population markers such that the population origin of each shrimp can be readily determined.

Objectives of our current study include selecting a genetic population marker, and then using the marker to identify genetic population structure of conspecific *P. monodon* in Thailand. Thereafter, we intend to use these markers to compare growth and survival of different population groups in commercial culture settings. We chose randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) for genetic marker identification because it is a simple and rapid method for generating genetic markers, and it has the added advantage that no prior knowledge is required of the genome under study (William et al., 1990; Hadrys et al., 1992). We will also

apply these techniques and findings to our recently established, closed life-cycle breeding program for *P. monodon* (Jarayabhand et al., 1998).

Materials and Methods

Sampling. One hundred and thirty-seven *P. monodon* were captured live from five locations: Satun (N = 26), Trang (N = 18), and Phangnga (N = 27) in the Andaman Sea; and Chumphon (N = 40), and Trat (N = 26) in the Gulf of Thailand (Fig. 1). Pleopods were removed from each shrimp and immediately placed on dry ice before storage at -80°C.

DNA extraction. Genomic DNA was extracted from frozen pleopods using the SDS-phenol/chloroform method (Klinbunga et al., 1996). Extracted DNA concentrations were spectrophotometrically determined using a SPECTRONIC® Genesys™ 5 spectrophotometer (MILTON ROY).

PCR amplification and agarose gel electrophoresis. Three decanucleotide primers (UBC 268, 5'-AGGCCGCTTA-3'; UBC 273, 5'-AATGTCGCCA-3'; and UBC 299, 5'-TGTCAGCGGA-3'), were used for intra-specific genetic structure analysis (Fritsch et al., 1993). PCR reactions were carried out in a 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.0001% gelatine, 100 µM of each dNTPs, 0.2 µM arbitrary primer, 25 ng DNA template and 1 unit of *Taq* DNA polymerase (William et al., 1990, Tassanakajon et al., 1997). Reactions were carried out in a Hybaid thermocycler for 35 cycles consisting of: 94°C for 15s; 36°C for 60s; and 72°C for 90s, followed by the final extension cycle at 72°C for 7 min. Resulting products were electrophoretically analyzed in 1.6% gels and viewed using a UV transluminator and ethidium bromide staining (Maniatis et al., 1982).

Data analysis. Each RAPD fragment was assigned a molecular length using a 100 bp ladder as the DNA standard and recorded in a binary matrix (0/1 where 0 = absent and 1 = present of a given band), for each individual. Similarity index values were calculated for each geographic area according to Nei and Li (1979). Indices of similarity between paired samples, corrected for within sample similarity were calculated (Lynch, 1990). Average genetic distance (D_{ij}) across all investigated primers was then converted using: $D_{ij} = 1 - \bar{S}_{ij}$; where $\bar{S}_{ij}$ is the average similarity index between samples for which the effects of within sample similarity has been subtracted.

A UPGMA dendrogram based on the genetic distance between samples was constructed (Sneath and Sokal, 1973), using Neighbor in Phylip 3.56c (Felsenstein, 1993). Geographic heterogeneity analysis was analyzed using a Monte Carlo simulation (Roff and Bentzen, 1989), implemented in REAP 4.0 (McElroy et al., 1991). An analysis of molecular variance (AMOVA; Excoffier et al., 1992), and F-statistics (Weir and Cocockerham, 1984), were carried out using Arlequin 1.1 (Schneider et al., 1997).

Results

Amplification of P. monodon DNA. A total of 53 RAPD fragments with molecular sizes of 200 to 1600 bp were consistently scored (Table 1). Average number of scorable bands per primer (13.7 to 15.3) was nearly identical in all samples. Percentage of polymorphic fragments within a geographic sample ranged from 46.7% (Trang) to 61.4% (Trat). Average similarity within Trat was lower than its proximal samples, Chumphon. Likewise, this index in Trang was also lower than that in Phangnga and Satun.

Numbers of RAPD genotypes generated from each primer were comparable (26, 30 and 32 genotypes from primers UBC 299, UBC 268 and UBC 273, respectively). Interestingly, UBC 268 gave a RAPD-amplified fragment of 260 bp found in 92.3% of the Trat sample (Fig. 2). This fragment was not observed in any Andaman Sea samples (Phangnga, Satun and Trang), but it was found in two *P. monodon* (5.0%) from Chumphon.

Genetic distance and geographic heterogeneity. Large genetic differences (d) were observed between Trat and other locations, including Chumphon ($d = 0.034$; Table 2). Surprisingly, paired comparisons of distances between Chumphon and each of the Andaman Sea populations indicated closer genetic relationships than between Chumphon and Trat. Genetic distances between samples within the Andaman Sea samples were much lower ($d < 0.005$). A UPGMA dendrogram divided the *P. monodon* populations into two groups, consisting of Trat and all other populations. Chumphon also grouped with the latter populations, and unexpectedly clustered most closely with Phangnga, located in the Andaman Sea (Fig. 3).

Overall comparisons of RAPD genotype frequencies revealed significant geographic heterogeneity among Thai *P. monodon* ($P < 0.0001$), and between regions ($P < 0.0001$). All primers used in our present study indicated geographic homology for all possible comparisons between Chumphon and each of the Andaman Sea samples ($P > 0.05$), but also indicated highly significant heterogeneity between Chumphon and Trat ($P < 0.001$). Accordingly, these five *P. monodon* populations can be regarded as three different stocks consisting of Trat (stock A), Chumphon (stock B), and Andaman Sea (stock C).

An AMOVA (treating Chumphon as a separated group from Trat), gave non-significant difference estimates of variance components among samples within groups ($F_{SC} = 0.009$, $P = 0.2687$), and among groups ($F_{CT} = 0.079$, $P = 0.1000$); but significant differences among sample within the total ($F_{ST} = 0.088$, $P < 0.0001$; Table 3).

Discussion

Stock domestication and closed life-cycle culture should be established using wild stock with high genetic diversity, including desirable culture traits (Klinbunga, 1999). Our RAPD-PCR results indicated high genetic diversity with Thai *P. monodon*. We

observed 78 RAPD genotypes using three octanucleotide primers. Inbreeding with Thai *P. monodon* is therefore not a major concern.

Garcia and Benzie (1995) identified three polymorphic RAPD markers from parents and offspring of six families using 14 different primers. Those markers were family-specific, and are useful markers for selective breeding programs at the family level. Identification of large numbers of genotypes and possible genetic markers by RAPD techniques demonstrates the value of this approach for monitoring genetic polymorphism with closed life-cycle propagation of *P. monodon*.

Tassanakajon et al. (1997) found that the primer UBC 428 yielded a population specific RAPD marker with Satun *P. monodon*. We intend to now verify growth and survival performance among different *P. monodon* stocks using the UBC 428 marker coupled with the 260 bp marker generated by UBC 268 from our present study. We are presently culturing two separated full-sib groups of *P. monodon* from Trat (40 families) and Satun (37 families) in our laboratory (Jarayabhand, 1998).

Population subdivisions in Penaeidae inferred from allozyme analyses were reported for: *Metapenaeus bennettae, M. macleayi, M. endeavouri, P. latisulcatus* (Mulley and Latter, 1981a and 1981b); *P. keraturus* (Mattoccia *et al.*, 1987); *P. merguiensis* (Daud, 1995); and *P. monodon* (Benzie *et al.*, 1992). Daud (1995) studied genetic variation and population structure of *P. monodon* collected from six locations in Malaysia using allozyme analysis, including: Kedah, Kumpung Pulau Sayak from the Andaman Sea (west); and Dungun, Kemaman, Pengareng and Sabah from the South China Sea (east). His results indicated genetic differentiation of *P. monodon* populations between the South China Sea and Straits of Malacca, but not within each region.

Klinbunga et al. (1999) determine genetic variation and population structure in *P. monodon* collected from Surat (a few hundred kilometers further south from Chumphon), Satun, and Trat using mtDNA-RFLP with 11 restriction endonucleases. They found high genetic diversity and significant geographic heterogeneity between the Andaman Sea and the Gulf of Thailand samples (mean nucleotide divergence between pairs of population = 0.26%, $P < 0.0001$). Nevertheless, they did not observe significant geographic heterogeneity between Surat and Trat ($P > 0.05$).

In our present study, we clearly demonstrated large genetic differences between *P. monodon* populations from different areas of Thailand. This finding agrees with Tassanakajon et al. (1997) who used RAPD analysis to examine genetic variation in three *P. monodon* populations. Geographic heterogeneity analysis showed significant genetic differences between Trat and Andaman Sea populations. *P. monodon* from these areas should therefore be regarded as separated stocks. Chumphon *P. monodon* showed closer relationships to all Andaman Sea populations, compared with Trat *P. monodon*. This could be the result of *P. monodon* farming and stock enhancement programs in this area. Accordingly, we regard Chumphon *P. monodon* as a different stock. Although sample sizes and number of primers used in our present study were small, overall results were in accord when respective populations were analyzed by

three homospecific microsatellites loci: CUPmo18, Di 25 and Di 27 (A. Tassanakajon, per. com.).

An ability to differentiate Chumphon *P. monodon* populations from Trat (both located in the Gulf of Thailand), using RAPD and microsatellite loci, but not mtDNA-RFLP suggests introgression of nuclear DNA from the Andaman Sea into Chumphon *P. monodon* through intra-specific hybridization. Currently, PCR-RFLP analysis of mitochondrial genes including cytochrome oxidase subunits I-II (COI-COII), 16S ribosomal DNA (16S rDNA) and NADH dehydrogenase subunit 5 (ND5) of the same sample set used in the present study is being carried out to clarify contradictory results from different genetic markers (S. Klinbunga, per. com.). It is premature at this time to conclude how much hybridization has occurred until additional investigation of mitochondrial genes based on PCR-RFLP is done covering larger areas of the Gulf of Thailand.

Massive numbers of *P. monodon* postlarvae (typically at PL_{15}) have been released annually at several locations in the Gulf of Thailand for stock enhancement purpose. Most of these PL were produced from Andaman Sea female broodstock (Klinbunga, 1996; Sodsuk. 1996). Fishery managers and shrimp culturists have traditionally shown little or no concern about effects of escapees, either accidental or intentional, from their facilities. Management practices include immediate terminate of culture stocks and release of infected *P. monodon* larvae or PL directly into the sea if hatchery stock show any serious symptoms of infection (Flegel et al., 1995). Undoubtedly, the anomalous gene pool found in Chumphon *P. monodon* resulted from these transplants and escapements .

Another explanation for intra-specific population differences between Chumphon and Trat *P. monodon* could be genetic isolation caused by prevailing surface currents in the Gulf of Thailand. Water currents flow counter-clockwise during the northeast monsoon, then completely reverse during the southwest monsoon (Dale, 1956).

Our present study indicates that RAPD-PCR is one potential technique for determining population structure and genetic markers in *P. monodon*. An inability to disassociate homozygous and heterozygous status by RAPD is compromised by its cost-effective and less time consuming to generate useful genetic markers in species that their genomes have not been well studied (Weising et al., 1995)

Acknowledgments

The authors thank Siriporn Pongsomboon for her crucial assistance with RAPD-PCR standardizations for our analyses. This research was subsidized by the National Center for Genetic Engineering and Biotechnology (BIOTEC) Grant No. BT-39-06-ATI-06-05, Thailand. A student grant was supported by the Thailand Research Fund (TRF) through Prof. Dr. P. Menasveta.

References

Asian Shrimp Culture Council (1996). Asian Shrimp News 1st quarter.

Benzie, J.A.H. (1994). Genetic and reproduction research on giant tiger prawns (*Penaeus monodon*): pond reared spawners achieved in Australia. *Aust. Biotech.* 4: 222-224.

Benzie, J.A.H., Frusher, S. and Ballment, E. (1992). Geographical variation in allozyme frequencies of populations of *Penaeus monodon* Crustacea: Decapoda) in Australia. *Aust. J. Mar. Freshwater Res.* 43: 715-725.

Benzie, J.A.H., Kenway, M. and Bechkenbach, A.T. (1997). Estimates for the heritability of size in juvenile *Penaeus monodon* prawn from half-sib mating. *Aquaculture.* 152: 49-53.

Dale, W.L. (1956). Wind and Drift currents in the South China Sea. *The Malaysian J. Trop. Geography.* 8: 1-31.

Daud, S.K. (1995). *Population Genetics of Penaeus monodon Fabricius and Penaeus merguiensis de Man in Malaysia.* PhD. thesis. University of Stirling, Scotland. 327 pp.

Excoffier, L., Smouse, P.E. and Quatto, J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application in human mitochondrial DNA data. *Genetics.* 131: 479-491

Felsenstein, J. (1993). *Phylip (Phylogenetic inference Package) version 3.5c.* Distributed by the author. Department of Genetics, University of Washington, Seattle.

Flegel, T., Fegan, D.F. and Sriurairatana, S. (1995).Environmental control of infectious shrimp diseases in Thailand, pp.65-79. In: *Diseases in Asian Aquaculture II* (M. Shariff, J.R. Arthur and R.P. Subasinghe, eds.) Fish Health Section, Asian Fisheries Society.

Fritsch. P., Hanson, M.A., Spore, C.D., Pack, P.E. and Reisenberg, L.H. (1993). Constancy of RAPD primer amplification strength among distantly related taxa of flowering plants. *Plant Mol.Biol.Rep.* 11:10-20.

Garcia, D. and Benzie, J.A.H. (1995) RAPD markers of potential use in penaeid prawn (*Penaeus monodon*). *Aquaculture.* 130: 137-144.

Hadrys, H., Balick, M. and Schierwater, B. (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* 1: 55-63.

Jarayabhand, P., Uraiwan, S., Klinbunga, S., Tassanakajon, A., Srimukda, P., Pattanachan, P., Panakulchaiwit, R. and Menasveta, P. (1998). Estimated heritability

for early growth rate of the black tiger prawn, *Penaeus monodon*, Fabricius, pp. 67-70. In *Advances in shrimp biotechnology* (T.W. Flegel, ed). National Center for Genetic Engineering and Biotechnology.

Klinbunga, S. (1996). Genetic variation and population structure of the giant tiger shrimp (*Penaeus monodon* Fabricius). Ph.D thesis, University of Stirling, Scotland.

Klinbunga, S. , Penman, D.J. and McAndrew, B.J. (1996). An improve protocol for total DNA isolation and visualisation of mtDNA RFLP (s) in tiger prawn, *Penaeus monodon. Thai J Aquat Sci.*, **3**, 36-41 (1996)

Klinbunga, S. , Penman, D.J. and McAndrew, B.J. and Tassanakajon, A. (1999). Mitochondrial DNA diversity in three populations of the giant tiger shrimp *Penaeus monodon. Mar. Biotechnol.*, 1 (in press).

Lynch, M. (1990). The similarity index and DNA fingerprinting. *Mol. Biol. Evol.* 7: 478-484

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Habor.

Mattoccia, M, Rosa, G.L., Matthaeis, E.D., Sbordoni, M.C. and Sbordoni, V. (1987). Patterns of genetic variability and differentiation in Mediterranean populations of *Penaeus kerathurus* (Crustacea, Decapoda), pp. 131-141. In: *Proceeding of the world symposium on selection, hybridization and genetic engineering in aquaculture vol. 2* (K. Tiews, ed.). H. Heenemann GmbH & Co., Berlin.

McElroy, D., Moran, P., Bermingham, E. and Kornfield, I. (1991). *REAP; The Restriction Enzyme Analysis Package, Version 4.0.*

Mulley, J.C. and Latter, B.D.H. (1981a). Geographic differentiation of Eastern Australian penaeid prawn populations. *Aust. J. Mar. Freshwater. Res.* 32: 889-895.

Mulley, J.C. and Latter, B.D.H. (1981b). Geographic differentiation of tropical Australian penaeid prawn populations. *Aust. J. Mar. Freshwater. Res.* 32: 897-906.

Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proc. Natl. Acad. Sci. USA.* 76: 5269-5273.

Roff, D.A. and Bentzen, P. (1989). The statistical analysis of mitochondrial DNA polymorphisms: X^2 and the problems of small samples. *Mol. Biol. Evol.* 6: 539-545.

Schneider, S., Kueffer, J-M, Roessli, D. and Excoffier, L. (1997). *Arlequin 1.1: A software for population genetic data analysis.* Geneva: Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Sneath, P.H.A. and Sokal, R.R. (1973). Numerical taxonomy. W.H. Freeman and Co., San Francisco.

Sodsuk, S. (1996). *Genetic Population Structure of Penaeus monodon Fabricius using Allozyme and Mitochondrial DNA Analysis*. PhD thesis. University of Stirling, Scotland. 277 pp.

Tassanakajon, A., Pongsomboon, S., Rimphanitchayakit, V., Jarayabhand, P. and Boonsaeng, V. (1997). Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild populations of the black tiger prawn (*Penaeus monodon*) in Thailand. *Mol.Mar.Biol.Biotechnol.* 6: 110-115.

Weir, B.S. and Cockerham, C.C. (1984). Estimation *F*-statistics for the analysis of population structure. *Evolution*. 38: 1358-1370.

Weising, K., Nybom, H., Wolff, K. and Meyer, W. (1995). DNA fingerprinting in plants and fungi. CRC Press, Boca Raton, FL.

William, G.K., Kubilik, A.R., Livak, K.L., Rafaski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. 18: 6531-6535.

Table 1. Total number of scored bands, percentage of polymorphic bands, and average similarity index for *Penaeus monodon* populations within each geographical area.

Sample site	Average scored bands per primer	Polymorphic bands (%)	Similarity within samples (S)
Chumphon	15.3	56.5	0.912
Trat	14.7	61.4	0.888
Phangnga	15.0	60.0	0.901
Satun	15.0	53.3	0.902
Trang	13.7	46.7	0.852

Table 2. Paired comparisons of genetic distance (below diagonal) for five, conspecific *Penaeus monodon* populations.

	Chumphon	Trat	Phangnga	Satun	Trang
Chumphon	-				
Trat	0.034	-			
Phangnga	-0.002	0.037	-		
Satun	0.017	0.039	-0.001	-	
Trang	0.001	0.038	0.003	0. 005	-

Table 3. Analysis of molecular variance (AMOVA) results when *Penaeus monodon* samples from Trat, Chumphon and the Gulf of Thailand (Phangnga, Satun and Trang) were treated as hierarchical groups.

Component	Variance	Percentage of variation	*F*-statiatics	Probability (P)
Among groups	0.0355	7.89	0.0789	0.1000
Among samples within groups	0.0039	0.87	0.0094	0.2687
Within samples	0.4105	91.24	-	-

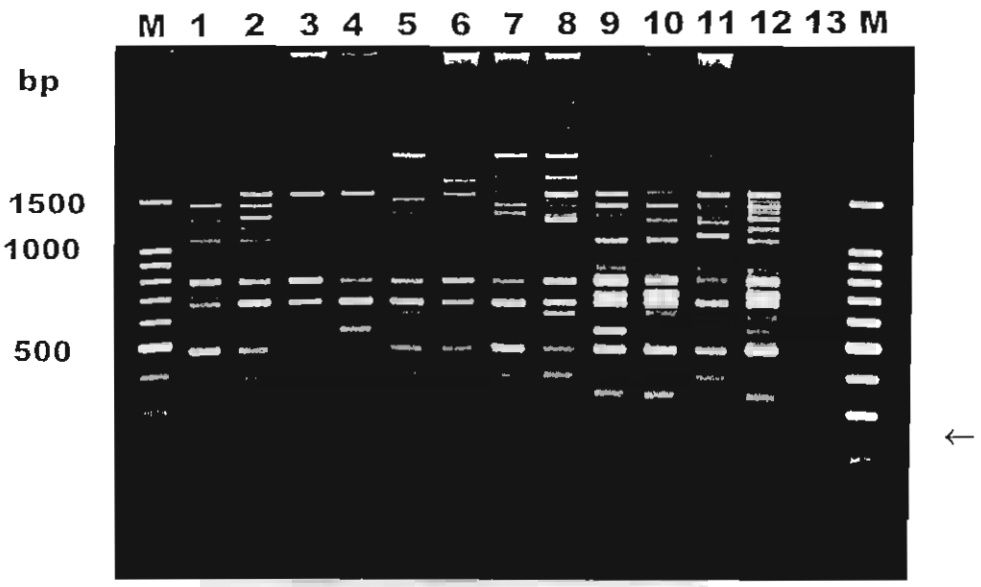
FIGURE LEGENDS

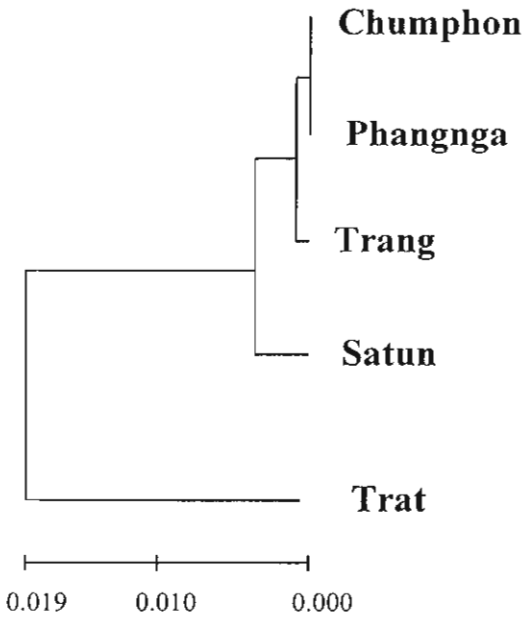
Figure 1. Map of Thailand showing sampling sites for five conspecific samples of *Penaeus monodon* used in this study.

Figure 2. An example of RAPD patterns for *Penaeus monodon* using UBC268 primer. A 100 bp ladder was used as a DNA marker (lane M). Lanes 1 and 2: *P. monodon* individuals from Satun. Lanes 3 and 4: *P. monodon* from Trang. Lanes 5-8: *P. monodon* from Trat. Lanes 9 and 10: *P. monodon* from Chumphon. Lanes 11 and 12: *P. monodon* from Phangnga. An arrow head indicates a 260 bp RAPD marker observed in almost all of the specimens from Trat, but not present with Andaman Sea *P. monodon*.

Figure 3. A UPGMA dendrogram illustration indicating relationships between *Penaeus monodon* from five locations in Thailand, based on genetic marker analyses.


THAILAND
Andaman Sea
Gulf of Thailand
Trat
Chumphon
Phangnga
Trang
Satun


M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
bp
1500
1000
500


Chumphon
Phangnga
Trang
Satun
Trat
0.019
0.010
0.000
Genetic distance

Invertebrate Reproduction and Development, 35:1 (1999) 9–17
Balaban, Philadelphia/Rehovot
0168-8170/99/$05.00 © 1999 Balaban

Monoclonal antibodies production specific to vitellin and vitellogenin of giant tiger prawn *Penaeus monodon*

SIWAPORN LONGYANT[1], PAISARN SITHIGORNGUL[2*], NITTAYA THAMPALERD[3], WEERAWAN SITHIGORNGUL[2] and PIAMSAK MENASVETA[1,4]

[1]*Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*Department of Biology, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand*
Tel. (662) 260-0127; Fax (662) 260-0128; email: paisarn@psm.swu.ac.th
[3]*Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand*
[4]*Marine Biotechnology Research Unit, BIOTEC, Bangkok 10400, Thailand*

Received 19 May 1998; Accepted 26 August 1998

Summary

Monoclonal antibodies specific to *Penaeus monodon* vitellin and vitellogenin were produced using crude ovarian extract from gravid *P. monodon* ovaries. After immunization and fusion of mouse spleen cells with P3X myeloma, hybridomas were selected by indirect immunoperoxidase ELISA against *P. monodon* ovarian extract, followed by dot-blotting against native and denatured proteins from ovarian extract, female haemolymph, and male haemolymph. Four hybridoma clones producing antibodies (PMV-11, 15, 22 and 64) were identified. They bound with ovarian extract proteins and with female haemolymph, but not with male haemolymph. One antibody (PMV-64) bound with both native and denatured proteins. Using Western Blot analysis of ovarian extract separated by PAGE, all four monoclonal antibodies bound with the same lipoglycoprotein band. Using Western Blot analysis of proteins separated by SDS-PAGE, PMV-64 antibody bound with Mw 80 and 83 kD proteins in ovarian extract, and with Mw 83 and 200 kD proteins in female haemolymph. All four monoclonal antibodies belong to the IgG1 subclass.

Key words: Crustacean, monoclonal antibodies, *Penaeus monodon*, vitellin, vitellogenin

Introduction

Ovarian maturation in crustaceans leading to mature oocyte formation involves several complex processes. Yolk accumulation, or vitellogenesis, is one such process. Vitellin, or lipoglycoprotein, is a major component of yolk (Wallace et al., 1967), and typically has two to eight subunits depending on Penaeid shrimp species (Table 1). With some species, however, such as *Penaeus monodon*, the molecular nature of this protein is still not well documented with respect to either size or subunit number (Quinitio et al., 1990; Chang et al., 1993; Chen and Chen, 1993). These differences in

*Corresponding author.

Table 1. Molecular weights of vitellin and vitellogenin subunits for six penaeid shrimp species

Species	Molecular weight, kD		References
	Vitellin	Vitellin subunits	
P. monodon	540	74, 83, 90, 104, 168	Quinitio et al., 1990
	ND	74, 83, 104, 168	Chen and Chen, 1993
	492	35, 45, 49, 58, 64, 68, 82, 91	Chang et al., 1993
	ND	45, 58, 74, 83, 104	This study
P. semisulcatus	283	86, 95	Tom et al., 1992
	ND	50, 63, 80, 90	Browdy et al., 1990
P. vannamei	289	61, 69	Tom et al., 1992
	ND	76, 97, 158	Quackenbush, 1989a
P. chinensis	380	40, 58, 78, 85, 105	Chang et al., 1996
	500	78, 85, 155	
Parapenaeus longirostris	ND	45, 66	Tom et al., 1987
Metapenaeus ensis	350	76, 102	Qiu et al., 1997

ND, not determined.

vitellin characterization may relate to differences in ovarian maturity and/or methodologies for vitellin purification used by different researchers (Qiu et al., 1997).

With crustaceans, vitellin is synthesized by ovaries (Eastman-Reks and Fingerman, 1985; Yano and Chinzei, 1987; Quackenbush, 1989a, 1989b; Browdy et al., 1990; Sagi et al., 1995). With vitellogenic crustaceans, a female-specific protein, vitellogenin, occurred in the haemolymph of all species studied (Wolin et al., 1973; Tom et al., 1987; Quackenbush and Keeley, 1988; Quackenbush, 1989a; Chang et al., 1994). Changes in vitellogenin concentrations in haemolymph were correlated with ovarian morphological changes where vitellogenin concentrations increased during ovarian development and reached their greatest concentration prior to maximum yolk accumulation in the oocytes (Quackenbush, 1989a). Vitellogenin then decreased markedly prior to ovulation and spawning (Byard and Aiken, 1984; Quinitio et al., 1989; Chang and Shih, 1995). The hepatopancreas has been shown to be partially involved as an extra-ovarian source of vitellogenin synthesis (Fainzilber et al., 1992, Shafir et al., 1992a, 1992b).

Vitellogenesis and ovarian maturation are under hormonal control (Hasegawa and Hirose, 1993), and thus vitellogenin concentrations in haemolymph may be used as an indirect indicator of regulating hormone activities. Hormonal control of crustacean vitellogenesis has been described, as follows. Vitellogenesis inhibiting hormone (VIH) has been isolated from eyestalks of American lobster *Homarus americanus* (Soyez et al., 1987), with a primary peptide structure of 77 residues. VIH is homologous with crustacean hyperglycemic and molt-inhibiting hormones (Soyez et al., 1991). Vitellogenesis stimulating hormone (VSH) has also been found in brain and thoracic ganglia of some crustaceans, including *Uca pugilator* (Eastman-Reks and Fingerman, 1985), and *Paratya compressa* (Takayanaki et al., 1986); however, the precise chemical nature of VSH and its mode of action remain unknown.

The objective of our present study was to develop monoclonal antibodies specific to vitellin and vitellogenin. This immunological tool could then be used to determine the haemolymph vitellogenin levels for studying the hormonal control of ovarian development in *P. monodon*, an economically important shrimp widely cultivated in Thailand. A monoclonal antibody approach was selected since this method has several advantages over alternatives such as use of antiserum. Monoclonal antibody production does not require high-purity antigens. A complex mixture of antigens can be used; and hybridoma clones which produce monoclonal antibodies against desired antigen can be selected during screening processes (Sithigorngul et al., 1989). Once established cell lines are obtained, homogeneous monospecific antibodies can be produced in virtually unlimited amounts. Due to its monospecificity, this diagnostic tool can be used to clarify the molecular nature of vitellin in *P. monodon*.

Materials and Methods

Source animal handling and initial preparations

Adult female *P. monodon* (80–120 g) were obtained from local fishermen in Cholburi Province, Gulf of Thailand. They were held in 1×5×1 m deep, rectangular concrete tanks with full-strength seawater (30‰) and ambient photoperiod. Commercial shrimp pellet diet was presented during the morning and evening. Water temperature was 26–28°C and was changed every morning. After acclimatizing for 3 days, prawns were bilaterally, eye ablated to induce ovarian development. Five to seven days after eye ablation, prawns with gravid ovaries were anesthetized using cold water (4°C), and haemolymph was collected via the arthrodial membrane of the fourth walking leg. Ovaries were removed by dissection and washed in cold, 0.15 M phosphate buffered saline pH 7.4 (PBS). Haemolymph was also collected from adult male *P. monodon* (60–80 g). Haemolymph and ovaries were frozen on dry ice and stored at −70°C.

Ovarian extract preparation

Individual ovaries were homogenized for 5 min in 0.5 mM EDTA in PBS (0.5 g/ml). The pellet and lipid layer were eliminated after centrifugation at 10,000 g and 4°C for 30 min. The protein content of the extract was determined by Bradford reagent (Bradford, 1976), adjusted to 10 mg/ml with PBS, then divided into 1 ml aliquots and stored at −70°C.

Immunization

Five BALB/c mice were injected with 0.5mg ovarian extract 1:1 mixed with complete Freund's adjuvant. At 2-week intervals they were re-injected with ovarian extract mixed with incomplete Freund's adjuvant for the second injection, or with the extract without adjuvant for the following injections. One week after the fourth injection, mouse anti-vitellin antisera were collected and tested against ovarian extract, and female and male haemolymph by double immodiffusion. The best performing mouse was boosted 1 week before hybridoma production. BALB/c mice were kindly supplied by the Animal House of the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand.

Hybridoma production

A cell fusion procedure was adapted from the method developed by Köhler and Milstein (1976), with modification described by Mosmann et al. (1979). A P3X myeloma cell line was used as the fusion partner. The P3X myeloma cell line was obtained from the Department of Virology, AFRIMS, Bangkok, Thailand. Fusion products from one mouse were plated on 16 microculture plates (96 wells). After identifying wells containing desired clones by the screening methods described below, cells were re-cloned at least twice by the limiting dilution method (Eshhar, 1985).

Screening methods

ELISA

Hybridoma cell lines were first screened by ELISA against ovarian extract. The ovarian extract (1 μg/well protein) was plated on Maxisorb microtiter plates (NUNC). Blotto (5% or 0.5% nonfat dry milk in PBS, Johnson et al., 1984) was used as blocking solution, antibody diluent, and washing solution. Antibody binding to plates was detected using horseradish peroxidase labeled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; Biorad) at 1:1000 dilution. Positive wells were further screened by dot-blotting.

Dot-blotting

Selected antigens, ovarian extract (1 mg/ml), female and male haemolymph in native forms, and SDS-mercaptoethanol treated forms were used for the second step of screening. All antigens (1 μl/spot) were applied to pieces of nitrocellulose membrane. After the membrane was baked at 60°C for 10 min, each piece was incubated in hybridoma conditioned media from each clone (1:20 in 5% Blotto) for 2 h. After extensive washing in diluted Blotto, the membrane was incubated in GAM-HRP (1:1000 in 5% Blotto with 40% male prawn haemolymph) for 2 h. The membrane was then washed as before and applied to substrate mixture (0.03% diaminobenzidine, 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS) for 5 min.

Selected hybridoma clones that bound with ovarian extract and with female haemolymph, but not binding with male haemolymph, were re-cloned and cryo-preserved for further characterization. Some hybridoma clones were injected into pristane primed mice for ascites fluid production.

Monoclonal antibodies characterization

Polyacrylamide gel electrophoresis and immuno-blotting

Polyacrylamide gel electrophoresis (PAGE) or

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed in slab gels in a mini-PROTEIN II electrophoresis apparatus (Bio-Rad) to identify vitellin and vitellogenin in the extract. Ovarian extract or haemolymph was applied to 5% gel for PAGE or 7.5% gel for SDS–PAGE according to the Bio-Rad Manual. Parts of the gel were cut off and visualized by staining with 0.1% Coomassie brilliant blue R250 for proteins, with Sudan black B for lipoproteins, or with periodic acid Schiff reagent (PAS) for glycoprotein (modified from Humason, 1979). Proteins in other parts of the gel were transferred to nitrocellulose sheet using Transblot apparatus (Bio-Rad) at 50 V for 2 h. The nitrocellulose sheet was then separated from the gel and quenched in 5% Blotto, cut into strips and assayed for antibody binding as described for dot-blotting assay.

Vitellin isolation from PAGE

To confirm the number of subunits of vitellin, glycolipoprotein bands from PAGE were cut off and homogenized in equal volume of 0.01% SDS, allowing the protein to dissolve for 12 h. The dissolved protein was separated from the gel by centrifugation and the gel pellet was re-extracted with the same solution for 12 h. Both supernatants were combined and concentrated by vacuum concentrator (Savants) and reapplied in SDS–PAGE as described above.

Vitellin and vitellogenin immunoprecipitation

Due to very high haemocyanin content and low proportion of vitellogenin in the haemolymph, the proportion of vitellogenin in the sample was increased by (immunoprecipitation) using female haemolymph mixed with pooled mouse anti-vitellin antiserum (4:1) collected previously before the fusion. After incubation at 4°C overnight, the precipitate of antibody antigen complex was collected by centrifugation (5000 g) and washed twice with PBS. The ovarian extract was prepared in the same manner as a control. Precipitates were then dissolved in treatment buffer and subjected to SDS–PAGE as described above.

Detectability of vitellin and vitellogenin by monoclonal antibodies specific to vitellin and vitellogenin

The range of vitellin concentrations that could be measured by these monoclonal antibodies was determined by competitive ELISA using the lowest concentration of each monoclonal antibody that gave maximal absorbance mixed with various dilutions of ovarian extract (initial concentration is 10 mg/ml) or female haemolymph, or male haemolymph, then processed as described in ELISA.

Class and subclass determination

The class and subclass of the mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA using a subisotyping kit, mouse (Calbiochem).

Results

After four immunizations with *P. monodon* ovarian extract, mouse sera from five mice produced strong precipitation band reactions to antigens in *P. monodon* ovarian extracts and to female haemolymph, but not to antigens in male haemolymph (Fig. 1). Precipitation bands from both antigens showed reaction of identity with all mouse anti-vitellin antisera (not shown). Antiserum from mouse No. 3 seemed to have the fewest numbers of weak precipitation bands with other antigens, so this mouse was used as the spleen cell donor for hybridoma production.

From one fusion, about 700 wells contained hybridoma cells from 16 microculture plates. The first screening on ELISA against ovarian extract yielded 73 positive wells with varying intensities. However, after screening with dot-blotting against ovarian extract, and female and male haemolymph in both native and SDS-treated antigens, only four hybridoma clones were identified (PMV-11, 15, 22 and 64). These clones produce antibodies that bound both ovarian extract and female haemolymph, but did not bind with male haemolymph. One antibody (PMV-64) bound with both native and denatured antigens (Fig. 2, Table 2). These four clones and a few other clones that showed different binding were re-cloned and used for further

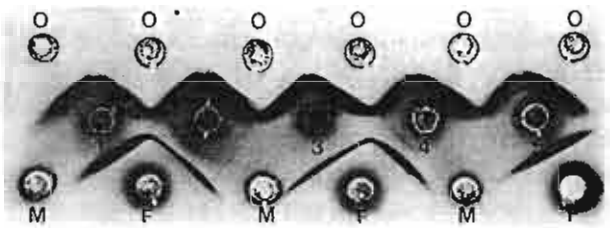


Fig. 1. Double immunodiffusion of five mouse anti-vitellin antisera (1–5) against *P. monodon* ovarian extract (O) and male (M) and female (F) haemolymph. The most dense precipitation bands occur between all mouse antisera with ovarian extract and with female haemolymph, but not with male haemolymph.

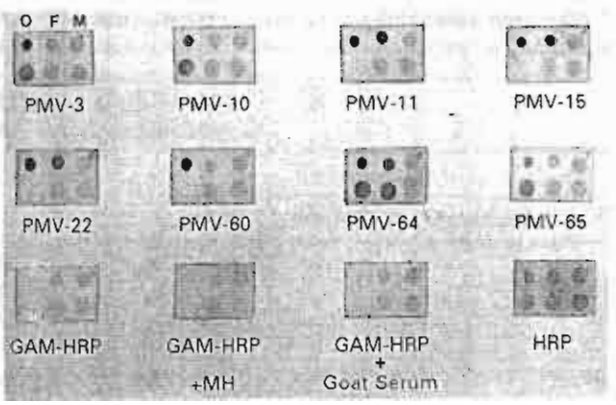


Fig. 2. Screening results of monoclonal antibodies by dot-blot. Each nitrocellulose sheet was treated with various monoclonal antibodies (PMV-...). Antigens in the vertical columns are *P. monodon*: ovarian extract (O), female (F) haemolymph and male (M) haemolymph. Antigens in the upper horizontal rows are untreated proteins, while antigens in the lower rows are SDS and mercaptoethanol treated proteins. Nonspecific binding of goat anti-mouse IgG-horseradish peroxidase conjugate (GAM-HRP) to haemolymph is substantial and cannot be blocked with normal goat serum (GAM-HRP + goat serum), but can be reduced by 40% male haemolymph (GAM-HRP+M). When nitrocellulose sheets were directly incubated with horseradish peroxidase (HRP), the enzyme bound strongly to proteins in all dots.

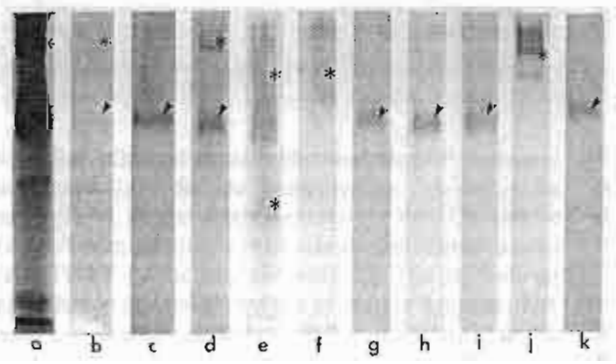


Fig. 3. PAGE and immunoblot analysis of *P. monodon* ovarian extract. Crude ovarian extract was separated by PAGE and visualized by staining with Coomassie blue (a), periodic acid Schiff (PAS) reagent (b), or Sudan black B (c); or it was transferred to nitrocellulose membrane and treated separately with mouse anti-vitellin antiserum (d), or with monoclonal antibodies; PMV-3 (e), PMV-10 (f), PMV-11 (g), PMV-15 (h), PMV-22, (i), PMV-60 (j), PMV-64 (k). The glycoprotein band (arrow head) was also recognized by PMV-11, 15, 22 and 64 monoclonal antibodies. Mouse anti-vitellin antiserum and other monoclonal antibodies recognized different proteins (asterisk) in the ovarian extract. About 20 μg/lane was used for glycolipoprotein staining and about 2 μg/lane was used for immunoblotting.

characterization and comparison of monoclonal antibodies by Western blot analysis of native protein (Figs. 2 and 3). All four monoclonal antibodies specific to vitellin and vitellogenin belonged to IgG1 subclass (Table 2).

During screening with dot-blotting, we observed substantial nonspecific staining of haemolymph spots with GAM-HRP (Fig. 2). This nonspecific staining was, however, considerably reduced by adding male haemolymph to GAM-HRP (40%), but nonspecific staining was not reduced by adding normal goat serum (Fig. 2). This background staining is due to nonspecific binding of haemolymph components to HRP, as shown by incubation of samples directly with HRP solution (0.01 mg/ml in Blotto; Sigma). To reduce nonspecific staining, GAM-HRP mixed with male haemolymph was used in the following tests.

Further characterization of native ovarian extract separated by PAGE using Western blot analysis showed that all four hybridoma clones (PMV-11, 15, 22 and 64) produced antibodies that bound with the same single band of glycolipoprotein (Fig. 3: g,h,i,k) and was also identified by PAS and Sudan black staining (Fig. 3: a,b,c). Since this is the primary constituent of ovarian extracts. It can be presumed to be vitellin. Mouse antiserum and other antibodies from other clones show different staining patterns. PMV-3 antibody and mouse anti-vitellin antiserum bound with the expected vitellin band, but also bound with other proteins as well (Fig. 3: d,e). PMV-10 and PMV-60 antibodies bound with high molecular weight protein (Fig. 3: f,j), while PMV-65 did not bind with any proteins (not shown). Western blot analysis of native female haemolymph was not conducted due to interference caused by excessive amounts of haemocyanin which interfered with movement of other proteins.

Further separation of the glycolipoprotein isolated from PAGE by SDS–PAGE revealed two major subunits with Mw 74 and 83 kD (band No. 2 and 3; Fig. 5: Aa), and three light staining subunits with Mw 45, 58 and 104 kD (band No. 5, 4, 1; Fig. 5: Aa). Characterization of SDS-treated vitellin by Western Blot was done only with PMV-64 monoclonal antibody since only this antibody can bind with SDS-treated antigens. PMV-64, monoclonal antibody interaction with ovarian extract, revealed a strong reaction at Mw 83 kD, and a lighter band with a smaller Mw 80 kD protein (Fig. 4: Ca). It produced only one band at Mw 83 kD with female haemolymph, and did not bind with any proteins in male haemolymph (Fig. 4: Cb,c). Mouse anti-vitellin antiserum revealed six strong

Table 2. Characterization of monoclonal antibodies specific to vitellin and vitellogenin, and range of vitellin concentrations that can be detected by the monoclonal antibodies on competitive ELISA

Hybridoma clone	Class and subclass	Dot-blot test				Range of vitellin concentration (μg/ml)
		Ovary extract		Female haemolymph		
		Native	Denatured	Native	Denatured	
PMV-3	IgM	+	+	-	-	ND
PMV-10	IgM	+	+	-	-	ND
PMV-11	IgG1	+	-	+	-	4–200
PMV-15	IgG1	+	-	+	-	0.25–20
PMV-22	IgG1	+	-	+	-	4–200
PMV-60	IgG1	+	-	-	-	ND
PMV-64	IgG1	+	+	+	+	4–200
PVM-65	IgM	+	+	-	-	ND

+, antibody can bind to the proteins. -, antibody cannot bind to the proteins. ND, not determined.

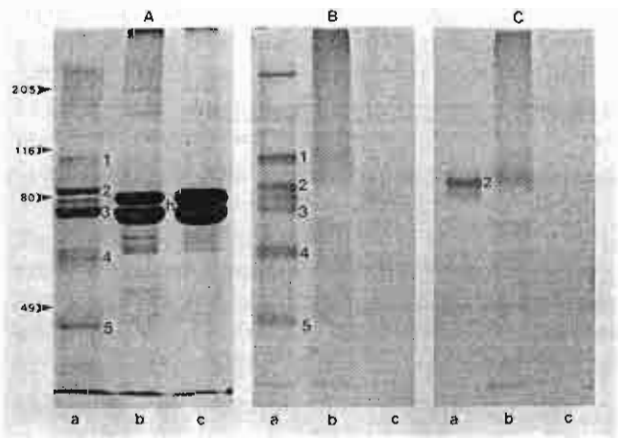


Fig. 4. SDS-PAGE and immunoblot analysis of *P. monodon* ovarian extract (a) female haemolymph (b) and male haemolymph (c). Preparations were stained with Coomassie blue (A), Western blotting with mouse anti-vitellin antiserum (B), or PMV-64 monoclonal antibody (C). The numbers on the left side are molecular weight of marker proteins. The numbers 1–5 are presumptive vitellin subunits, h is the haemocyanin subunits. Protein content was about 20 μg/lane (a) and 50 μg/lane (b,c) for Coomassie blue staining, and about 2 μg/lane (a) and 5 μg/lane (b,c) for Western blotting.

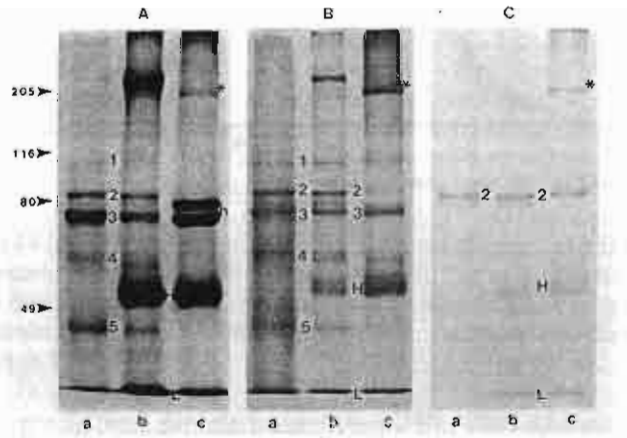


Fig. 5. SDS-PAGE and immunoblot analysis of isolated proteins from PAGE-glycolipoprotein band (a), immunoprecipitation of ovarian extract (b), and immunoprecipitation of female haemolymph (c). Preparations were stained with Coomassie blue (A), Western blotting with mouse anti-vitellin antiserum (B), or PMV-64 monoclonal antibody (C). The numbers on the left side are molecular weight of marker proteins. The numbers 1–5 are presumptive vitellin subunits, h is haemocyanin subunits, H and L are heavy and light chains of the mouse immunoglobulin. * indicates the extra vitellin immunoreactive protein which was not found in ovarian extract. Protein content was about 20 μg/lane (a) and 50 μg/lane (b,c) for Coomassie blue staining, and about 2 μg/lane (a) and 5 μg/lane (b,c) for Western blotting.

immunoreactive bands Mw 45, 58, 74, 83, 104 and 215 kD in ovarian extract, but did not produce any bands in either male or female haemolymph (Fig. 4B), which indicated that the proportion of vitellogenin in female haemolymph is very low beyond the binding capacity of mouse anti-vitellin antiserum produced from injection of native vitellin. The Mw 215 kD protein is not a vitellin subunit since we did not observe this protein with the SDS–PAGE of glycolipoprotein isolated from PAGE (Fig. 5: Aa, Ba).

Immunoprecipitated ovarian proteins had similar patterns of protein staining as ovarian extract (Fig. 5: Bb). However, with immunoprecipitated female haemolymph, Mw 74, 83 and 200 kD proteins were revealed by mouse anti-vitellin antiserum, and Mw 83 and 200 kD proteins were recognized by PMV-64 antibody (Fig. 5: Bc, Cc).

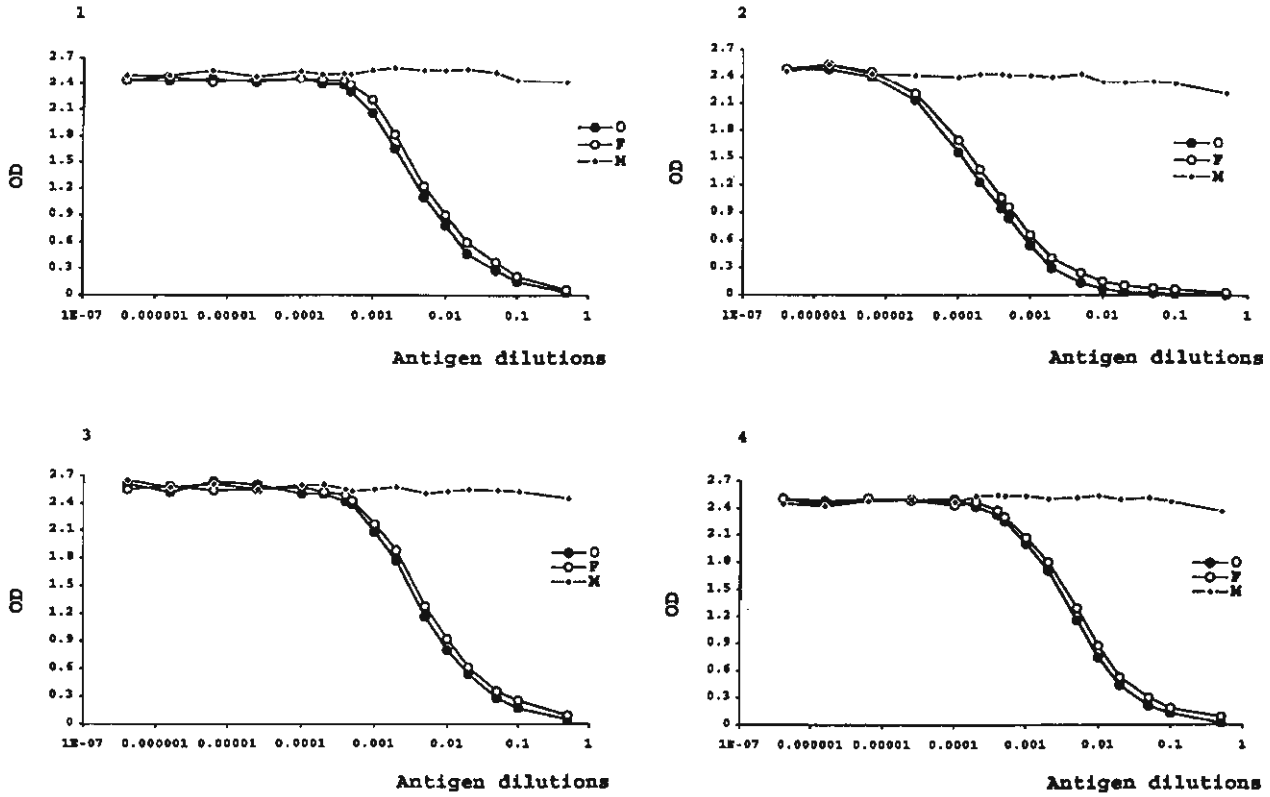


Fig. 6. Competitive ELISA of PMV-11 (1), 15 (2), 22 (3) and 64 (4) monoclonal antibodies with various antigens; ovarian extract (O, initial protein concentration 10 mg/ml), female haemolymph (F), and male haemolymph (M). The antigens were diluted and evaluated for binding inhibition of antibodies to ovarian extract fixed on the wells. The concentration of the ascites fluids of PMV-11, 15 and 64 monoclonal antibodies are 1:100,000, 1:45,000 and 1:50,000, consecutively. The concentration of culture fluid of PMV-22 monoclonal antibody is 1:500.

Competitive ELISA demonstrated that vitellogenin in female haemolymph can completely inhibit binding of all monoclonal antibodies to fixed vitellin similar to vitellin itself, whereas male haemolymph did not show any affects. The range of vitellin that can be measured by this method is 0.25–200 μg/ml (Table 2, Fig. 6).

Discussion

We isolated four hybridoma clones which produced antibodies that bound with both vitellin and vitellogenin, from fusion of P3X myeloma and spleen cells of a mice immunized with native, *P. monodon* ovarian extract. Only one antibody (PMV-64), however, bound with both native and SDS-treated proteins. This antibody bound with Mw 83 kD protein in ovarian extract and in female haemolymph. A smaller protein (80 kD) in ovarian extract was also recognized by the antibody. This latter protein was not previously reported from *P. monodon*, and it is possible that it may be a degradation product of the Mw 83 kD protein since it was present in minute amounts. With precipitated female haemolymph, PMV-64 antibody recognized an extra protein at Mw 200 kD, but no similar Mw protein in the ovary. This is evidence that this Mw 200 kD protein is a precursor that underwent hydrolysis to Mw 83 kD (Chang et al., 1994) and Mw 74 kD vitellogenin subunits before incorporation into oocytes, since it showed immunological reaction similarity to Mw 83 and 74 kD proteins in female haemolymph and ovarian extract.

SDS-PAGE analysis of glycolipoprotein isolated from PAGE revealed that this protein consisted of two major subunits of Mw 74 and 83 kD, and three minor subunits Mw 45, 58 and 104 kD. The 168 kD protein was not detected with this preparation. Even though this Mw protein can be visualized slightly in crude ovarian extract, it was not immunoreactive with mouse antiserum (Fig. 4). Mouse anti-vitellin antiserum revealed six immunoreactive bands with Mw 215, 104, 83, 74, 58 and 45 kD, but no protein in female and male haemolymph. This occurred because crude ovarian extract in native form was used for immunization, antibody against another cell component

(Mw 215 kD protein) was also present, and antibody specific to the denatured form of 83 kD protein was very weak in mouse anti-vitellin antiserum. Consequently, binding of antibodies in mouse anti-vitellin antiserum to denatured protein was much less pronounced than binding of PMV-64 antibody. With female haemolymph, the vitellogenin content was very low and could not be increased by loading more sample. Vitellogenin detection could, however, be improved by immuno-precipitation of vitellogenin with mouse anti-vitellin antiserum. This result revealed 83 and 200 kD with PMV-64 antibody, and 74, 83 and 200 kD with mouse anti-vitellin antiserum. It is possible that the 83 and 74 KD vitellin subunits may be derived from 200 kD vitellogenin in haemolymph. Neither monoclonal antibodies nor mouse anti-vitellin recognized a 168 kD vitellin subunit (Quinitio et al., 1990; Chen and Chen, 1993), nor did the SDS–PAGE of protein isolated from lipoglycoprotein of PAGE detect a 168 kD protein. We found vitellin consisting of only five major subunits, 45, 58, 74, 83 and 104 kD, in the isolated glycolipoprotein separated by PAGE. Our findings agree in part with other studies (Quinitio et al. 1990; Chen and Chen, 1993; Chang et al., 1993; Table 1). With some penaeid species, two subunits of vitellin were found (Tom et al.,1987, 1992; Qiu et al., 1997), as well as three or four subunits (Quackenbush, 1989a; Browdy et al., 1990; Chang et al., 1996; Table 1). Monoclonal antibodies specific to other subunits may help resolve this issue and reveal the production site of each vitellin subunit.

Most monoclonal antibodies specific to vitellin and vitellogenin that we produced bound with native forms of antigens, which was not surprising since the antigen used in this study was a native form. Immunization of SDS-treated antigens is an alternative way of obtaining antibodies which can bind with both native and denatured antigens, and can be used in broader aspects. PMV-64 monoclonal antibody can bind with both native and denatured forms of vitellin and vitellogenin, thus it was more desirable choice for determining vitellogenin concentration by competitive ELISA in our study.

It is noteworthy that native haemocyanin bound with various proteins such as HRP and mouse antibody. This causes substantial, nonspecific staining with the dot-blotting assay. It also occurred with immunoprecipitate of vitellin and vitellogenin antibody complexes (Fig. 5: Ab), and the precipitate in double immunodiffusion (not shown). Possibly, this abundant protein is not only an oxygen carrier, but also plays a major role in immunity since it can bind to a broad spectrum of alien, organic molecules.

Acknowledgments

We thank Dr. Arlo W. Fast of the University of Hawaii for kindly editing this manuscript. This work was supported by a student scholarship from the Thailand Research Fund (TRF) to S. Longyant under supervision of P. Menasveta, and by BIOTEC of National Science and Technology Development Agency (NSTDA) grants BT-39-06-API-17-23 to P. Sithigorngul.

References

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72 (1976) 533–544.

Browdy, C.L., Fainzilber, M., Tom, M., Loya, L. and Lubzens, E., Vitellin synthesis in relation to oogenesis *in vitro*-incubated ovaries of *Penaeus semisulcatus* (Crustacea, Decapoda, Penaeidae). J. Exp. Zool., 255 (1990) 205–215.

Byard, E.H. and Aiken, D.E., The relationship between molting, reproduction, and haemolymph female-specific protein in the lobster, *Homarus americanus*. Comp. Biochem. Physiol., 77A (1984) 749–757.

Chang, C.F., Lee, F. and Huang, Y., Purification and characterization of vitellin from the mature ovaries of prawn, *Penaeus monodon*. Comp. Biochem. Physiol., 105B (1993) 409–414.

Chang, C.F., Lee, F., Huang, Y. and Hong, T., Purification and characterization of the female-specific protein (vitellogenin) in mature female haemolymph of the prawn, *Penaeus monodon*. Invert. Reprod. Develop., 25 (1994) 185–192.

Chang, C.F., Jeng, S., Lin, M. and Tin, Y., Purification and characterization of vitellin from the mature ovaries of prawn, *Penaeus chinensis*. Invert. Reprod. Develop., 29 (1996) 87–93.

Chang, C.F. and Shih, T.W., Reproductive cycle of ovarian development and vitellogenin profiles in the freshwater prawns, *Macrobrachium rosenbergii*. Invert. Reprod. Develop., 27 (1995) 11–20.

Chen, C. and Chen, S., Isolation and partial characterization of vitellin from the egg of the giant tiger prawn, *Penaeus monodon*. Comp. Biochem. Physiol., 106B (1993) 141–146.

Eastman-Reks, S.B. and Fingerman, M., *In vitro* synthesis of vitellin by the ovary of the fiddle crab, *Uca pugilator*. J. Exp. Zool., 233 (1985) 111–116.

Eshnar, Z. Monoclonal antibody strategy and technique. In : Hybridoma Technology in Bioscience and Medicine, T.A. Springer (ed.). Pleenum Press, New York, 1985, pp. 1–42.

Fanzilber, M., Tom, M., Shafir, S., Applebuem, S.W., and Lubzen, E., Is there extraovarian synthesis of vitellogenin in penaeid shrimp? Bio. Bull., 183 (1992) 233–241.

Hasegawa, Y., and Hirose, E., Hormonal control of sexual differentiation and reproduction in crustacea.. Amer. Zool., 33 (1993) 403–411.

Humason, G.L., The Animal Tissue Techniques. W.H. Freeman, San Francisco, 1979.

Johnson, D.A., Guatsch, J.W., Sportman, J.R., and Elder, J.H., Improve technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech., 1 (1984) 3–8.

Köhler, G. and Milstein, C., Derivation of specific antibody-producing tissue culture and tumor cell fusion. Eur. J. Immunol., 6 (1976) 511–519.

Mosmann, T.R., Bauman, R., and Williamson, A.R., Mutations affecting immunoglobulin light chain secretion by myeloma cells I. Functional analysis by cell fusion. Eur. J. Immunol., 9 (1979) 511–516.

Qiu, Y.W., Ng, T.B. and Chu, K.H., Purification and characterization of vitellin from the ovaries of the shrimp *Metapenaeus ensis* (Crustacea: Decapoda: Penaeidae). Invert. Reprod. Develop., 31 (1997) 217–223.

Quackenbush, L.S. and Keeley, L.L., Regulation of vitellogenesis in the fiddle crab, *Uca pugilator*. Biol. Bull., 175 (1988) 321–331.

Quackenbush, L.S., Vitellogenesis in the shrimp, *Penaeus vannamei*: *in vitro* studies of the isolated hepatopancreas and ovary. Comp. Biochem. Physiol., 94B (1989a) 253–261.

Quackenbush, L.S., Yolk protein production in the marine shrimp, *Penaeus vannamei*. J. Crustacean Biol., 9 (1989b) 509–516.

Quinitio, E.T., Hara, A., Yamauchi, K, Mitzuchima T. and Fuji, A., Identification and characterization of vitellin in a hermaphrodite shrimp, *Pandarus kessleri*. Comp. Biochem. Physiol., 94B (1989) 445–451.

Quinitio, E.T., Hara, A., Yamauchi, K. and Fuji, A., Isolation and characterization of vitellin from the ovary of *Penaeus monodon*. Invert. Reprod. Develop., 17 (1990) 221–227.

Sagi, A., Soroka, Y., Snir, E., Chomsky, O., Calderon, J. and Milner, Y., Ovarian protein synthesis in the prawn *Macrobrachium rosenbergii*: Does ovarian vitellin synthesis exist? Invert. Reprod. Develop., 27 (1995) 41–47.

Shafir, S., Tom, M., Ovadia, M. and Lubzens, E., Protein, vitellogenin and vitellin levels, in the haemolymph and ovaries during ovarian development in *Penaeus semisulcatus* (de Haan). Biol. Bull., 183 (1992a) 394–400.

Shafir, S., Ovadia, M. and Tom, M., *In vivo* incorporation of labeled methionine into protein, vitellogenin and vitellin in females of the penaeid shrimp *Penaeus semisulcatus* de Haan. Biol. Bull., 183 (1992b) 242–247.

Sithigorngul, P., Cowden, C., Guastella, J. and Stretton, A.O.W., Generation of monoclonal antibodies against a nematode peptide extract: another approach for identifying unknown peptides. J. Comp. Neurol., 284 (1989) 389–397.

Soyez, D., Van Deijnen, J.E. and Martin, M. Isolation and characterization of a vitellogenensis-inhibiting factor from sinus glands of the lobster, *Homarus americanus*. J. Exp. Zool., 244 (1987) 479–484.

Soyez, D., Le Caer, J.P., Noel, P.Y. and Rossier, J., Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster *Homarus americanus*. Neuropeptides, 20 (1991) 25–32.

Takayanagi, H., Yamamoto, Y. and Takeda N., An ovary-stimulating factor in the shrimp, *Paratya compressa*. J. Exp. Zool., 240 (1986) 203–209.

Tom, M., Goren, M. and Ovadia, M., Purification and partial characterization of vitellin from the ovaries of *Parapenaeus longirostri* (Crustacea, Decapoda, Penaeidae). Comp. Biochem. Physiol., 87B (1987) 17–23.

Tom, M., Fingerman, M., Hayes, T.K., Johnson, V., Kerner, B. and Lubzens, E., A comparative study of the ovarian proteins from the two penaeid shrimps, *Penaeus semisulcatus* de Haan and *Penaeus vanamei* (Boone). Comp. Biochem. Physiol., 102B (1992) 483–490.

Wallace, R.A., Walker, S.L. and Hauschka, P.V., Crustacean lipovitellin isolation and characterization of the major high density lipoprotein from the eggs of decapods. Biochemistry, 6 (1967) 1582–1590.

Wolin, E.M., Laufer, H. and Albertini, D.F., Uptake of the yolk protein, lipovitellin, by developing crustacean oocytes. Dev. Biol., 35 (1973) 160–170.

Yano, I. and Chinzei, Y., Ovary is the site of vitellogenin synthesis in Kuruma prawn, *Penaeus japonicus*. Comp. Biochem. Physiol., 86B (1987) 213–218.

Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin subunits

SIWAPORN LONGYANT[1,2], PAISARN SITHIGORNGUL[2*], NITAYA THAMMAPALERD[3], WEERAWAN SITHIGORNGUL[2] and PIAMSAK MENASVETA[1,4]

[1] Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

[2] Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand.

[3] Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

[4] Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand and Marine Biotechnology Research Unit, BIOTEC, Bangkok 10400, Thailand.

Text: 18 pages,
3 tables,
5 figures.

Abbreviated title: Monoclonal antibodies production and characterization of vitellin.

Key words: Crustacean, monoclonal antibodies, *Penaeus monodon*, vitellin, vitellogenin, subunits, characterization.

* Corresponding author:

Dr. Paisarn Sithigorngul
Department of Biology
Faculty of Science
Srinakharinwirot University
Sukhumvit 23, Bangkok 10110
Thailand

Tel. No. (662) 260-0127
Fax. No. (662) 260-0128
E-mail <paisarn@psm.swu.ac.th>

Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin subunits

Summary

Monoclonal antibodies specific to *Penaeus monodon* vitellin subunits were produced from mouse immunized with sodium dodecyl sulfate (SDS) treated ovarian extract prepared from gravid ovaries. After fusion of mouse spleen cells with P3X myeloma, hybridomas were selected by indirect immunoperoxidase ELISA against *P. monodon* ovarian extract. This was followed by dot-blotting against native and denatured proteins from ovarian extract, female haemolymph, and male haemolymph then by dot-blotting against each vitellin subunit. Hybridoma clones producing antibodies specific to each of vitellin subunits, molecular mass of 83, 74 , 104 and 58, 104 and 45 kD, antibodies specific to the 215 kD protein - an oocyte specific protein, and one monoclonal antibody specific to haemocyanin were isolated. All monoclonal antibodies can bind to both native and denatured proteins. Western blot analysis of ovarian extract and female haemolymph from gravid ovary prawns separated by PAGE and SDS-PAGE revealed five vitellin subunits, molecular mass of 104, 83, 74, 58 and 45 kD in ovarian extract, and four vitellogenin related polypeptides, molecular mass of 200, 104, 83 and 74 kD in the female haemolymph. From the immunoreactive relationships among these proteins it could be assumed that vitellogenin may be released into the haemolymph in two forms, 200 and 74 kD, then the 200 kD polypeptide was either processed into the 104 and 83 kD polypeptides, or directly taken up into the oocyte. In the oocyte, the 104 kD protein would be further cleaved into 58 and 45 kD polypeptides while the 74 kD protein would undergo slight modification or remained unchanged. Western blot analysis of vitellin subunits at various stages of ovarian development revealed that the 200 kD protein appeared in the oocyte during early ovarian development and the 45 and 58 kD proteins appeared during the late development.

Key words: Crustacean, monoclonal antibodies, *Penaeus monodon*, vitellin, vitellogenin, subunits, characterization.

Introduction

Vitellin, or lipoglycoprotein, is a major component of yolk protein in the oocyte (Wallace et al., 1967), and typically consists of two to eight subunits depending on the authors and penaeid shrimp species (Table 1). With some species, however, namely *Penaeus monodon*, the molecular nature of vitellin and its precursor-vitellogenin is still not well documented with respect to either size or subunit number (Quinitio et al., 1990; Chang et al., 1993, 1994; Chen and Chen, 1993, 1994). These differences in vitellin characterization may relate to differences in ovarian maturity and/or methodologies for vitellin purification used by different researchers (Qiu et al.,1997). Recently, the monoclonal antibodies against vitellin were generated from mouse immunized with ovarian extract and 5 vitellin subunits which are slightly different from those in previous studies were described (Longyant et al., 1999); however, most monoclonal antibodies obtained in that study can bind to native proteins and therefore can not be used for characterization of all of the vitellin and vitellogenin subunits. In order to obtain antibodies that can bind to all denatured vitellin subunits, in the present study, the ovarian extract was denatured with sodium dodecyl sulfate before immunization. We were able to isolate monoclonal antibodies specific to each subunit of vitellin as well as another oocyte specific protein and haemocyanin. These antibodies were used for characterization of vitellin and vitellogenin subunits.

Materials and Methods

Source animal handling and initial preparations: Adult female *P. monodon* (80-120 g) were obtained from local farms around Bangkok. They were held in 5000 liter round tanks with sea water (30 $^{o}/_{oo}$) at 26-28 °C and ambient photoperiod. The prawns were fed with squid or small mussels twice daily. Three days after molting, female prawns were bilaterally eye-ablated to induce ovarian development. Seven to ten days after eye-ablation, 5 prawns with gravid ovaries (gonadosomatic index > 4% with dark grayish green color) were anesthetized using cold sea water (4 °C) and haemolymph was collected via the arthrodial membrane of the fourth walking leg. Ovaries were removed by dissection and washed in cold, 0.15 M phosphate buffered saline (PBS) pH 7.4. Haemolymph was also collected, in the same manner, from normal adult male *P. monodon* (60-80 g). Haemolymph and ovaries were frozen on dry ice and stored at -70 °C.

In order to determine the presence of various vitellin subunits at different stages of ovarian development, ovaries and haemolymph from individual prawn with ovaries at different developmental stages were also collected for SDS-PAGE and Western blot analysis of vitellin subunits as described below, and the haemolymph vitellogenin levels were determined by competitive ELISA as described previously (Longyant et al., 1999).

Ovarian extract preparation: Pooled ovaries from 5 prawns were homogenized for 5 min in 0.5 mM EDTA in PBS (0.5 g/ml). The pellet and lipid layer were eliminated after centrifugation at 10,000g and 4 °C for 30 min. The protein concentration of the extract was determined by Bradford assay (Bradford, 1976), adjusted to 10 mg/ml with PBS, then the extract was divided into 1 ml aliquots and stored at -70 °C.

Immunization: Ovarian extract was denatured by mixing 1:1 with 2X treatment buffer (for SDS-PAGE), boiled for 1 minute and dialysed against 4 changes of PBS for 48 hrs. Four Swiss mice were injected with 0.5 mg SDS-treated ovarian extract 1:1 (V:V) mixed with complete Freund's adjuvant. At two week

intervals, they were re-injected with ovarian extract mixed with incomplete Freund's adjuvant for the second injection, or with the extract without adjuvant for the following injections. One week after the fourth injection, mouse anti-vitellin antisera were collected and tested against ovarian extract, and female and male haemolymph by double immunodiffusion. The best performing mouse was boosted one week before hybridoma production.

Hybridoma production: A cell fusion procedure was adapted from the method developed by Köhler and Milstein (1976), with modification described by Mosmann et al. (1979). A P3X myeloma cell line was used as the fusion partner. The P3X myeloma cell line was obtained from Department of Virology, AFRIMS, Bangkok, Thailand. Fusion products from one mouse were plated on 23 microculture plates (96 wells, NUNC). After identifying wells containing desired clones by the screening methods described below, cells were re-cloned at least twice by the limiting dilution method (Eshnar, 1985).

Screening methods

ELISA: Hybridoma cell lines were first screened by ELISA against ovarian extract. The ovarian extract (1 ug/well protein) was plated on Maxisorb microtiter plates (NUNC). Blotto (5% or 0.5% nonfat dry milk in PBS) (Johnson et al., 1984) was used as blocking solution, antibody diluent, and washing solution. Antibody binding to plates was detected using horseradish peroxidase labeled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; Biorad) at 1:1000 dilution. Positive wells were further screened by dot-blotting.

Dot-blotting: Selected antigens, ovarian extract (1 mg/ml), female and male haemolymph in native forms, and SDS-mercaptoethanol treated forms were used for the second step of screening. All antigens (1 ul/spot) were applied to the same piece of nitrocellulose membrane. After the membrane was baked at 60°C for 10 min, each piece was incubated in hybridoma conditioned media from each clone (1:20 in 5% blotto) for 2 hr. After extensive washing in diluted blotto, the membrane was incubated in GAM-HRP (1:1000 in 5% blotto with 40%

male prawn haemolymph) for 2 hr. The membrane was then washed as before and exposed to the substrate mixture (0.03% diaminobenzidine, 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS) for 5 min.

Selected hybridoma clones were further screened by dot-blotting against 5 vitellin subunits and the 215 kD protein eluted from ovarian extract separated by SDS-PAGE (as described below) then processed in the same manner (dot-blotting) as described above.

Selected hybridoma clones were re-cloned and cryopreserved. Some hybridoma clones were injected into pristane primed mice for ascites fluid production.

Monoclonal antibody characterization

Polyacrylamide gel electrophoresis and immunoblotting: Polyacrylamide gel electrophoresis (PAGE) or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels in a mini-PROTEIN II electrophoresis apparatus (Bio-Rad) to identify vitellin and vitellogenin in the extract. Ovarian extract and haemolymph were applied to 5% gel for PAGE or 7.5% gel for SDS-PAGE according to the Bio-Rad Manual. Parts of the gel were cut off and visualized by staining with 0.1% Coomassie brilliant blue R250 for proteins, with Sudan black B for lipoproteins, or with periodic acid Schiff reagent (PAS) for glycoproteins (modified from Humason, 1979). Proteins in other parts of the gel were transferred to nitrocellulose sheet using Transblot apparatus (Bio-Rad) at 50 V for 3 hr. The nitrocellulose sheet was then separated from the gel and quenched in 5% blotto, cut into strips and assayed for antibody binding as described for the dot-blotting assay.

Isolation of ovarian proteins from SDS-PAGE: After ovarian extracts were separated by SDS-PAGE and stained with Coomassie blue, the desired protein bands were cut off, destained and then homogenized in an equal volume of 0.01% SDS, allowing the protein to be dissolved for 12 hr. The dissolved protein was separated from the gel by centrifugation and the gel pellet was re-

extracted with the same solution for 12 hr. Both supernatants were combined and concentrated in a vacuum concentrator (Savant). The vitellin subunits and oocyte specific protein from SDS-PAGE were used for screening hybridoma clones using dot-blotting as described above.

Class and subclass determination: The class and subclass of the mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA using a subisotyping kit for mouse (Zymed Laboratories).

Results

After the fourth immunization with *P. monodon* ovarian extract treated with SDS, the mouse anti-vitellin antisera from four mice produced strong precipitation bands to antigens in *P. monodon* ovarian extracts and to female haemolymph, but not to antigens in male haemolymph. All antisera bound to the denatured ovarian extract better than to the normal ovarian extract. Precipitation bands from ovarian extract and female haemolymph showed reaction of identity with all mouse anti-vitellin antisera (not shown). All mice showed similar responses; one of these mice was used as the spleen cell donor for hybridoma production.

From one fusion, every well containing hybridoma colonies ranged from 1-8 colonies/well in 23 microculture plates. The first screening on ELISA against ovarian extract yielded over 300 positive wells with varying immunoreactive intensities. Since there were so many positive wells containing several colonies, only about 200 wells containing a few hybridoma colonies were further screened by dot-blotting against ovarian extract, and female and male haemolymph using both native and SDS-treated antigens (Fig. 1). The wells showing strong immunoreactivities (70 wells) were further screened by dot-blotting against vitellin subunits and recloned. Twenty hybridoma clones were re-cloned successfully and grouped into 6 categories according to their binding capabilities (Fig. 2; Table 2). All monoclonal antibodies obtained from this experiment bound to both native and denatured antigens (Fig. 1, Table 2). In Western blot analysis of ovarian extract separated by PAGE (Fig. 3), all four groups of monoclonal antibodies specific to vitellin and vitellogenin bound to the same lipoglycoprotein band while the other monoclonal antibodies (PMVS-106 and other clones) bound to an oocyte specific protein, and PMVS-22 monoclonal antibody showed light staining to a smear of highly negative charged protein.

In Western blot analysis of ovarian extract separated by SDS-PAGE, all four groups of monoclonal antibodies specific to vitellin bound to each

vitellin subunit (Fig. 4A-E lane a), 83 kD (PMVS-93), 74 kD (PMVS-140), 104 and 58 kD (PMVS-109), and 104 and 45 kD (PMVS-158). The other monoclonal antibodies (PMVS-106 and other clones; Fig. 4F lane a) bound to the oocyte specific protein molecular mass of 215 kD, and another monoclonal antibody (PMVS-22) bound to both haemocyanin subunits (Fig. 4G lane a).

In Western blot analysis of female haemolymph separated by SDS-PAGE (Fig. 4 lane b), a high molecular weight vitellogenin subunit (200 kD), was recognized by PMVS-93, 109 and 158 monoclonal antibodies. These antibodies also recognized the vitellogenin subunits of molecular mass of 83 or 104 kD. The 58 and 45 kD vitellin related proteins were not observed in haemolymph (Fig. 4B, D and E lane b). The PMVS-140 antibody recognized only the vitellogenin of molecular mass of 74 kD (Fig. 4C lane b). The PMVS-106 antibody did not show specific binding to any haemolymph proteins (Fig. 4F lane b) and PMVS-22 antibody bound to both subunits of haemocyanin (Fig. 4G lane b).

Western blot analysis of ovarian extracts prepared from individual prawn at different stages of ovarian development (Fig. 5, Table 3) revealed that none of the proteins in resting stage ovary reacted with monoclonal antibodies specific to vitellin and oocyte specific protein (Fig. 5B-F lane a). In developing stages, the major vitellin subunits, molecular mass of 200, 104, 83, and 74 kD were recognized while in the ripening stage the 200 kD tended to disappear with the gradual appearance of the 58 and 45 kD subunits. The presence of the 200 kD protein in the ovarian extract seemed to be unrelated to the haemolymph vitellogenin levels but, rather, related to the early development stage of the ovary (Fig. 5A-E lane b-e, Table 3).

Isotypes and subisotypes of the monoclonal antibodies mostly (15 out of 20 clones) belonged to the IgG1 isotype (PMVS-10, 11, 18, 43, 50, 52, 53, 72, 93, 97, 122, 137, 140, 157 and 158), only four of them (PMVS-22, 106, 109 and 121) belonged to the IgG2a subisotype and only one (PMVS-42) belonged to the IgG2b subisotype.

Discussion

From one fusion of P3X myeloma and spleen cells of a mouse immunized with the denatured ovarian extract of *Penaeus monodon*, we isolated a complete set of monoclonal antibodies specific to vitellin subunits. One group of hybridoma clones produced antibodies that bound to oocyte specific protein and one hybridoma clone produced antibody that bound to both subunits of haemocyanin. All of the monoclonal antibodies could bind to both native and denatured proteins which was not surprising since the immunogens used were denatured proteins; therefore, the production of monoclonal antibodies by immunization with denatured immunogens yields more broadly useful monoclonal antibodies than using native proteins as reported previously (Longyant et al., 1999; Table 2).

The monoclonal antibodies specific to various vitellin subunits obtained from this study are comparable to the antisera made against purified vitellin subunits in another previous study (Chen and Chen, 1993). Two groups of monoclonal antibodies (PMVS-93 and 140) recognize each single subunit of vitellin, the 83 and 74 kD subunits respectively. They are equivalent to anti-Ep3 and anti-Ep4 antisera (Chen and Chen, 1993). The other two groups of monoclonal antibodies (PMVS-109 and 158) recognize the same subunit (104 kD) which is equivalent to anti-Ep2 antiserum. However, PMVS-109 and 158 monoclonal antibodies recognize different small subunits (58 and 45 kD), indicating that the two monoclonal antibodies are specific to different epitopes on the 104 kD protein and that the 58 and 45 kD proteins may be derived from the 104 kD protein. The Ep2 and Ep3 antisera crossreact with the 168 kD protein in haemolymph in the same manner as PMVS-93, 109 and 158 monoclonal antibodies in this study crossreact with the 200 kD haemolymph protein. Therefore, it is quite certain that the 168 or 170 kD polypeptides described in other studies (Quinitio et al., 1990; Chen and Chen, 1993, 1994: Chang et al., 1994) are the same as the 200 kD polypeptide in our study. The difference in size is due to variations in calculation since their studies

used a wide range of molecular weight standard proteins (170 and 340 kD). Also, the SDS-PAGE results (Chen and Chen, 1993) clearly shown that the 168 kD polypeptide migrated more slowly than the 170 kD standard protein; therefore, this protein must be bigger than 170 kD. In our study, this protein migrated slightly faster than the 205 kD standard protein (Fig 4A & 5A). From the immunoreactive crossreactivity (Chen and Chen, 1993), anti-Ep2 (104 kD) and anti-Ep3 (83 kD) antisera crossreact with the 168 kD protein which indicates that the 104 and 83 kD proteins derived from the cleavage of the 168 kD protein (which is unlikely); thus, this polypeptide must be larger than 168 kD.

In haemolymph from female prawn with gravid ovaries, the vitellogenin subunits (vitellin related proteins), molecular mass of 200, 104, 83 and 74 kD (the 104 and 83 kD are slightly larger than the counterpart vitellin subunits) were recognized by PMVS-93, PMVS-140, PMVS-109 and PMVS-158 monoclonal antibodies (Fig. 4) which is similar to a previous report on *P. monodon* that haemolymph contained four egg yolk peptides of 168, 104, 83 and 74 kD (Chen and Chen, 1994). The immunoreactive relationships among those vitellin subunits suggest that the 200 kD protein is the precursor and either cleaved to yield the 104 and 83 kD proteins in the haemolymph before or directly after being taken up into the oocyte. Then, in the oocyte, the 104 kD subunit may be further cleaved to yield two smaller 58 and 45 kD subunits which share the same epitopes with the 104 kD subunit. This result shows the superior characteristics of the monoclonal antibodies that can be used to differentiate between different epitopes on the same molecule.

The 74 kD subunit does not share any epitope with the 200 kD protein, in common with the anti-Ep4 antiserum which does not crossreact with the 168 kD polypeptide (Chen and Chen, 1993). This protein may be synthesized, released, and then taken up without or with minor modification (Fig. 3). This vitellogenin subunit (74 kD) was not reported in the isolation of haemolymph vitellogenin by Sudan black staining and sucrose density gradient *in P.*

monodon (Chang et al., 1994) and *P. chinensis* (Chang and Jeng, 1995). It is possible that, in haemolymph, this subunit may be not associated with the 200 (168), 104 and 83 (93) kD proteins. Moreover, the size of the 74 kD protein is also very close to the size of the high abundant small subunit of haemocyanin, and the co-migration of these two proteins in SDS-PAGE may cause difficulty in identification of this subunit.

This study confirms previous findings from SDS-PAGE analysis of the glycolipoprotein isolated from PAGE, revealing that, in gravid *P. monodon* ovary, vitellin consists of 5 major subunits molecular mass of 45, 58, 74, 83 and 104 kD (Longyant et al., 1999) which is similar to the vitellin of *P. chinensis* consisting of 5 major subunits molecular mass of 40, 58, 78, 85, and 105 kD (Chang et al., 1996). The 168 kD protein in the ovarian extracts of other studies (Quinitio et al., 1990; Chang et al., 1993) was not detected in this and the previous studies (Longyant et al., 1999) on *P. monodon*. As discussed above the 168 kD protein may be the same as the 200 kD protein found in the haemolymph in our study and was found during early developmental stages of the ovaries. In studies of vitellogenin synthesis in ovarian tissue *in vitro*, the 168 and 74 kD proteins were secreted into the medium (Chen and Chen, 1994). It is possible that during this highly active stage of ovarian development the 168 (200) kD protein may be taken up directly and slowly undergo processing to the 104 and 83 kD with maturation progress. The accumulation of the 58 and 45 kD hydrolysis products increased with the reduction of the high molecular weight forms. The other possibility is that the 200 kD protein was a contaminant from the haemolymph surrounding the oocyte follicles in the ovary; this is unlikely since in the gravid ovary with high haemolymph vitellogenin levels the 200 kD protein was not observed in ovarian extract (Fig. 5B, D and E lane d & e, Table 3). The immunoreactivity of the PMVS-106 revealed that the 215 kD protein is synthesized in ovary only during oocyte maturation, not in resting ovary, and that this protein may undergo processing to smaller molecular forms as well since different

immunoreactive small molecular forms were also detected in all stages of ovarian development (Fig. 5F). Thus, interpretations of the incorporation of radioactive amino acid using anti-vitellin antiserum must be made with caution, since this protein is synthesized during ovarian development and most antisera against purified vitellin may contain considerable amounts of the antibodies specific to other proteins as shown in previous experiments (Quinitio et al., 1990).

Eight small subunits of vitellin including the 45 and 58 kD polypeptides were observed in another study of this species (Chang et al., 1993). In this case, it is possible that these small subunits may have been generated during sample preparation, since in this preparation the ovaries were homogenized in Tris buffer without EDTA or other protease inhibitors. Then, after homogenization, the ovarian extract was also dialysed without protease inhibitors. Similar confusion in the identification of vitellin subunits has also occurred in other species such as *P. semisulcatus* (Browdy et al., 1990; Tom et al., 1992; Khayat et al., 1994; Lubzen et al., 1997: Table 1); however, only 2-3 subunits of vitellin has been reported for most penaeid shrimp species (Table 1).

From the immunoreactive equivalent of the vitellogenin and vitellin subunits, our results suggested that vitellogenin may be synthesized and released into the haemolymph in two different molecular forms, the 200 and 74 kD proteins, then the 200 kD protein is processed into either the 104 and 83 kD before or after being taken up into the oocyte. Finally, as maturation progresses, the 104 kD fragment is further processed into the 58 and 45 kD subunits. The 74 kD protein was taken up and remained in the oocyte with little or no further modification. However, the relationship between the stage of ovarian development and haemolymph vitellogenin level in the samples shown here seem to be uncorrelated. In the freshwater prawn *Macrobrachium rosenbergii*, the vitellogenin level increased during ovarian maturation and then sharply dropped in the ripe ovary (Derelle et al., 1986), however, the

haemolymph vitellogenin levels at each gonadosomatic index are highly variated in this species (Lee and Chang, 1997). More detailed investigations of the relationship between vitellogenin levels and stage of ovarian development are in progress.

The site of vitellogenin synthesis is still in question. If vitellogenin was synthesized in the ovaries then secreted into haemolymph before being taken up by the developing oocytes as proposed by several authors (Yano and Chinzei, 1987; Lubzens et al., 1997), there are still questions about which cells in the ovary (the oocyte or follicular cell, or other cell) synthesize vitellogenin. Another question is why vitellogenin has to be secreted into the haemolymph before being taken into the oocytes which are in close proximity and are in a very active stage for vitellogenin uptake. In preliminary experiments using various monoclonal antibodies against each subunit for immunocytochemical localization, we were not able to observe any vitellin immunoreactive substance in either follicular cell or hepatopancreas of vitellogenic prawn.

This study showed that the monoclonal antiboby technique can generate highly and monospecific antibodies to each of vitellin subunits without the requirement for purification of the antigens. Antibodies with different specificity can be used for characterization of the molecular nature of the molecule while making antisera to each subunit would be a very tedious task (since a slight impurity in the immunogen would cause mis-segregation of the the molecules). Monoclonal antibodies specific to each subunit can be used as a tool to investigate the production site of vitellogenin subunits and to follow molecular processing of the vitellin during ovarian development as well as larval development.

Acknowledgments

We deeply appreciate Dr. Chris Airriess, Department of Zoology, University of British Columbia, Canada for kind editing this manuscript. This work was supported by a student scholarship from Thailand Research Fund (TRF) to S. Longyant under supervision of P. Menasveta, and by BIOTEC of National Science and Technology Development Agency (NSTDA) grants BT-39-06-API-17-23 to P. Sithigorngul.

References

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72 (1976):533-544.

Browdy, C.L., Fainzilber, M., Tom, M., Loya, L. and Lubzens, E., Vitellin synthesis in relation to oogenesis *in vitro*-incubated ovaries of *Penaeus semisulcatus* (Crustacea, Decapoda, Penaeidae). J. Exp. Zool.,255 (1990):205-215.

Chang, C.F., Lee, F. and Huang, Y., Purification and characterization of vitellin from the mature ovaries of prawn, *Penaeus monodon*. Comp. Biochem. Physiol., 105B (1993):409-414.

Chang, C.F., Lee, F., Huang, Y. and Hong, T., Purification and characterization of the female-specific protein (vitellogenin) in mature female haemolymph of the prawn, *Penaeus monodon*. Invert. Reprod. Develop., 25 (1994):185-192.

Chang, C. and Jeng, S. Isolation and characterization of the female-specific protein (vitellogenin) in mature female haemolymph of the prawn *Penaeus monodon*. Comp. Biochem. Physiol., 112B (1995):257-263.

Chang, C.F., Jeng, S., Lin, M. and Tin, Y., Purification and characterization of vitellin from the mature ovaries of prawn, *Penaeus chinensis*. Invert. Reprod. Develop., 29 (1996):87-93.

Chen, C. and Chen, S., Isolation and partial characterization of vitellin from the egg of the giant tiger prawn, *Penaeus monodon*. Comp. Biochem. Physiol., 106B (1993):141-146.

Chen, C. and Chen, S. Vitellogenensis in the giant tiger prawn, *Penaeus monodon* Fabicius, 1798. Comp. Biochem. Physiol., 107B (1994):453-460.

Derelle, E., Grosclaude, J., Meusy, J.J., Jun'era, H. and Martin, M. ELISA tritation of vitellogenin and vitellin in the freshwater prawn, *Macrobrachium rosenbergii*, with monoclonal antibody. Comp. Biochem. Physiol., 85B (1986):1-4.

Eshnar, Z., Monoclonal antibody strategy and technique. In : Hybridoma technology in bioscience and medicine, T.A. Springer, ed., Pleenum Press, New York, 1985, p1-42.

Humason, G.L., The animal tissue techniques, W.H. Freeman and Company, San Francisco, 1979, 661 pp.

Johnson, D.A., Guatsch, J.W., Sportman, J.R., and Elder, J.H., Improve technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech., (1984) 1:3-8.

Khayat, M., Lubzen, E., Tietz, A., and Funkenstein, B., Cell-free synthesis of vitellin in the shrimp *Penaeus semisulcatus* (de Haan). Gen. Comp. Endocrinol. 93 (1994): 205-213.

Köhler, G. and Milstein, C., Derivation of specific antibody-producing tissue culture and tumor cell fusion. Eur. J. Immunol., 6 (1976): 511-519.

Lee, F. and Chang, C. The concentration of vitellogenin (vitellin) and protein in haemolymph, ovary and hepatopancreas in different ovarian stages of the freshwater prawn, *Macrobrachium rosenbergii*. Comp. Biochem. Physiol. 117A (1977):433-439.

Longyant, S., P. Sithigorngul, N. Thampalerd, W. Sithigorngul and P. Menasveta, Monoclonal antibodies production specific to vitellin and vitellogenin of giant tiger prawn *Penaeus monodon*. Invert. Reprod. Dev., 35 (1999):9-17.

Lubzens, E., Ravid, T., Khayat, M., Daube, N., and Tietz, A. Isolation and characterization of lipoproteins from the haemolymph and ovary of the penaeid shrimp *Penaeus semisulcatus* (de Haan): Apoprotein and lipids. J. Exp. Zool., 278 (1997):339-348.

Mosmann, T.R., Bauman, R., and Williamson, A.R., Mutations affecting immunoglobulin light chain secretion by myeloma cells. I: Functional analysis by cell fusion. Eur. J. Immunol., 9 (1979):511-516.

Qiu, Y.W., Ng, T.B. and Chu, K.H., Purification and characterization of vitellin from the ovaries of the shrimp *Metapenaeus ensis* (Crustacea: Decapoda: Penaeidae). Invert. Reprod. Dev., 31:1-3 (1997) :217-223

Quackenbush, L.S., Vitellogenesis in the shrimp, *Penaeus vannamei*: *in vitro* studies of the isolated hepatopancreas and ovary. Comp. Biochem. Physiol., 94B (1989):253-261.

Quinitio, E.T., Hara, A., Yamauchi, K. and Fuji, A., Isolation and characterization of vitellin from the ovary of *Penaeus monodon*. Invert. Reprod. Dev., 17 (1990):221-227.

Tom, M., Goren, M. and Ovadia, M., Purification and partial characterization of vitellin from the ovaries of *Parapenaeus longirostri* (Crustacea, Decapoda, Penaeidae) Comp. Biochem. Physiol., 87B (1987):17-23.

Tom, M., Fingerman, M., Hayes, T.K., Johnson, V., Kerner, B. and Lubzens, E., A comparative study of the ovarian proteins from the two penaeid shrimps, *Penaeus semisulcatus* de Haan and *Penaeus vanamei* (Boone) Comp. Biochem. Physiol., 102B (1992):483-490.

Wallace, R. A., Walker, S.L. and Hauschka, P.V., Crustacean lipovitellin isolation and characterization of the major high density lipoprotein from the eggs of decapods. Biochemistry., 6 (1967):1582-1590.

Yano, I and Chinzei, Y., Ovary is the site of vitellogenin synthesis in Kuruma prawn, *Penaeus japonicus*. Comp. Biochem. Physiol., 86B (1987):213-218.

Table 1. Molecular mass of vitellin and vitellogenin subunits reported in six penaeid shrimp species.

Species		Molecular mass (kD)		References
		Holo protein	Subunits	
P. monodon	Vitellin	540	74[3], 83[2] 90, 104[1], 168*	Quinitio et al., 1990.
		ND	74[3], 83[2],104[1], 168*	Chen and Chen, 1993.
		492	35, 45[5], 49, 58[4], 64, 68, 82[2], 91	Chang et al., 1993.
		ND	45[5], 58[4], 74[3], 83[2], 104[1]	Longyant et al., 1999.
	Vitellogenin	ND	74[3], 83[2], 104[1], 168*	Chen and Chen, 1994.
		ND	82[2], 170*	Chang et al., 1994.
		ND	74[3], 83[2], 104[1], 200*	This study.
P. chinensis	Vitellin	380	40, 58, 78, 85, 105	Chang et al., 1996.
		500	78, 85, 155	
	Vitellogenin	ND	85, 191	Chang and Jeng, 1995.
P. semisulcatus	Vitellin	283	50, 63, 80, 90	Browdy et al., 1990.
		283	86, 95	Tom et al., 1992.
		ND	80, 96, 158	Khayat et al., 1994
		ND	80, 120, 200	Lubzens et al., 1997.
	Vitellogenin	ND	80, 120, 200	Lubzens et al., 1997.
P. vannamei	Vitellin	289	61, 69	Tom et al., 1992.
		ND	76, 95, 97, 103	Quackenbush, 1989.
Parapenaeus longirostris	Vitellin	ND	45, 66	Tom et al., 1987.
Metapenaeus ensis	Vitellin	350	76, 102	Qiu et al., 1997.

ND = Not determined

The superscripts indicated the equivalent vitellin or vitellogenin subunits found by other studies.

Table 2 Specificity of monoclonal antibodies tested by dot-blot and Western blot obtained from this study (b) compared to the previous study (a) (Longyant et al., 1999)

Monoclonal antibodies obtained from mice immunized with	Dot-blot test								
	Ovarian extract		Female haemolymph		Vitellin subunits (kD)				
	Native	Denatured	Native	Denatured	104	83	74	58	45
a. Native vitellin									
PMV-11, 15, 22	+	-	+	-	?	?	?	?	?
PMV-64	+	+	+	+	-	+	-	-	-
b. Denatured vitellin									
PMVS-10, 18, 50, 53, 93, 97	+	+	+	+	-	+	-	-	-
PMVS-11, 43, 72, 121, 137, 140	+	+	+	+	-	-	+	-	-
PMVS-52, 109	+	+	+	+	+	-	-	+	-
PMVS-158	+	+	+	+	+	-	-	-	+
PMVS-42, 106, 122, 157	+	+	-	-	Oocyte specific protein				
PMVS-22	-	-	+	+	Haemocyanin				

The underlined clones are the representative antibodies from each group that used for further analysis.

+ = bind, - = not bind and ? = not known.

Table 3. Vitellin subunits found in various stages of ovarian development

Stage of development	GSI	Vitellogenin levels	Oocyte specific protein and vitellin subunits (kD)						
	%	(mg/ml)	215	200	104	83	74	58	45
a. Resting	0.54	0	-	-	-	-	-	-	-
b. Early Developing	2.84	5.06	+	±	±	±	++	-	-
c. Developing	3.23	0.37	+++	++	++	++	+++	-	-
d. Ripe	5.78	0.87	+	±	++	++	+++	±	±
e. Ripe	5.59	4.54	++	-	+	++	+++	++	++

The intensity of the immunoreactivity was scored :

- = undetectable, ± = slightly detectable, and + = cleary detectable with three levels of intensity.

Figure 1. Screening results of 20 monoclonal antibodies by dot-blot against native and denatured antigens. Each nitrocellulose sheet was treated with various monoclonal antibodies (PMVS-...). Antigens in the vertical columns are *P. monodon*: ovarian extract (O); female (F) and male (M) haemolymph. Proteins in the upper horizontal rows were untreated, while proteins in the lower rows were SDS and mercaptoethanol treated.

Figure 2. Screening results of monoclonal antibodies by dot-blot against each vitellin subunit (1 = 104 kD, 2 = 83 kD, 3 = 74 kD, 4 = 58 kD and 5 = 45 kD subunits) and the oocyte specific protein (0 = 215 kD). The illustrations show only one representative from each group of monoclonal antibodies.

Figure 3. PAGE and immunoblot analysis of *P. monodon* ovarian extract. Crude ovarian extract was separated by PAGE and visualized by staining with Coomassie blue (a), periodic acid Schiff (PAS) reagent (b), or Sudan black B (c); or it was transferred to nitrocellulose membrane and treated separately with monoclonal antibodies; PMVS-93 (d), PMVS-140 (e), PMVS-109 (f), PMVS-158 (g), PMVS-106 (h) and PMVS-22 (i). About 20 ug protein/lane was used for glycolipoprotein staining (a-c) and about 2 ug protein/lane was used for immunoblotting (d-i).

Figure 4. SDS-PAGE and immunoblot analysis of *P. monodon* ovarian extract (a) and female haemolymph (b). Preparations were stained with Coomassie blue (A); the far left lane contained molecular standard proteins (s). The proteins were transferred to a nitrocellulose membrane and immunoblotted with various monoclonal antibodies, PMVS-93 (B), PMVS-140 (C), PMVS-109 (D), PMVS-158 (E), PMVS-106 (F), PMVS-22 (G). The numbers on the left side are the molecular weights of the marker proteins. The numbers (0-5) between lane a and b are ovarian specific protein and vitellin subunits (molecular mass of 215, 104, 83, 74, 58, 45 kD respectively), h is haemocyanin subunits, and * indicates

the 200 kD vitellogenin precursor. Protein content was about 20 ug/lane (a) and 50 ug/lane (b) for Coomassie blue staining, and about 2 ug/lane (a) and 5 ug/lane (b; except in G lane b =1 ug/lane) for immunoblotting.

Figure 5. SDS-PAGE and immunoblot analysis of *P. monodon* ovarian extract prepared from different stages of development: (a) resting stage, (b) early developing, (c) developing, (d) ripe ovary with a low haemolymph vitellogenin level, (e) ripe ovary with a high vitellogenin level. The preparation was stained with Coomassie blue (A), the far left lane (s) are standard marker proteins. The proteins were transferred and immunoblotted with various monoclonal antibodies: PMVS-93 (B), PMVS-140 (C), PMVS-109 (D), PMVS-158 (E) and PMVS-106 (F). The legends in the figure are the same as in figure 4. Protein content was about 25 ug/lane for Coomassie blue staining, and about 1 ug/lane for immunobloting.

Figure 1.

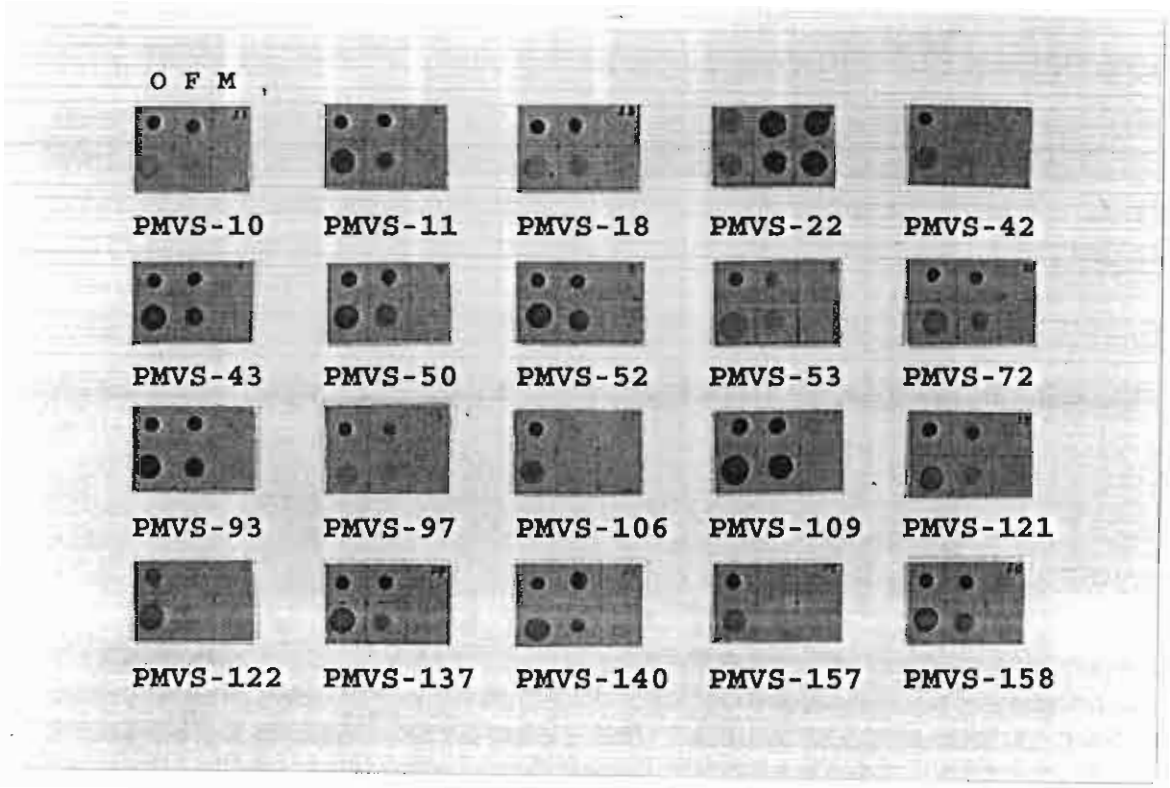


Figure 2.

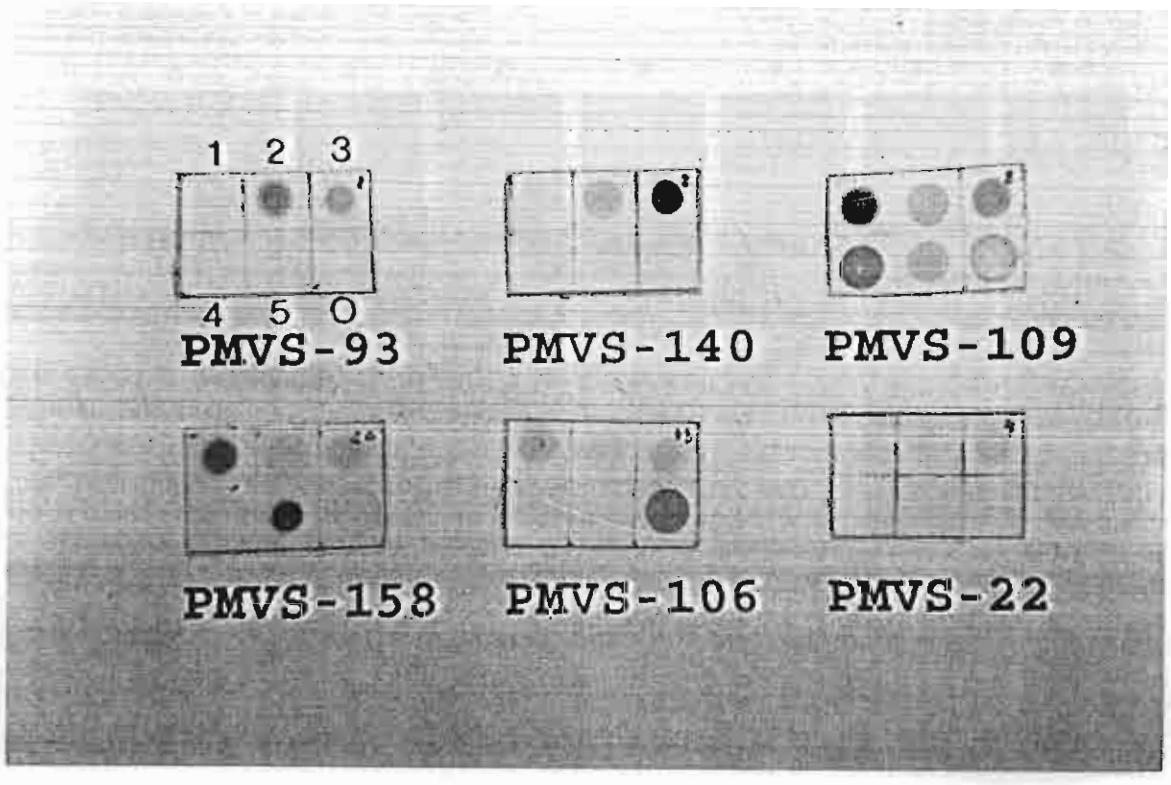


Figure 3.

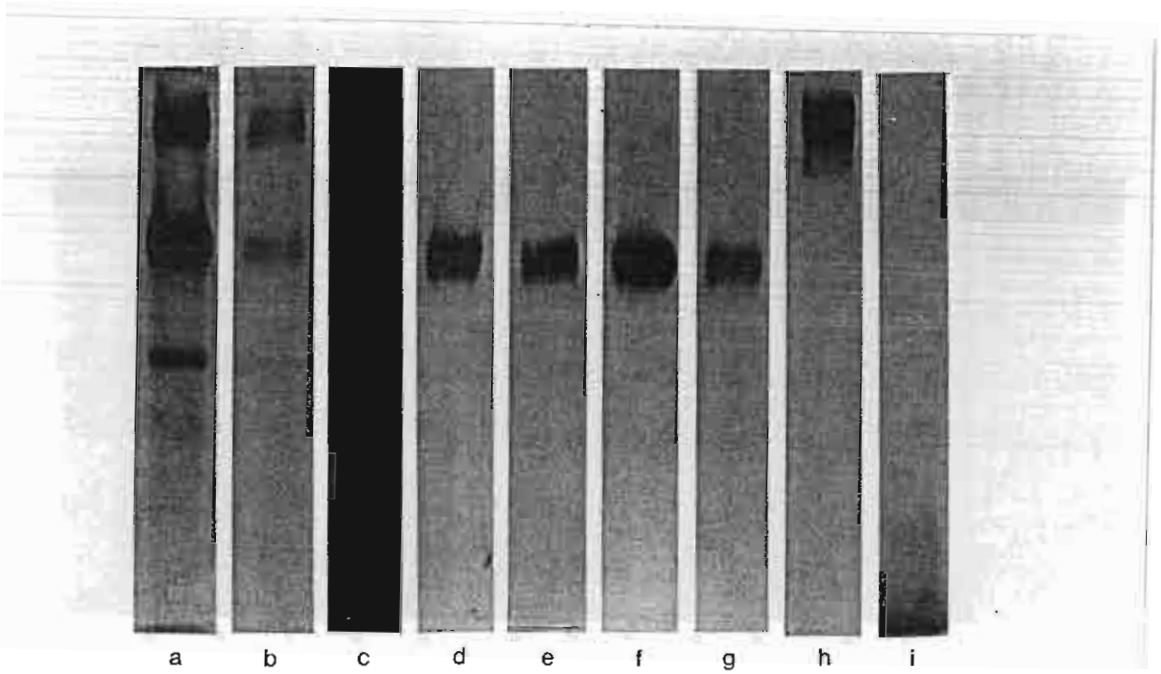


Figure 4.

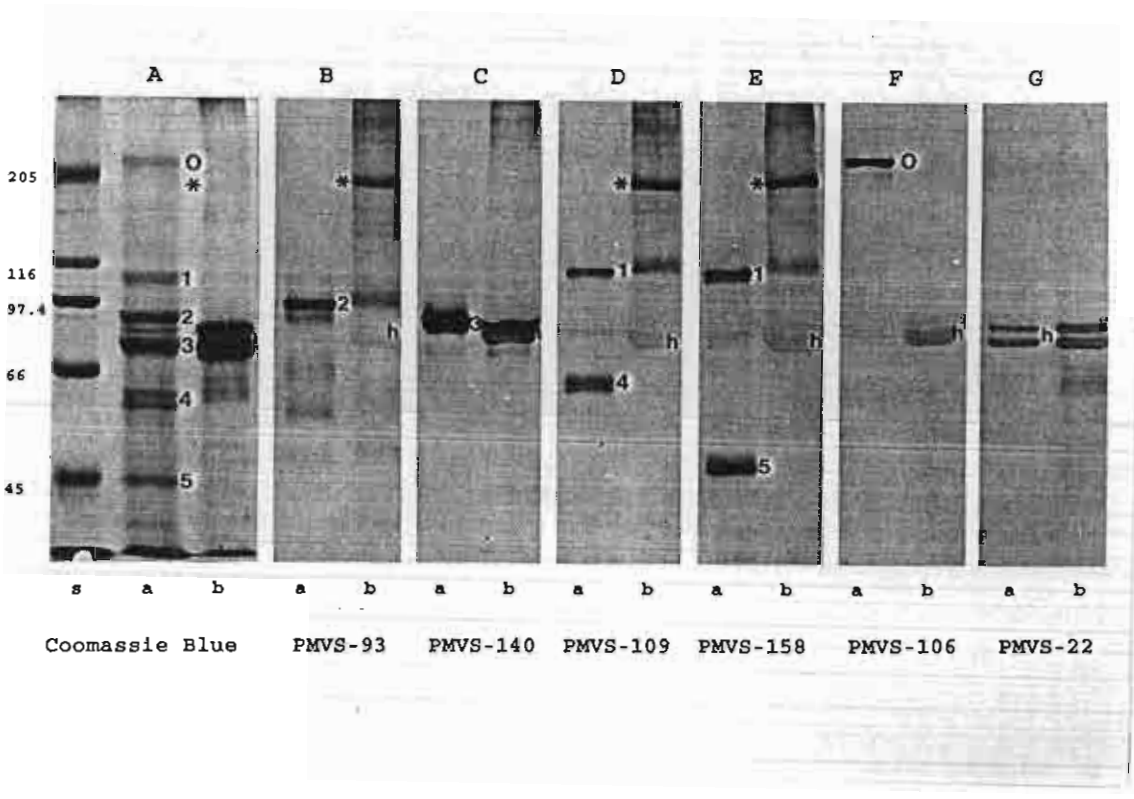
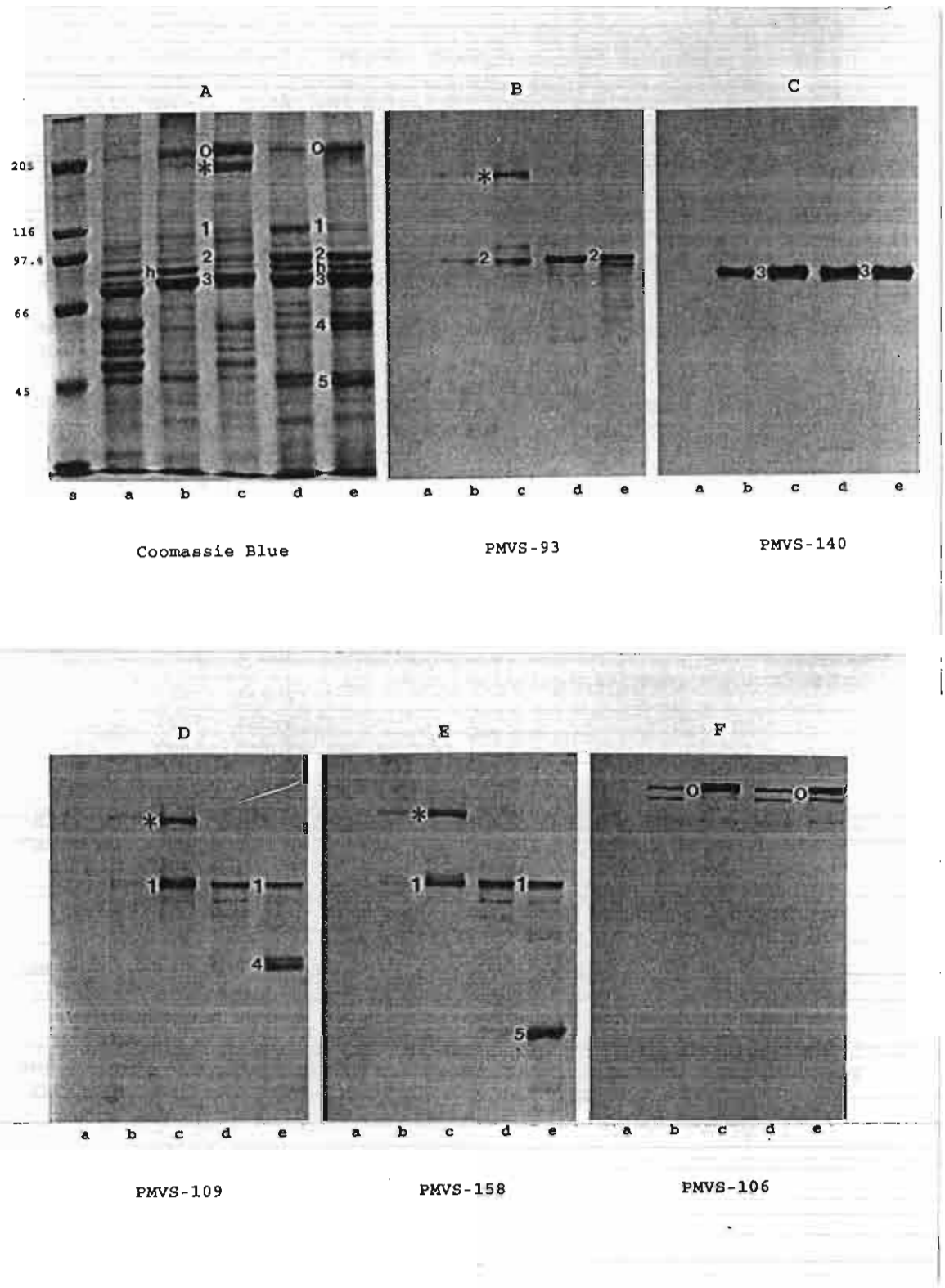


Figure 5.



GST 0.5 1.84 3.23 5.98 5.59

O 5.06 .37 .87 4.54

Development of a monoclonal antibody specific to yellow head virus: Establishing an immunological-basis for specific diagnosis of viral infection in *Penaeus monodon*

Paisarn Sithigorngul[1*], Phudhi Chauychuwong[2], Weerawan Sithigorngul[1], Siwaporn Longyant[1], Parin Chaivisuthangkura[1], Piamsak Menasaveta[2,3,4].

[1]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand.

[2]Marine Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, Ministry of Science, Technology and Environment, Bangkok, 10400, Thailand.

[3]Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand.

[4]Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.

*Corresponding author:

Dr. Paisarn Sithigorngul
Department of Biology,
Faculty of Science
Srinakharinwirot University,
Sukhumvit 23, Bangkok 10110,
Thailand.

Tel. No. (662) 260-0127
Fax. No. (662) 260-0128
E-mail <paisarn@psm.swu.ac.th>

Text 9 Pages
5 Figures
- Tables

Abbreviated title: Development of a monoclonal antibody: diagnostic test for viral infection

Key words: Dot-blot, immunocytochemistry, monoclonal antibodies, SEMBV, *Penaeus monodon*, viral infection, YHV.

Development of a monoclonal antibody specific to yellow head virus: Establishing an immunological-basis for specific diagnosis of viral infection in *Penaeus monodon*

Abstract

A monoclonal antibody specific for viral antigen was produced from a mouse immunized with *Penaeus monodon* gill extract prepared from SEMBV artificially infected shrimps. One clone designated SEM-3-2B specifically bound to native and SDS treated viral antigens. Immunocytochemical studies of infected gills revealed viral immunoreactivities in epithelial cell cytoplasm and in haemocytes. No antibody binding was observed in gills from non-infected shrimp. However, immunocytochemical examination of tissues from single viral infected shrimps revealed that the antibody reacted to tissues from YHV infected shrimps but did not bind to the tissues from SEMBV infected shrimps. Western blot analysis indicated that the antibody reacted with a 135 kD glycoprotein of YHV. These results suggested that viral preparations utilized in this study were obtained from shrimps infected with both viruses. Preliminary work with dot-blotting using indirect immunoperoxidase assay revealed that the antibody was able to detect viral antigen in diluted haemolymph up to 1:50 dilutions as well as in ammonium sulfate precipitated haemolymph up to 1:1000 dilutions. In addition, viral immunoreactivities were observed in a few cells of gill muscles, ovaries, hepatopancreas and lymphoid organs of asymptomatic shrimps using immunocytochemical techniques. This evidence suggested that this antibody can be used to identify mild viral infection in shrimp and therefore provides a promising and effective tool for routine viral diagnosis.

Key words: Dot-blot, immunocytochemistry, monoclonal antibodies, SEMBV, *Penaeus monodon*, viral infection, YHV.

Introduction

Shrimp diseases, especially viral infections have caused major economic disasters in Thai shrimp farming during the past decade. Yellow-head virus (YHV) and systemic ectodermal and mesodermal baculovirus (SEMBV), two highly pathogenic viruses have caused massive mortality of *Penaeus monodon*, the principal penaeid species cultured commercially in Thailand (Flegel et al., 1997). Recent attempts to prevent and control these two diseases relied primarily on detection and diagnosis of infected animals. PCR is now widely used to detect SEMBV, while a RT-PCR test is under development for YHV detection (Wongteerasupaya et al., 1997, Lightner and Redman, 1998). Even though both PCR techniques are highly sensitive for virus detection, there are practical limitations for their widespread commercial application. These limitations include needs for special equipment and highly trained personnel. Consequently, assay costs are expensive, which limits PCR use.

An alternative method to PCR is histopathological examination and symptomatic diagnosis (Wongteerasupaya et al., 1995; Lightner and Redman 1998; Mohan et al., 1998; Park et al., 1998), but this also requires experienced personnel. In addition, early infection diagnosis is difficult and unreliable with this approach. Moreover, field samples of shrimp are usually not well preserved and pathogenic symptoms may be incorrectly assigned to other causes such as bacterial infection. These limitations are the main reasons for incomplete epidemiological data for SEMBV and YHV infections.

Immunological techniques are widely used for diagnosis of many viral diseases with both human medicine and agricultural applications. Beside high sensitivity and accuracy, immunological methods can be simplified to obtain results quickly at relatively low costs. Pregnancy strip test for HCG is one such application of this technique.

Several attempts to produce antisera against YHV and SEMBV have been reported recently (Nadala et al., 1997 and 1998; Loh et al., 1998). However, the antisera assay (Western blot analysis) is not highly sensitive and is not easily applied during field examinations. Several laboratories have been trying to produce monoclonal antibodies (MAbs), but successful development of MAbs with sufficient specificity for diagnosis applications has not yet been reported. Moreover, problems with MAbs specificity have been reported with both IHHNV (Lightner and Redman, 1998) and YHV (Kasornchandra-personal communications). In both cases, specificity problems were related to obtaining IgM during production of MAbs.

Our work presented herein describes our successful development of highly specific MAbs against YHV from preparations made from shrimps artificially infected with SEMBV and preliminary detection of YHV by dot-ELISA and immunocytochemistry.

Materials and Methods

Viral infection and preparation. Juvenile *P. monodon* (25-30 g) from farms near Bangkok were held in 300-l fiberglass tanks with 20-ppt seawater and ambient photoperiod. SEMBV (kindly provided by Aquatic Animal Health Research Institute, Royal Thai Department of Fisheries, Bangkok) was injected via the arthrodial membrane of the second walking leg as a diluted viral suspension (1:10) at 50-ul/shrimp.

When infected shrimp showed signs of heavy infection such as feeding cessation and slow movement, haemolymph was drawn from the arthrodial membrane of the fourth walking leg using a syringe with 22G needle. Haemolymph was diluted to double volume with modified Alsever solution, centrifuged at 3,000 g to eliminate haemocyte and stored at -20°C.

Gills from moribund shrimp were also collected, fixed in Bouin or Davidson's fixative solution for immunocytochemical study, or frozen for viral preparation. Approximately 5 g of gills from infected shrimp were homogenized in 40-ml of 2X PBS (0.3M phosphate buffered saline pH 7.2 modified from Hudson and Hay, 1976) using a homogenizer (IKA lab Technik). Cell debris was removed by centrifugation at 10,000 g for 1-hr at 4°C. The supernatant was subsequently centrifuged at 100,000 g for 1-hr at 4°C using an ultra-centrifuge (Sorvall Pro 80). The pellet was resuspended in 2-ml of 2X PBS, divided into small aliquots and stored at -80°C. Haemolymph and gill preparations from non-infected shrimp were prepared in the same manner.

Immunization. Three Swiss mice were intraperitoneally injected with viral infected gill extract mixed with complete Freund's adjuvant in a 1:1 ratio. They were subsequently injected with virus infected gill extract mixed with incomplete Freund's adjuvant 3 times at 2-week intervals. One week after the third injection, mouse antiserum was collected and tested against non-infected and infected gill extracts by dot-blot. The best performing mouse was boosted three days before hybridoma production.

Hybridoma production. A cell fusion procedure was adapted from the method developed by Kohler and Milstein (1976), with modifications described by Mosmann et al. (1979). A P3X myeloma cell line was used as a fusion partner. Fusion products from one mouse were plated on 30 microculture plates (96 wells). After identification of the positive clones by screening methods described below, cells were recloned by limited dilution method.

Screening methods

a. Dot-blotting. Gill extracts (10 mg/ml) from non-infected and infected shrimp in both untreated (native) and SDS-mercapto-ethanol treated forms were used for screening. Gill extract antigens (1-ul/spot) were applied to nitrocellulose membrane. The membrane was baked at 60°C for 10-min and subsequently incubated in hybridoma conditioned media from each clone (1:20 in 5% Blotto) for 8-hr. After extensive washing in diluted Blotto, the membrane was incubated in horseradish peroxidase labelled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; Biorad) at 1:1000 dilution for 8-hr. The membrane was washed in Blotto and incubated in a substrate mixture containing 0.03% diaminobenzidine, 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS for 5-min. Hybridoma clones that displayed immunoreactivity against infected gill extract, but did not react with gill extract from non-infected shrimp were confirmed for the viral specificity by immunocytochemistry, recloned and cryopreserved for further investigation.

b. Immunocytochemistry. Gills from infected and non-infected live shrimp were dissected, and fixed in Bouin fixative solution for 24-hr, and processed for paraffin sectioning. Serial sections (8-um thickness) were prepared and processed for indirect immunoperoxidase

staining using various MAbs and GAM-HRP diluted to 1:1000 with 10% calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% H_2O_2 in PBS. Preparations were counter-stained with haematoxylin and eosin Y, dehydrated in graded ethanol series, cleared in xylene and mounted in permount. Viral localization in various tissues from non-infected and infected shrimp was also determined in the same manner.

To confirm the specificity of the MAb, tissue sections from shrimps infected with single virus (YHV or SEMBV) confirmed by PCR (kindly provided by Dr. Tim Flegel) were also determined by immunocytochemistry as described above.

c. Detectability of viral antigen by monoclonal antibodies. Haemolymph from infected shrimp were serially diluted with haemolymph from non-infected shrimp. Approximately 1-ul of each dilution was applied on nitrocellulose strip and processed for dot-ELISA as described above. Final dilutions observed by this method were determined by comparing with negative controls, which were haemolymphs from non-infected shrimp.

d. Viral isolation by ammonium sulfate precipitation. Viral particles in various dilutions of haemolymph were precipitated with 33% ammonium sulfate for 2-hr at 4°C to concentrate viral particles and eliminate some protein contamination from haemolymph. The precipitant was separated by centrifugation at 3000 rpm with a clinical centrifuge, washed once with 33% ammonium sulfate and then resuspended in PBS. The supernatants, both prior to and after washing were combined. Saturated ammonium sulfate was added to the supernatant for the final concentration of 40% and incubated for 1-hr. The precipitant was collected as described above. Viral presence in the pellet was determined by dot-blotting and Western blot.

e. SDS-PAGE and Western blot analysis. Haemolymph and gill extract from non-infected and infected shrimps were separated by 7.5% SDS-PAGE according to the method described by BioRad manual. Samples were electrophoresed for 6 hr at 30 V and gels were stained using Coomassie Brilliant Blue R-250.

For Western blotting, the samples separated by SDS-PAGE were blotted onto nitrocellulose membranes (BioRad). The nitrocellulose membrane was incubated in 5% Blotto for 10 min, treated with 1:200 SEM 3-2B conditioned media for 4 hr and then processed as described above in Dot-blotting.

For locating the relative position of the SEM-3-2B immunoreactive bands, the same nitrocellulose membrane was reprobed with MAb specific to haemocyanin subunits PMVS-22 (Longyant et al., in press) and developed in the substrate without cobalt choride.

f. Class and subclass determination. Class and subclass of mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP).

Results

Fusion trials produced about 600 wells containing hybridoma cells, from 30 micro-culture plates. The first screening by dot-blotting against extracts from non-infected and infected gills in both native and SDS treated forms revealed two positive hybridoma clones. These two clones, namely SEM-3-2B and SEM-219 produced

antibodies that reacted with infected gill extracts in both native and denatured forms, and showed low cross-reactivity with gill extracts from non-infected shrimp (Fig. 1). Using immunocytochemical screening, both antibodies showed cytoplasm immuno-staining in a large numbers of cells of infected gill tissues. However, in some cases the antibody reacted with a few cells in gill tissue from normal appearing shrimp (Fig. 5). These results suggested that some normal appearing shrimps used in our trials had mild viral infections. Antibodies produced from many other clones that showed reactivity against gill extracts from both non-infected and infected shrimp were discarded.

Two hybridoma clones, SEM-3-2B and SEM-219 were re-cloned, expanded, and cryopreserved. SEM-3-2B antibody produced more intense staining on dot-blotting than SEM-219 (Fig. 1); we thus used SEM-3-2B antibody for further study.

In dot-blotting of serially diluted haemolymph from infected shrimp using indirect immunoperoxidase method, the SEM-3-2B antibodies detected the antigen with strong reactivity up to 1:50 dilutions. When infected haemolymph was diluted by haemolymph from non-infected shrimp, concentrated by ammonium sulfate precipitation, viral presence was detected up to 1:1000-1:1500 dilutions (Fig. 2). However, precise quantitative analysis of haemolymph virus used in this experiment is still in progress.

Immunocytochemical studies of gill tissues from infected shrimp clearly revealed that viral immunoreactivities were found in the cytoplasm of the infected epithelial cells and haemocytes with condensed or pycnotic nuclei which are the typical characteristics of YHV infection (Fig. 4A, Chantanachookin et al., 1993; Flegel et al., 1997). The hypertrophied nuclei and Cowdry A-type nuclear inclusions, typical for SEMBV infection (Flegel et al., 1997; Tapay et al., 1997) were also observed but were not immunoreacted with the SEM-3-2B antibody (Fig. 4A).

Immunocytochemical studies of various tissues from shrimps with single infection of YHV or SEMBV (PCR and histopathological confirmed by Dr. Tim Flegel) revealed that only tissues from YHV infected shrimps but not SEMBV infected shrimps were immunoreacted with the antibody (data not shown).

Western blot analysis of haemolymph, 33% ammonium sulfate precipitated haemolymph and gill extract from infected shrimps exhibited a single band at 135 kD (Fig. 3) corresponded to the molecular mass of YHV enveloped glycoprotein (Nadala et al., 1997). Therefore, it is quite certain that the SEM3-2B is indeed specific to YHV not SEMBV.

As shown in figure 3, SEM-3-2B antibody had a high specificity to viral antigen and showed exceptionally low cross-reactivity to various proteins from shrimp tissues. Even though Coomassie blue staining of SDS-PAGE and Western blot analysis using MAb specific to haemocyanin-PMVS-22 revealed that various proteins were present in the mixture utilized in this analysis and immunization (Fig. 3).

No SEM 3-2B immunoreactivity was observed in either haemolymph from non-infected shrimps or 40% ammonium sulfated precipitated haemolymph (after 33% precipitation) from infected shrimps (Fig. 3). This evidence indicated that most of the viral particles were readily

separated by 33% ammonium sulfate treatment. The similar results also obtained with dot-blotting experiments (Fig.2B)

In gill tissues from asymptomatic shrimps, a few infected cells were observed using immunocytochemistry. In the same preparations, intensive infections in some muscle cells were found and some of these muscle cells had atrophied as a result of infection (Fig. 5A).

Various tissues from normal appearing adult female shrimps (age 8-12 months) from different sources were examined for the presence of viral infection. We found that adult female shrimps from many ponds without any previous history of disease had a mild viral infection at different levels. Viral immunoreactivities were found in interstitial cells, hemocytes between ovarian follicle and between hepatopancreatic tubules and in several cell types of the lymphoid organ (Fig. 5B, C&D).

Both SEM-3-2B and SEM-219 antibodies belonged to IgG2a subclass.

Discussion

Even though our trials produced relatively low fusion yields (typical yields in the previous experiment are >3,000 clones/fusion with other antigens; Longyant et al., in press), we did obtain one hybridoma clone, namely SEM3-2B. This clone produced MAb with high specificity to viral antigen. Improved hybridoma yields should produce greater numbers of specific antibodies.

Immunocytochemical studies using MAbs SEM-3-2B against gill preparations revealed that the immunoreactivities were observed in epithelial cells and haemocytes with the typical characteristics of YHV infection rather than those of SEMBV infection (Chantanachookin et al., 1993, Flegel et al., 1997, Tapay et al., 1997). Immunocytochemistry and Western blot analysis of shrimp tissues infected with single virus indicated that the SEM 3-2B antibody was specifically against YHV enveloped glycoprotein. Therefore, the YHV antigens in our preparation may derived from naturally YHV infected shrimps used for artificial SEMBV infection. The dual infection of these two viruses were previously reported (Mohan et al., 1998; Park et al., 1998).

Using indirect ELISA combined with ammonium sulfate precipitation of virus in haemolymph, our observed sensitivity for haemolymph viral detection was at 1:1000 dilutions. However, precise viral quantification is in progress. Modified methods using sandwich ELISA combined with two or three amplification steps should increase sensitivity several folds.

Western blot analysis data strongly suggested that this antibody can be used to detect small amount of viral antigen in a mixture containing a large amount of various proteins. This indicated that a highly specific monoclonal antibody can be obtained, even though a tiny amount of desired antigen is present in a partially purified protein mixture utilized for immunization.

In naturally infected asymptomatic shrimps, viral immunoreactivities were observed in gill muscle cells, interstitial cells, hemocytes between hepatopancreatic tubules, and in ovarian follicle. However, in artificially acute viral infected shrimps, it is quite interesting that we did not observe viral immunoreactivity

in the muscle fiber. Heavy infection was detected only in interstitial cells between muscle fibers. It is possible that in the naturally infected asymptomatic shrimps, the virus may present in muscle cells as a mild latent infection.

Using immunocytochemical techniques, we were able to demonstrate a low level of viral infections in shrimps from ponds without any previous history of infection. We found viral immunoreactivities in shrimp tissues that have never been identified for viral infections. Therefore, this technique is superior to typical histology examination. The antibody can detect viral infection at the early stages, and may at least partially replaced PCR screening for infected shrimp.

The SEM-3-2B MAb from our study belonged to a subclass IgG2a which was different from IgM obtained by other laboratories working on crustacean viruses. The IgM antibodies non-specifically reacted with components of non-infected shrimp, resulting in false positive reactions with non-infected shrimp tissue samples in ELISA-based assay (Lightner and Redman, 1998). It is promising that monoclonal antibodies use will become much more common in shrimp disease diagnosis because of its high sensitivity, rapid results, versatility, low costs, and simplicity. Further development and application of monoclonal antibody-based tests will greatly benefit commercial shrimp culture by providing routine diagnosis for modestly equipped laboratories in the field.

Acknowledgements

We deeply appreciate Dr. Tim Flegel for kindly providing YHV and SEMBV infected tissues for specificity test of the antibody, and his suggestions. We thank Dr. Arlo W. Fast of the University of Hawaii for kindly editing this manuscript. This work was partially supported by Thailand Research Fund (TRF) Senior Scholar to Dr. Piamsak Menasaveta.

Literature Cited

Chantanachookin C, Boonyaratpalin S, Kasornchandra J, Direkbusarakom S, Ekpanithapong U, Supamataya K, Sriurairatana, K and Flegel K (1993) Histology and ultrastructure reveal a new granulosis-like virus in *Penaeus monodon* affected by yellow head disease. Dis Aquat Org 17:145-157.

Flegel TW, Boonyaratpalin S and Withyachumnarnkul B (1997) Progress in research on yellow-head virus and white-spot virus in Thailand. In T.W. Flegel and I.H. MacRae (eds), Diseases in Aquaculture III., Asian Fisheries Society, Manila, Philippines. P 285-302.

Hudson L and Hay FC (1976) Practical Immunology, Blackwell Scientific Publications, London U.K. 274 pgs.

Köhler G and Milstein C (1976) Derivative of specific antibody-producing tissue culture and tumor cell fusion. European J Immunol 6:511-519.

Lighner DV and Redman RM (1998) Shrimp disease and current diagnosis methods. Aquacultures 164:201-220.

Loh PC, Cesar E, Nadala B, Tapay LM and lu Y (1998) Recent developments in immunological-based and cell culture protocols for the specific detection of shrimp viral pathogens. In Advance in Shrimp Biotechnology, Proceedings to the Special Session on Shrimp Biotechnology 5th Asian Fisheries Forum, The National Center for Genetic Engineering and Biotechnology, Thailand, p. 255-259.

Longyant, S, Sithigorngul S, Thampalerd N, Sithigorngul W, and menasaveta P (in press) Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin subunits. Invert Reprod Dev.

Mohan CV, Shankar KM, Kulkarni S and Sudha PM (1998) Histopathology of cultured shrimp showing gross signs of yellow-head syndrome and white spot syndrome during 1994 Indian Epizootics. Dis Aquat Org 34:9-12.

Mosmann TR, Bauman R and Williamson AR (1979) Mutations affecting immunoglobulin light chain secretion by myeloma cells I. Functional analysis by cell fusion. Eur J Immunol 9:511-516.

Nadala ECB Jr, Tapay LM and Loh PC (1997) Yellow-head virus: a rhabdovirus-like pathogen of penaeid shrimp. Dis Aquat Org 31:141-146.

Nadala ECB Jr, Tapay LM and Loh PC (1998) Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp. Dis Aquat Org 33:221-229.

Park JH, Lee YS, Lee S and Lee Y (1998) An infectious viral disease of penaeid shrimp newly found in Korea. Dis Aquat Org 34:71-75.

Tapay LM, Lu Y, Gose RB , Brock JA and Loh PC (1997) Infection of white-spot Baculovirus-like virus (WSBV) in two species of penaeid shrimp *Penaeus stylirostris* (Stimpson) and *P. vanamei* (Boone). Diseases in Asian Aquaculture III. Asian Fisheries Society, Manila, Philippines p 297-302.

Wongteerasupaya C, Vicker JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B and Flegel T (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon.* Dis Aquat Org 21:69-77.

Wongteerasupaya C, Thongchuea W, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B and Flegel T (1997) Detection of yellow-head virus (YHV) for *Penaeus monodon* by RT-PCR amplification. Dis Aquat Org 31:181-186.

Figure Legends

Figure 1. Monoclonal antibodies (MAbs) screening with Dot-blotting. Samples of three MAbs obtained during this study.

Figure 2. Dot-blotting of infected haemolymph serially diluted with haemolymph from non-infected shrimp treated with SEM-3-2B antibody. Diluted haemolymphs were applied directly (A), or concentrated by 33% ammonium sulfate precipitation (B). Only the supernatant of the first dilution was further precipitated with 40% ammonium sulfate. Numbers on the left columns are dilution factors and the "0" represents the haemolymph from non-infected shrimp used as a diluent.

Figure 3. SDS-PAGE and Western blot analysis of haemolymphs and gill extract from *P. monodon*, haemolymph from infected shrimp (a), 33% ammonium sulfate precipitate haemolymphs from non infected (b) and infected (c) shrimps, 40% ammonium sulfate precipitated haemolymph after 33% precipitation (d) and gill extract from infected shrimps (e). Preparations were stained with Coomassie blue (A) or Western blotting with SEM-3-2B MAb (gray staining) and reprobed with PMVS-22 MAb, the antibody specific to haemocyanin (brown staining). The numbers on the left side are molecular weight of marker proteins (s).

Figure 4. Immunocytochemistry of gill tissues from infected (A) and non-infected (B) shrimps treated with SEM-3-2B antibody. Both epithelial cells and hemocytes were severely infected with the YHV (Y) and SEMBV (S). The YHV infected cells were immunoreactive (brown staining) and showed condensed chromatin and pycnotic nuclei. The SEMBV infected cells were not immunoreactive and showed Cowdry A-type nuclear inclusions. Viral immunoreactivities were also demonstrated in hemolymph and in some parts of the gills. Immunoreactivity in gills from non-infected shrimps was not observed with this antibody in this preparation.

Figure 5. Immunocytochemical localizations of viral immunoreactivities observed in various tissues from asymptomatic naturally infected shrimp. (A) Gill muscle: infected muscle fibers (m) and in atrophied muscle fibers (arrow). (B) Hepatopancreas: viral immunoreactivities in cells between tubules (arrow) and a hemocyte. (C) Ovary: viral immunoreactivities in an interfollicular cell (arrow) and a hemocyte (h), but not in oocyte (o). (D) Other: Viral immunoreactivities in lymphoid organ cords with some parts of the ground substance (arrow), and other cells (arrow head).

Figure 1.

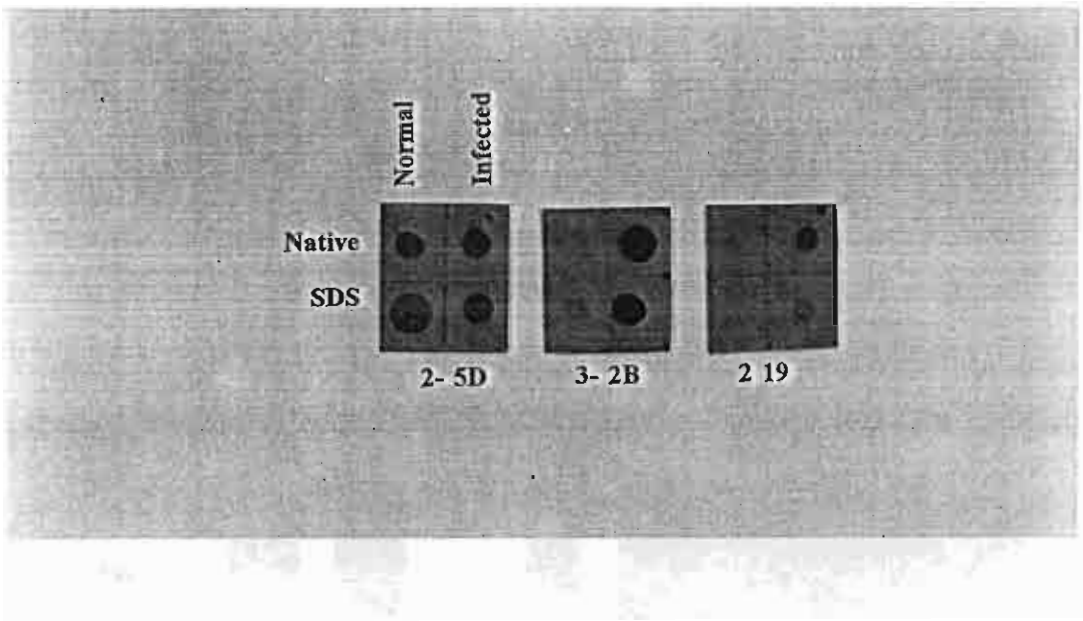


Figure 2.

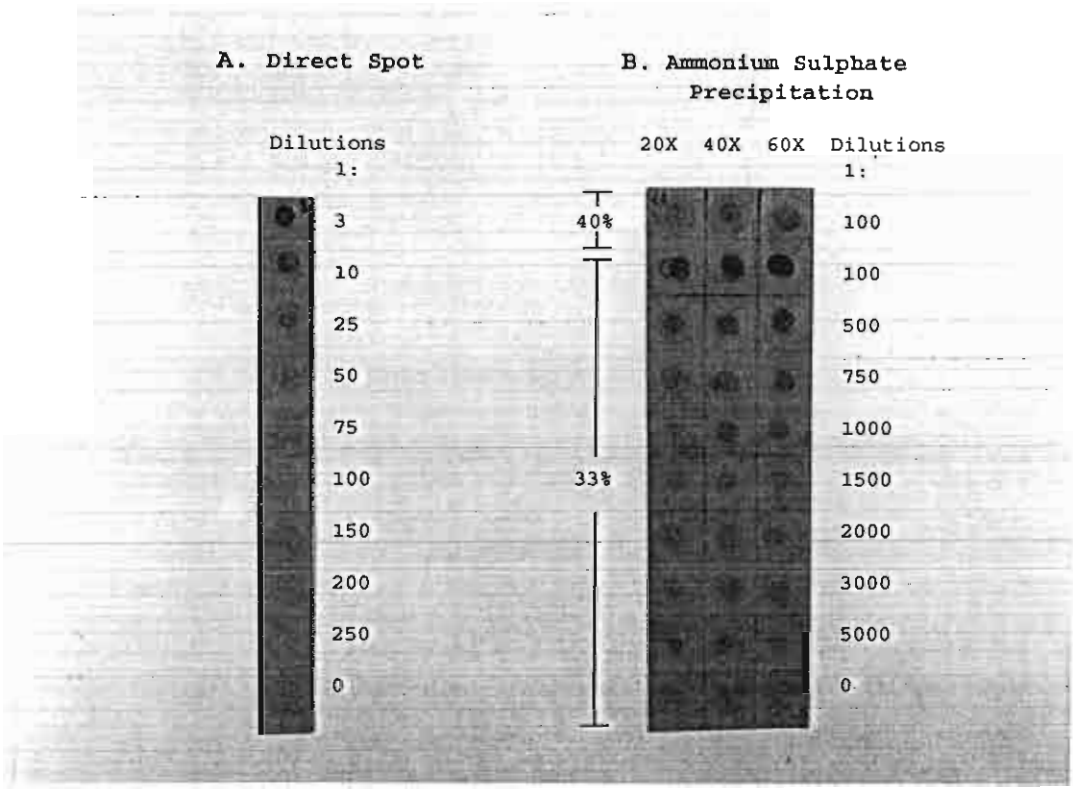


Figure 3.

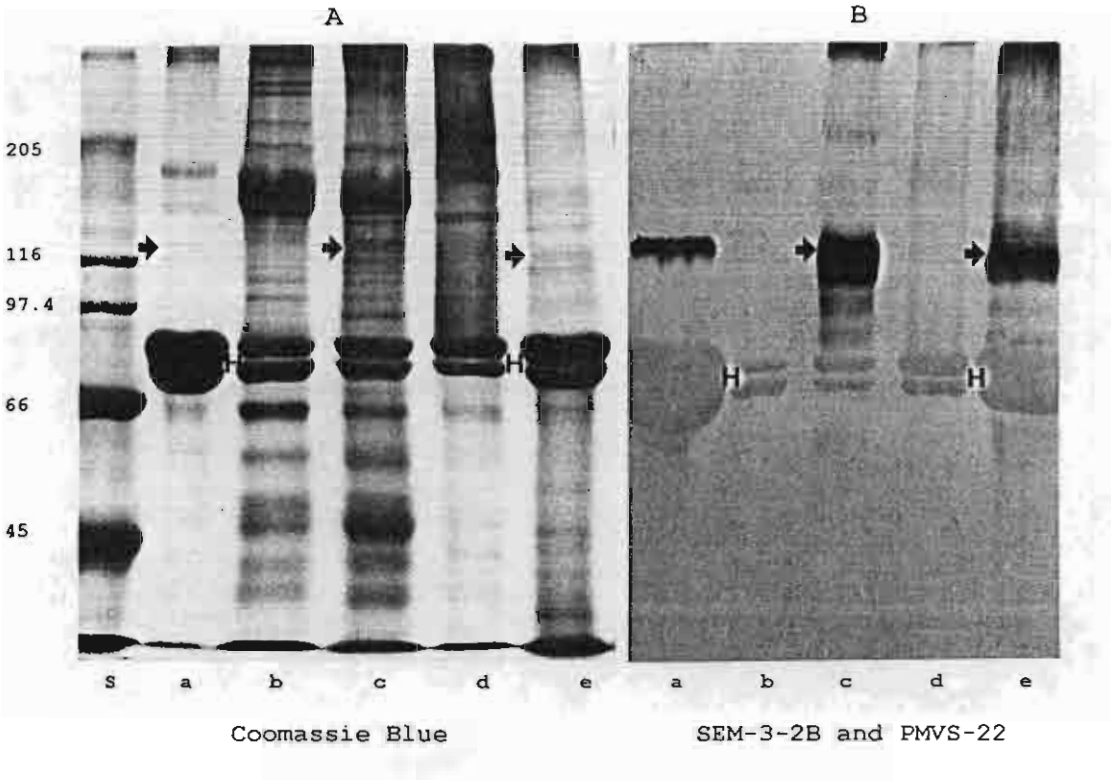

A
B
205
116
97.4
66
45
H
H
H
H
S
a
b
c
d
e
a
b
c
d
e
Coomassie Blue
SEM-3-2B and PMVS-22

Figure 4.

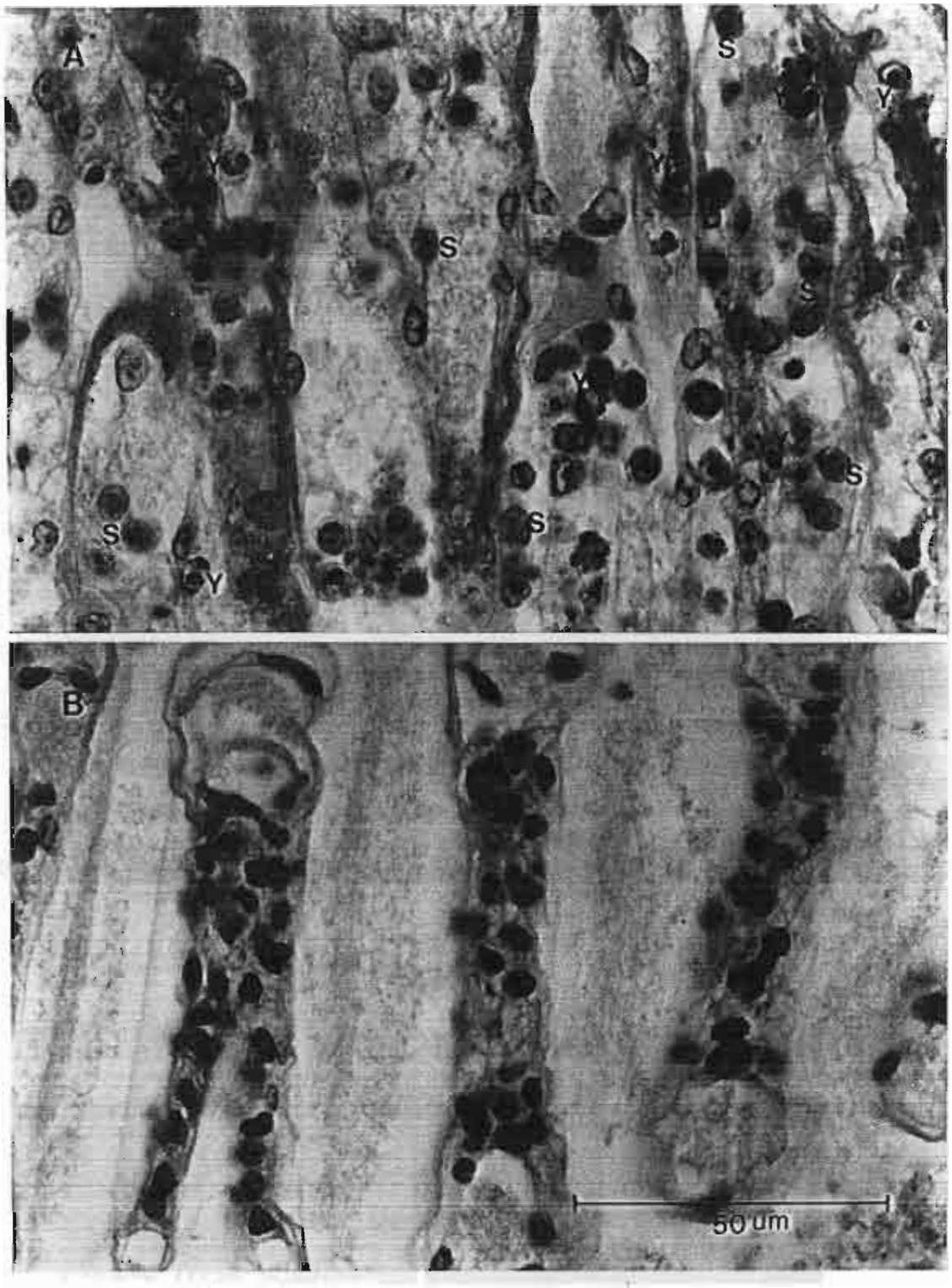
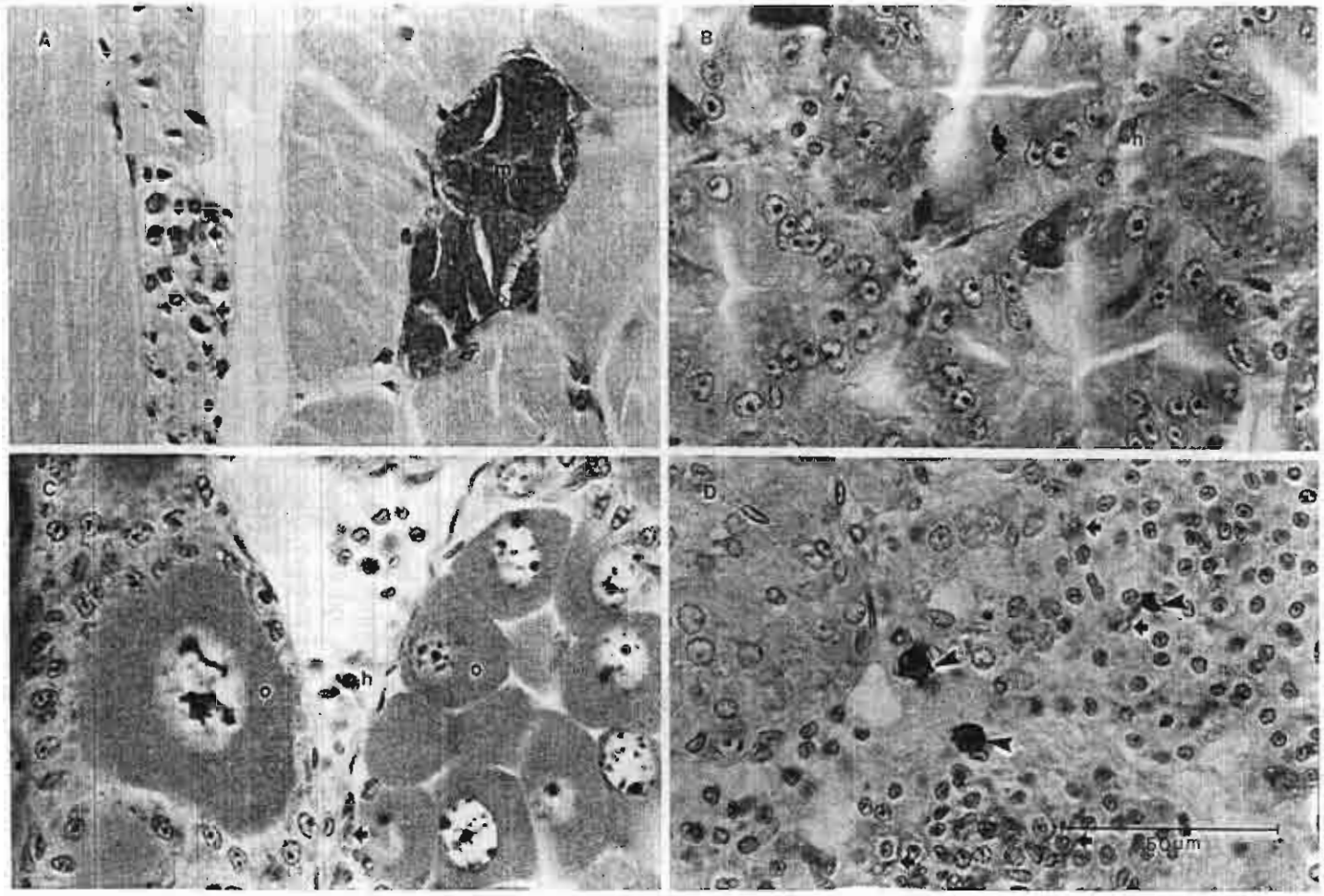


Figure 5.



cDNA cloning of neurohormones from the eyestalk of *Penaeus monodon*, Fabricius

Narongsak Puanglarp[1], Sirawut Klinbunga[1] and Piamsak Menasveta[1,2]

[1] Marine Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, Rama VI Road, Bangkok 10400, Thailand

[2] Aquatic Resources Research Institute, Chulalongkorn University, Phayathai Road, Bangkok 10330, Thailand

Abstract

Three neurohormones, crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), and gonad-inhibiting hormone (GIH) play important roles in growth, molting and reproduction of crustaceans. Cloning and characterization of these hormones will be useful for a further study on the manipulation of functions and mechanisms of these neurohormone in economically important species like *Penaeus monodon*. Furthermore, the application of these will improve efficiency of shrimp aquaculture.

RT-PCR was conducted from mRNA templates extracted from eyestalk of *P.monodon* and *P.semisalcatus*. The primers used in this study were designed from the published sequences of those three hormone genes as follow. MIH primers were designed from that of *P. vannamei* and *P. japonicus* ; CHH primers were designed from that of *P.japonicus*; and GIH primers were designed from that of *Homarus americanus*. Only one band was obtained from the RT-PCR of *P.monodon* with MIH primers designed from *P.vannamei*. The size was approximately 400 bp. After comparing the sequences of the resulting product with those of previously reports of MIH genes, the product should not be MIH coding gene. In addition, the PCR using genomic DNA as templates has been conducted The results revealed a few products from both *P.monodon* and *P. semisalcatus* have been amplified. Some of the appropriate size products have been sequenced and compared to previously reported sequences. A product from PCR of *P.semisalcatus* genomic DNA showed relatively high similarity to that of MIH gene from *P.vannamei*.

Introduction

Black tiger prawn (*Penaeus monodon*, Fabricius) culture is one of the most important agricultural industries in Thailand. For the last few decades, new cultural technologies have been adopted and consistently developed from extensive to semi-extensive and now intensive systems. Nevertheless, *P.monodon* culture is encountering a reduction of quality seed supply. Currently, *P. monodon* seeds are obtained through the mature females caught in the open sea. The ovarian development of wild females can be further stimulated by unilateral eyestalk ablation (Chen and Chen, 1994). By doing that, the productions of 3 important neurohormones in the eyestalk are sufficiently eliminated. These neurohormones are Crustacean hyperglycemic hormone (CHH), Molt-inhibiting hormone (MIH), and Gonad-inhibiting hormone (GIH) which play the important role in the regulation of blood sugar level, molting cycle and reproductive organ, respectively (Keller, R 1992 and De Kleijn and Van Herp, 1995). To prevent over-exploitation of the wild *P. monodon* females, pond-reared females without well-developed ovaries are an alternative source to be used for culture activity. In order to gain the improvement on the seed production from the captive *P. monodon*, the research on the molecular mechanisms of these 3 hormones is essentially required. In order to obtain some basic knowledge on molecular biology of these hormones in the eyestalk of giant tiger prawn, *P. monodon*, the pilot study was conducted to identify the genes of CHH, MIH and GIH using RT-PCR and PCR techniques.

Materials and Methods

Animals

Specimens of black tiger prawn (*P. monodon*) were purchased from local fishermen in southern and eastern coasts of Thailand and some were obtained from prawn culture farm in Ratchaburi province. The animals were kept at laboratory condition (26-28 ppt aerated seawater and the ambient temperature) and fed with artificial feeds twice a day.

RNA isolation

Prawns in the intermolt stage were collected and subjected to the RNA isolation. Ten eyestalks were ablated from the animals with sharp scissors and the optic ganglia were removed. The eyestalks were rinsed briefly in 70% cold ethanol and then ground to a fine power in liquid nitrogen using mortar and pestle. Total RNA from eyestalks was prepared using TRIzol Reagent (Life Technologies). Briefly, the eyestalk power was added to 10 ml of TRIzol Reagent, followed by the addition of chloroform (0.2 ml/1ml TRIzol reagent). The sample was mixed vigorously and incubated for 10 min at room temperature. The mixture was centrifuged at 12,000xg for 10 min and the aqueous phase containing total RNA was obtained. Total RNA was recovered after ethanol precipitation. The purified mRNA was either extracted directly from eyestalk tissues or isolated from total RNA using PolyATtract System 1000 kit (Promega).

PCR amplification of MIH, CHH, and GIH

Genomic DNA purified from each individual of *P.monodon*, *P.semisalcatus*, *P. vannamei*, and *P.japonicus* was used as DNA template for PCR reaction. Each reaction was performed in a final concentration of 1x PCR buffer (10mM Tris-HCl, pH 8.3 and 50mM KCl). 1.5-3 0 mM MgCl, 0.8 µM dNTP, 0.4-1.0 pmol downstream and upstream primers, and 0.5-1.0 U Taq Polymerase. The PCR conditions included 35 cycles with denaturing temperature at 94°C for 1 min, annealing temperature at 45-50°C for 1 min, and extending temperature at 72°C for 1 min. The final extension step was further incubated at 72°C for 10 min. PCR products were monitored by running on 0.8% agarose gel containing ethidium bromide.

DNA cloning and sequencing

The PCR products were purified either by phenol/chloroform extraction or Wizard PCR Preps DNA Purification System (Promega). Each DNA fragment was then ligated into pGEM-T vectors (Promega) and transformed to *E.coli* JM 109 competent cells Transformed cells were cultured on LB plate containing 50 µg/ml ampicilin, 40 µg/ml x-gal, and 0.5 mM IPTG. The recombinant plasmid containing DNA target was purified by alkali mini-prep method and sequenced using an automated sequencer ABI 373 (Biosystems)

Results

RT-PCR amplification of MIH, CHH, and GIH

The RT-PCR reactions was conducted to amplify MIH, CHH and GIH from total RNA and mRNA extracted from *P. monodon* using primers CHH I & II, MIH I & II, GIH-HO-F1&R1, GIH-HO-F2&R1, GIH-HO-F1&R2, and GIH-HO-F2&R2, respectively. The results of all RT-PCR using total RNA as templates showed no specific DNA product (smear) on 0.8% agarose gel (picture not shown). Similarly, the product of RT-PCR from mRNA also revealed no clear bands, except the product of MIH I & II shown at the size of 400 bp on 0.8% agarose gel (Figure 1). The DNA fragment was then cloned and sequenced After the sequenced DNA was compared (BLAST) to the known genes in gene database (SwissProt) (Altschul. Stephen F., Warren Gish, Webb Miller, Eugene W. Myers. and David J Lipman, 1990), the result showed no similarity to any of known MIH genes.

Primer construction

Primers used in this experiment were designed based on the reported DNA and amino acid sequences of CHH, MIH and GIH from a number of crustacean species (Table 1).

Table 1. Primers used in PCR and RT-PCR reaction for amplifying CHH, MIH and GIH genes from *P. monodon*.

Primer	Sequence	Source
MIH I	5' CACCTTCGACCACTCCTGCA 3'	*P.vannamei* (Sun, P.S., 1994)
MIH II	5' GGATAGCGCAGAATTAGCCA 3'	*P.vannamei* (Sun, P.S., 1994)
CHH I	5' CTTCGCATGTTGTCAGCGCT 3'	*P.japonicus* (Ohira et al., 1997a)
CHH II	5' CTTCCCGACTGTCTGCATGG 3'	*P.japonicus* (Ohira et al., 1997a)
MIH-PJ-F1	5' GAAAGTAGTGCGAGTGTGTGAGGAT 3'	*P.japonicus* (Ohira et al., 1997b)
MIH-PJ-R1	5' TCAGGAACCATTCGTTGTAGAAGCA 3'	*P.japonicus* (Ohira et al., 1997b)
GIH-HO-F1	5' GGITTYWSIGTCARMGIGTITGGYT 3'	*H.americanus* (Soyez,D. et al,1991)
GIH-HO-R1	5'GTRTGRAARCARTCYTTYTTRCACAT 3'	*H.americanus* (Soyez,D. et al,1991)
GIH-HO-F2	5' TGGTTYACIAAYGAYGARTGYCC 3'	*H.americanus* (Soyez,D. et al,1991)
GIH-HO-R2	5' ACCCAYTTICKRAAYTGATCRATYTC 3'	*H.americanus* (Soyez,D. et al,1991)

DNA isolation

DNA was prepared from pleopod muscles of the prawn using the Phenol/Chloroform extraction method described by Klinbunga et al. (1996)

Pleopods were dissected and homogenized in digestion buffer (100 mM Tris-OH, 200 mM EDTA, 250 mM NaCl, 200 µg/ml Proteinase-K, pH 8.0, and 1% SDS) prior to incubation at 55°C for 2 h. Sample was then extracted 3 times with phenol and 2 times with chloroform/isoamylalcohol. Genomic DNA was recovered by ethanol precipitation.

RT-PCR amplification of MIH, CHH, and GIH

Purified total RNA or mRNA was subjected to the RT-PCR amplification using Access RT-PCR System (Promega). The reaction was performed in a 50µl reaction mixture containing, 1x AMV/Tfl Reaction Buffer, 0.2 mM dNTP, 1 pmol (1µM) each of downstream and upstream primers, 1 mM $MgSO_4$, 5 U AMV Reverse Transcriptase, 5 U Tfl DNA polymerase, 10 ng – 1µg of RNA sample. To create the first strand cDNA, the mixture was first incubated at 48°C for 45 min, followed by denaturing step at 94°C for 2 min. For second strand cDNA synthesis and PCR amplification, forty cycle of PCR amplification was carried out in a HYBAID OMNIGENE DNA thermal cycler as follows: denaturation at 94°C, 30 s; annealing at 60°C, 1 min; extension at 68°C, 2 min; final extension at 72°C for 10 min. The amplified cDNA was recovered and concentrated with Wizard PCR'Preps DNA Purification System (Promega).

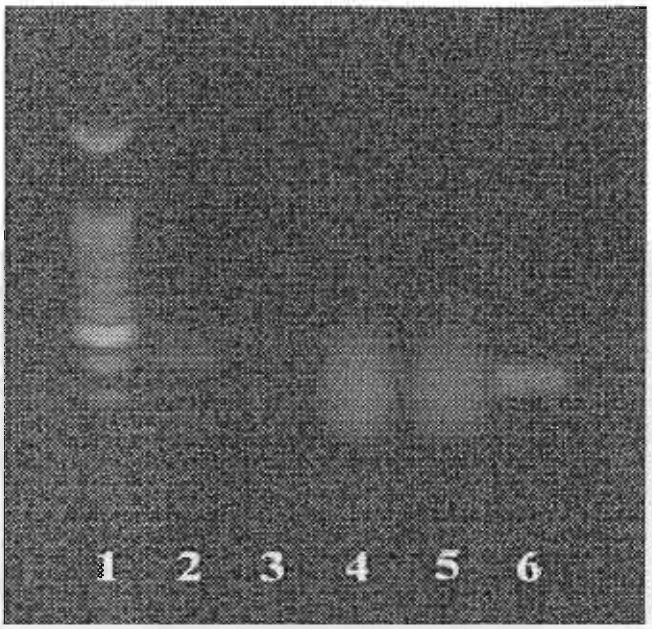


Figure 1 RT-PCR of MIH gene from *P. monodon* using total RNA (lane 4 and 5) and mRNA (lane 2 and 3) extracted from the eyestalks as the template and MIH I + II (lane 2 and 4) and MIH-PJ-F1 + MIH-PJ-R1 (lane 3 nad 5) as primers. A 100 bp DNA ladder (lane 1) and a positive control (lane 7) are included.

PCR amplification of CHH, MIH and GIH using genomicDNA as templates

Sixteen DNA fragments were isolated from PCR products using 7 separated pairs of primers. Eight of these DNA fragments were cloned and sequenced. After comparing with the known DNA sequences from gene database (BLAST), none of these showed acceptable similarity to the reported sequences of CHH, MIH, and GIH genes. Figure 2 shows examples of the PCR products electrophoresed through 0.8% Agarose gel. The results of all PCR amplifications on the genomic DNA of *P.monodon* are shown in table 2.

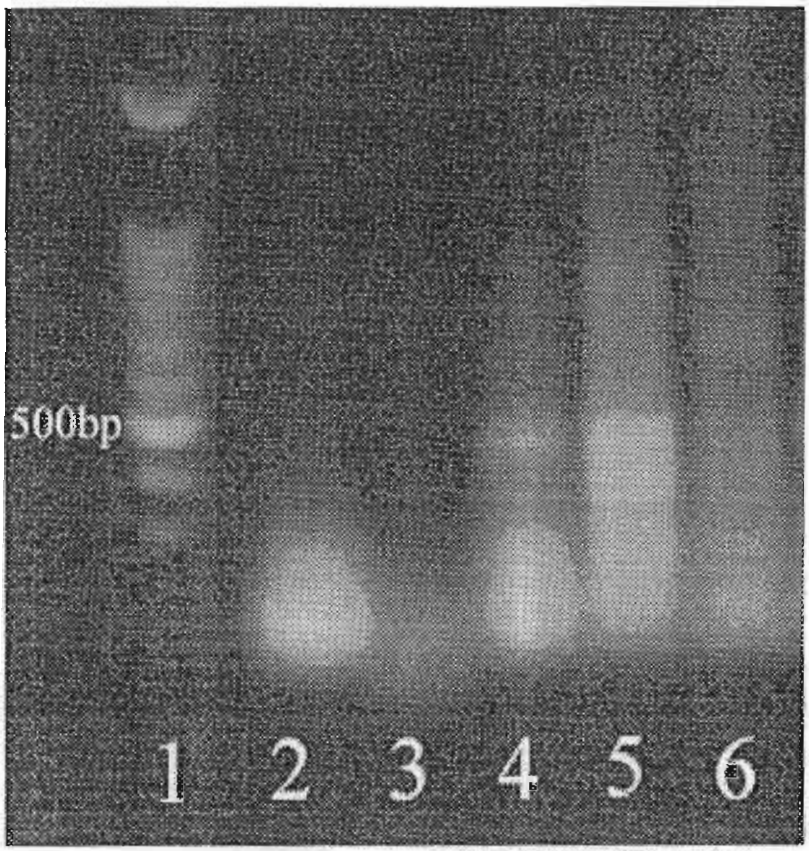


Figure 2. PCR amplification of CHH (CHH I + II, lane 3), MIH (MIH I + II, lane 4 and MIH-PJ-F1 + MIH-PJ-R1, lane 5) and GIH (GIH-HO-F1 + GIH-HO-R1, lane 6) using genomic DNA of *P.monodon* as template. Lane 1 and 2 are a 100 bp ladder and negative control, respectively.

Table 2. Products of DNA amplification from *P. monodon* using various primers.

Primers	Expected size (bp)	RT-PCR		PCR
		Total RNA	mRNA	Genomic DNA
CHH I + CHH II	360	smear	-	-
MIH I + MIH II	213	smear	*Band 1 (400bp)	*Band 1 (400bp) *Band 2 (200bp)
MIH-PJ-F1 + MIH-PJ-R1	100	smear	-	Band 1 (800bp) Band 2 (500bp) Band 3 (400bp) *Band 4 (250bp)
GIH-HO-F1 + GIH-HO-R1	221	smear	-	Band 1 (700bp) *Band 2 (400bp)
GIH-HO-F1 + GIH-HO-R2	290	smear	-	Band 1 (600bp) *Band 2 (500bp) *Band 3 (400bp) *Band 4 (300bp) Band 5 (200bp)
GIH-HO-F2+ GIH-HO-R1	140	smear	-	Band 1 (1000bp) Band 2 (900bp) Band 3 (700bp) Band 4 (600bp)
GIH-HO-F2 + GIH-HO-R2	209	Smear	-	-

Asterisks (*) indicate DNA fragments which have been sequenced and compared (BLAST) to DNA sequence database (SWISSPROT).

PCR products of *P. vannamei* and *P. semisulcatus* genomic DNA using MIH I and MIH II primers

In order to make DNA probe for the MIH gene. MIH I and II primers designed from known sequence of *P. vannamei* MIH gene, together with the genomic DNA from *P. vannamei* were used for PCR amplification. Genomic DNA from other species of penaeid shrimps, such as *P. semisulcatus*, was also used as DNA template. The result (Figure 3) of PCR reaction shows an amplified product of about 400 bp in the reaction of *P. vannamei* and three bands of PCR products (200, 400, and 750 bp) in the reaction of *P. semisulcatus*. The 400 bp fragment from *P.vannamei* was cloned and sequenced. The

DNA sequence of *P.vannamei* showed complete similarity with the reported cDNA of MIH gene. Moreover, the DNA fragment was interrupted by 236 bp of untranslated DNA sequence. This reveals the existence of intron in MIH gene of *P. vannamei* (Figure 4)

The 200 bp and 500 bp fragments from *P.semisulcatus* were also cloned and sequenced (Figure 5). After comparing with gene database, the sequence of 200 bp fragment contained high similarity to the reported MIH gene from most crustaceans. There was also an untranslated region at the 3' of the fragment (Figure 5), locating at the same position as the intron in *P. vannamei*. The sequence of 500 bp fragment shows perfect match with the 200 bp fragment at the 3' terminal and also contains precusor region similar to the precusor generally found in CHH/MIH/GIH family genes (Figure 5). The MIH sequence obtained from *P.semisulcatus* shows high similarity to the reported MIH gene from *P.vannamei* and that of other crustacean species (Figure 6).

Figure 3. PCR products resulted from MIH I and MIH II primers when *P. vannamei* (lane 3 and 4) and *P. semisulcatus* (lane 5) genomic DNA was used as templates. A 200 bp fragment (lane 5) was reamplified using the same primers (lane 6). Lane 1 and 7 are λ-Hind III DNA marker and a 100 bp ladder, respectively.

```
  Thr Phe Asp His Ser Cys Lys Gly Ile Tyr Asp Arg Glu Leu
C ACC TTC GAC CAC TCC TGC AAG GGC ATT TAC GAC CGG GAG TTG

  Phe Arg Lys Leu Asp Arg Val Cys Glu Asp Cys Tyr Asn Val
  TTC AGA AAG CTG GAC CGC GTC TGC GAG GAC TGT TAC AAC GTG

  Phe Arg Glu Asp Lys Val Ala Thr Glu Cys Lys **********
  TTC CGC GAG GAT AAG GTG GCC ACC GAG TGC AAG TAAGAGCCGCG
**************************************************
GATTCTGGCACTTTGTTCTCCTGTCTCTCTCTCAGTTTGTCTCACCATTTAAGTTGA
**************************************************
TTCTTATATAAGACGGGTTATTTTGTTTGACTTATTTTCTGTTATGTTCTTTTTTTT
**************************************************
ACGTTTTAGTTGTTTTTATTTCTTCTTTTATTTATAATGATAATTTGTACATTACAT
**************************************************
TGTTCTTATTTTTTTCTCTTTTTCCCATTCCTTCTCCCTTTACGAAAAAACAGG

Ser Asn Cys Phe Val Asn Lys Arg Phe Asn Val Cys Val Ala Asp
TCC AAT TGC TTC GTG AAT AAG AGG TTC AAT GTC TGT GTG GCT GAT

Leu Arg His Asp Val Ser Arg Phe Leu Lys Met Ala Asn Ser Ala
CTC AGA CAT GAT GTC AGC CGC TTT CTG AAA ATG GCT AAT TCT GCG

Leu Ser
CTA TCC
```

Figure 4 DNA sequence of MIH amplified by primer MIH-I and MIH-II using *P. vanamei* genomic DNA as template.

a) >200 bp

```
  Thr Phe Asp His Ser Cys Lys Gly Ile Tyr Asp Arg Glu Leu
C ACC TTC GAC CAC TCC TGC AAG GGC ATC TAC GAC CGG GAG CTC
  Phe Arg Lys Leu Asp Arg Val Cys Glu Asp Cys Tyr Asn Leu
  TTC AGA AAG CTG GAC CGC GTC TGT GAG GAC TGC TAC AAC CTG
  Tyr Arg Lys Pro Tyr Val Ala Thr Glu Cys Lys **********
  TAC CGC AAG CCN TAC GTC GCC ACC GAG TGC AAG TAAGGGCGTCG
**************************************************
CCGAGTCTCTCTTTGTGCATGTCGTCATTTAAGTTGGAATTTTACAAGTGACAGTTT
**************************
ATCTCGTGTGGCTAATTCTGCGCTATCC
```

b) > 400 bp

```
His Phe  ?   ?   ?  Leu Gln Val Cys Ser Ala Ala Leu Val Ser
CAC TTT NGA CNA NTC CTG CAG GTG TGC TCC GCC GCC CTG GTG TCG
Leu Leu Val Leu Ala Leu Ser Ser Arg Ser Ala Phe Ala Arg Ser
CTG CTG GTG CTG GCT TTG TCG TCC CGC AGC GCT TTC GCC CGC TCC
Val Asp Gly Val Gly Arg Leu Glu Lys Leu Leu Ser Ser Ser Ser
GTG GAC GGC GTG GGG CGC CTT GAG AAG CTG TTG TCG TCC TCG TCT
Ser Ser Ser Ser Gly Ser Ser Ser Pro Leu Leu Ala Phe Gly Asp
TCG TCT TCG TCA GGC TCT TCC TCT CCC CTG CTT GCC TTC GGC GAC
Asp His Ser Val Asn Lys Arg Asp Thr Phe Asp His Ser Cys Lys
GAC CAC AGC GTG AAC AAG CGC GAC ACC TTC GAC CAC TCC TGC AAG
Gly Ile Tyr Asp Arg Glu Leu Phe Arg Lys Leu Asp Arg Val Cys
GGC ATC TAC GAC CGG GAG CTC TTC AGA AAG CTG GAC CGC GTC TGT
Glu Asp Cys Tyr Asn Leu Tyr Arg Lys Pro Tyr Val Ala Thr Glu
GAG GAC TGC TAC AAC CTG TAC CGC AAG CCN TAC GTC GCC ACC GAG
Cys Lys **************************************************
TGC AAG TAAGGGCGTCGCCGAGTCTCTCTTTGTGCATGTCGTCATTTAAGTTGGAAT
******************************************
TTTACAAGTGACAGTTTATCTCGTGTGGCTAATTCTGCGCTATCC
```

Figure 5. Two DNA sequences of PCR products amplified by primer MIH-I and MIH-II using *P. semisulcatus* genomic DNA as template. a) shows the 200 bp band and b) 500 bp band. Underline indicates the translated region of MIH gene and asterisk (*) indicates the un-translated region.

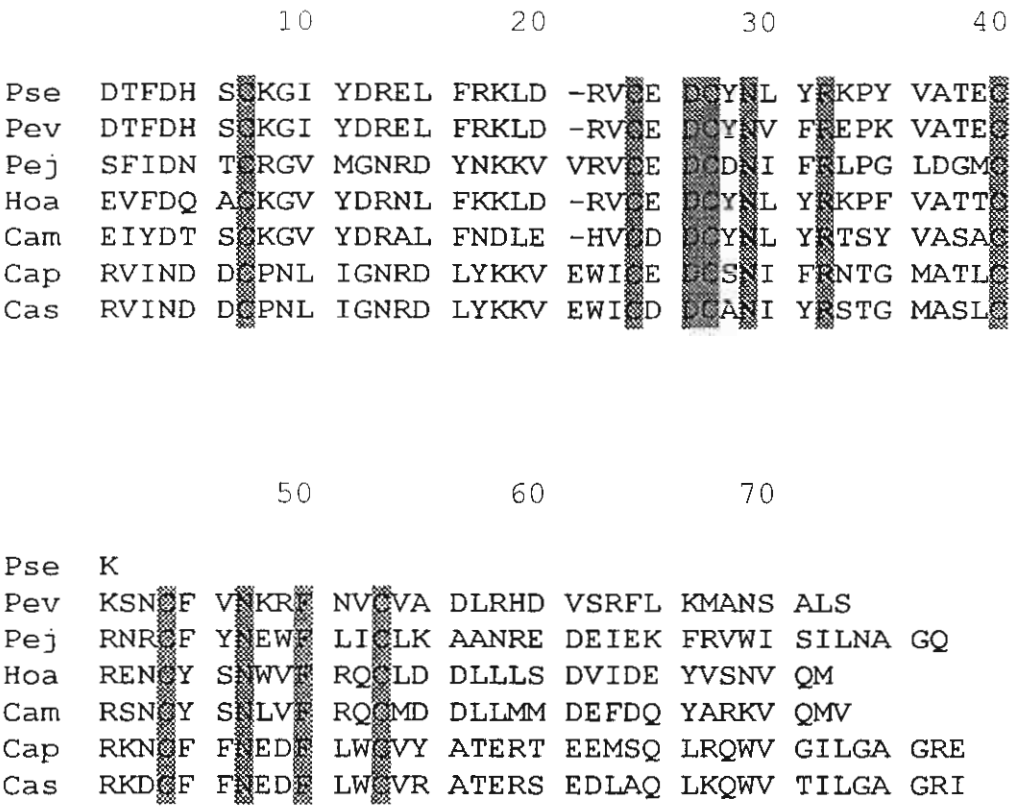


Figure 6. Alignment of the deduced amino acid sequence for *P. semisulcatus* MIH with that of other crustaceans Abbliviations: Pev, *Penaeus vannamei* (White shrimp); Pej, *Penaeus japonicus* (Kuruma shrimp); Hoa, *Homarus americanus* (Lobster); Cam, *Carcinus maenus* (Shore crab); Cap, *Cancer pagurus* (Crab); and Cas, *Callinectes sapidus* (Blue crab)

Discussion

The use of RT-PCR with primers derived from partial amino acid sequences has failed to perform the amplification of CHH, MIH and GIH cDNA from *P.monodon*. Only a single DNA product (400 bp) has been obtained from the RT-PCR with MIH I and II primers. The sequence showed no identity to the known MIH gene. This could be explained that either the conserved sequences of the hormone genes using for primer design were not specific enough or the total RNA and mRNA used in the reaction did not contain the target RNA. Further trial on improving more specific primers and the condition of reaction are required

A number of PCR products, amplified from genomic DNA of *P.monodon*, were determined (17 fragments), some which were close to the expected sizes were cloned and partially sequenced (7 fragments). So far, none of those sequences were identity to the known sequences of CHH, MIH and GIH. There are ten more DNA fragments to be further sequenced. However, the possibility of finding the target sequences among these fragment was uncertain. Therefore, an addition experiment was conducted on the basis of using DNA probe amplified from the original species from which the specific primers were designed. In the initial attempt, the *P.vannamei* genomic DNA together with MIH I & II primers, which were also designed from *P.vannamei* MIH gene, were applied to the PCR reaction The PCR reaction yielded an unexpected product of 450 bp instead of 213bp. After cloning and sequencing, the result revealed that the genomic DNA sequence of *P.vannamei* MIH gene were interrupted by the 236 bp of un-translated DNA region. The existence of intron in MIH gene of *P.vannamei* and *P.semisulcatus* is similar to the MIH gene of the crab, Charybdis feriatus (Chan, et al., 1998). However, the complete genomic DNA sequence is needed to clarify the number of intron in *P.vannamei* and *P. semisulcatus* MIH gene.

Further study

The result reported here is an initial study on the molecular structures of three important neurohormones which control the growth and reproduction system in penaid shrimps. The project has not reached the aim of the experiment. Therefore, further study need to be conducted in the following objectives:

-improve the specific primers and the right contion of PCR and RT-PCR reaction
-determine the specificity of DNA probe by performing Northern and Southern blotting
-create cDNA and genomic DNA libraries from the specific tissues of P.monodon.
-sequence and determine the gene structure and expression of these hormone genes.

-study on the neurohormones from other species
-apply the knowledge to improve the aquaculture techniques which concern the growth rate and maturation in the commercial shrimp

Once the primary structure of CHH, MIH and GIH genes have been identified, the function and the role of these hormones will be revealed. This knowledge will be much valuable to the shrimp aquaculture industry. In the future, the eye ablation technique to increase the maturation rate in shrimp may no longer needed. This will be helpful for the shrimp culture industry and the preservation of shrimp broodstock in natural habitat.

Acknowledgements

Many thanks to Asso.Prof.Dr. Padermsak Jarayabhand for providing the shrimp broodstocks of *P. monodon* and *P.japonicus* specimens, Asst.Prof.Dr. Chalor Limsuwan, and Mr. Payung Patarakulchai for providing cultured *P. monodon*. Also thanks to Dr. Ana Rogue for providing *P.vannamei* specimens. This work was financially supported partly by a grant from Thailand Research Fund granted to Prof. Dr Piamsak Menasveta and partly by National Center for Genetic Engineering and Biotechnology.

References

Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Chan. Siu-Ming, Xiao-Guang Chen, and Pei-Li Gu. 1998. PCR cloning and expression of the molt-inhibiting hormone gene for the crab (Chyrybdis feriatus). Gene. 244, 23-33.

Chen, Che-Chun and Shiu-Nan Chen, 1994. Vitellogenesis in the giant tiger prawn, Penaeus monodon Fabricius, 1789. Comp. Biochem. Physiol. 107B, 453-460.

De Kleijn, Dominique P. V. and Francois Van Herp, 1995. Molecular biology of neurohormone precursors in the eyestalk of Crustacea. Comp. Biochem. Physiol. 112B, 573-579.

Keller, R., 1992. Crustacean neuropeptides: structures, functions and comparative aspects. Experimentia (Basel) 48, 439-448.

Klinbunga, S., Penman, D.J. and McAndrew, B.J., 1996. An improved protocol for total DNA isolation and visualisation of mtDNA RFLP(s) in tiger prawn, Penaeus monodon. Thai. J.Aquat. Sci. 3, 36-41.

Ohira, T., T. Watanabe, H. nagasawa, and K. Aida., 1997a. Cloning and sequence analysis of a cDNA encoding a crustacean hyperglycemic hormone from the Kuruma prawn Penaeus japonicus. Mol. Mar. Biol. Biotechnol. 6(1), 59-63.

Ohira, T., T Watanabe, H. nagasawa, and K. Aida. 1997b. Molecular cloning of a molt-inhibiting hormone cDNA from the kuruma prawn Penaeus japonicus. Zoolog. Sci. 14(5), 785-789.

Soyez,D., J. P. Le Caer, P. Y. Noel and J. Rossier ,1991. Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster homarus americanus. Neuropeptides. 20, 25-32.

Sun, P.S., 1994. Molecular cloning and sequence analysis of a cDNA encoding a molt-inhibiting hormone-like neuropeptide from the white shrimp Penaeus vannamei. Mol. Mar. Biol. Biotechnol. 3(1), 1-6.

Appendix 1. Partial sequences of DNA fragments obtained from PCR and RT-PCR using cDNA and genomic DNA from *P.monodon* as templates

1. The sequence of genomic DNA obtained from PCR using primer MIH-PJ-F1 and MIH-J-R1

1.1 Band 1: (250 bp)

TGTAgAAGCA TGAGGTGAGG GAAATAGACC GGAACTTGTT CAGTGCTAGG
CTTTGGTATG GGTACGATGA TGCTTGTAGT CCACACCTGA GGTATGCATC
CTTTCTGAAG GCAAAGATTA TAAAGTAgAA GCAAGGGATT GCCAGGGACC
TTCTANAgAA gACAGAgAAT GGAGTATGTG ATTCCATCCT CACACACTC

2. The sequence of genomic DNA obtained from PCR using primer MIH I and MIH II

2.1 Band 1: (400 bp)

GAATTCGATT CACCTTCGAC CACTCCTGCA TCCTCCACTT TGGTAAATGG
ATGTCCGTTA CCTATGGGTG GAGGTTCTCA CCGAGACGGA CGTCGATGCC
AAGTGCACCA AGTACCTGGA CACTATCACT ATCGCCTACC ACAACTTCTT
TCCGTAGAAg ACCAGTCGAT GTCACCCCGC CGACCCGCCC TGGATCACGG
ATAGTATCAA GCGCCTCATG AAGCAGCGGA ACCGCGCCCA CCACTCACAT
AATGGCACAT GCTACAAATC ACTCTGAAAT AAAGCATGCA AAGAAgAATT
TCTACCCTAG CAAATTACGA CACCTCaAGG GACAAGGACT CCAACAAACA
GTTTGGCCAG ATCAAGCAAT TATATTGCAT TAACCAAGAA NGCTAGCAGC
CTCCCCTGTG TCAGCTATCT TTCCAGCCAA GCAGCCACAG AGCTGGTGAA
CCAGCACCTG CTAGCGCCTT CACCGCCTTG AcATCACCTC CCTGCAAGCT
TACCTGCTAg TACAAACCGC TGTGCCAACC ACCCATGTaC ACCAAGTCTG
CCGTAAGTAC AGCAgTGCCG TTCTAAAANG TCNGTTACCC ACTGGCTAAT
TCTGCCTA

2.2 Band 2 : (550 bp)

GAATTCGATT GGATAGCGCA GAATTAGCCA GCACAGGCCA GAGGACACGA
TGTCTAAGCA TGACACTCTC AGACACCACA GACGCCATAT AGTGTTAGTG
TGCCTTATCT TCGCCAGGAG TGCATGACAT CACTCTCGCC GAGCTGTTCT
TGGAGCTTGT aTGCAGGATG CGGTGACGCG GTTTCGCAAG GGCTAGTGTG
TGAGATANAG CTGTaAAGAG GGAACGGAGG AGGCAGAgAG CGGCGAAgAT
GAACAGAGTA AACAAAATAA TATTGATAAT ATTGCTATTA GTTATAGTGA
CCCACGTGAT GCGATTGATC AAAACATGAA GTAAATCATA TCAACAAAAA
TCATCATCAG TAACTGCAGC AATCGTGAGA AGTTATAATG ACATAgAgTA
ACTGCTGCCA TTATCGTTAC CACTGTAACA CTAATGACGA TGGTTGAAAA
ACTATAAGAT AATATTAATA ATGTaGCAAA AGTTGTCCCA ATAAGCATTC
ACCTTCATCT TAACAGCACA GTANGACAgT AAATGTgCTT ACCCCTTACC
CCCTTCTTCa CTGCCTCaGA AGAACCATGA CGTCCCNGAN ACAGGAAATT
CNTTTGG

3. The sequence of cDNA obtained from RT-PCR using primer MIH I and MIH II

3.1 Band 1: (405 bp)

CACCTTCGACCACTCCTGCACAGACACACCCACCCCCAAGAATGGGAAGTGTTCACTAT
TTTGAGGACCAGACTCTTTAAGCACTCTGAATTCTCTCGTGTGATTGTTGAGCATGAAC
TTGAACCTCCTCAAGTTTCACTAGTTTACGCACACTTATTGTAACTTGATGGAAAGGTC
TGACAAAAGTGTTAGAGTTCGAGTCAAAATCTGCTCACAGTGGCCCCAGTACTTTCAAA
GTTAGTGATGATTTTTCTCACAAACACAGCCAACACCATAGATTCATCATTCAGCCCAA
GTATCTCTGATAGGCGGCGATAGAACTTAGAGGTTTTGTGAACCTGATCACCTACATAG
ATCTTGCGGAACTGCTCAAAAAAAGAGATGGGGGCTAATTCTGCCCTATCC

4 The sequence of genomic DNA obtained from PCR using primer GIH-HO-F1 and GIH-HO-R1

4.1 Band 2 : (400 bp)

GGGTTCTCGGTGCAGCGGGTGTGGTTCTGTCACGGGCTAAATATGGCTTTTATATCGCT
GGCAACATGGACATGCTCACAGCTTCTAGTGATCTGTGGAGGAAAGTCAAACAGGATCT
CGTCAAAGGGGAATCGTTAGGGAATGCCCTAGTCCTCAAGTGTGAGAACCATCCCCAAA
ACCTGCAGTCTGTGAAGTGTTATGAAGATTTCTTGGCTAAAAGTCCCCAAGGAGGTTGC
CTCTTGCAATGTGACCAAAAACTGCCTGACTGTGATCATCTCTGTGAGTGGAGGTGCCA
TGTGAAAGACATGCATCACACGAAGTACAAGTGCAGTAAACCTTGTTCTAAGGTGAGGT
GTGATTTAAACCATCCCTGTCCAAAATTATGCTGGGAAAAATGTGGGCCCCTGTGGAAC
CATCATAACCAAGACCTTTCCGTGTGGCCACACACCTACACTGGCTTGTAAAGAGAAAG
CTTGCCAACATTAGTAGAAAGAGTGATGGCACATTGCAGCACAGAGTACTGNACCTTGt
aCTACAGGAAACTATATCTTGtCCAGTgAGTCAAAGTCGCTTGCTGGGCTATGTGNAGC
TTAATGCCgCCCAAAGACCgACATAAACTCTGTCAAAGGTATCCTAtCGCAAACGcTCA
cTTGTCATAAAGGaTTCCgAGgATGTG

5. The sequence of genomic DNA obtained from PCR using primer GIH-HO-F1 and GIH-HO-R2

5.1 Band 2 : 500 bp

ACCCACTTGCTAAACTGATCGATCTCCACCATCGTGATTGACTCAACGTTTTTATGTCG
CGTTACTTCACGCAGCATGGCACCGTCGCCGCCGCCGATAATCAGCACATGTTTCGCGT
GACCATGGGCCAGTAGCGGAACATGGGTCATCATCTCATGATAGATAAACTCGTCGCGC
TCGGTGGTTTGTACTACGCCATCCAGCGCCATTACGCGACCAAGTGCAGCGTTCTCAAA
AATGATCAGATCCTGGTGATCGGTCTTTTCATGATACAGAACGTTATCTACCGCAAAGT
ACTGCCCAAACTGGTCGTGTAGCGTTTCATGCCACTGTTTTTTTTCGGCCATGGGTTGA
TACCTCCTTTGTTAACACCCGTAAAAAAAGGGCGCAACATAATAGCTAACATTGACCGC
GGATGCACGGTCAATATTTCTACAAAAAGGTATCAGGGATTATTTGACGTAGGCAAGCA
GGCTTAAGGAATCGCGGGCCAAGGCTTTGCATTTTTTAACGACGGGAATGCCAATGCCG
CTGAAATCGCGGTAACTGTCTTCCCGAAGGCTTTCATGTCAAAGGTGTTCGTAATTACT
GANGTCCNCTGGTTTTGCTGAA

5.2 Band 3 : 400 bp

GGGTTCTGGGTGCAACGGGTGTGGCTGTTGGTACAACGGCTCCTGAGCGGCTGGCTGCT
GATAAGTTTGCTCAGTCTGGTATGTAGACTGTGGAGCAAAAGTGGATTGCTGCTCTTCG
GCTTGCCAGGCGTTACCTGCCACCGGTTGTTCTGGCGCAGGGGCATAATACGGCTGTTG
CGCAGGTTGTTCAGCTGCAGGTGCATAATACGGCTGCTGCGGCTGTACTGGTTGTTGCA
GCGGCTCATTATATTGCACTGCAGGCTGCGCATATTGTGACTGCTGTGGGTAACCTTCC
GGTGCAGGAGCAATAACCGGCTCTCCCGTTTGTGGACCCGGTACAGGCTGCCAGGCTAC
TGTAGGTTGCGCAGGTGGAACATCAACAGAAGCAACAGGCGGCGTCTGAATCACAGGTT
CAACCGGCGCAACCCAACTTTGTGTCGCCGTGGTAACAACAACTGCTACAGCGACAGGT
TCNGTAATTGGCGCACCGTTTAATAATGGATCGTATTCGTCATATTCTGGCTGCCTTTN
CACNAATGCCCCAAAAATAAGAACTTCTTCCGGGTCGGCAAACCACACCCTCTTGCCAC
CCAAAAACCCAA

5.3 Band 4 : 300 bp

GGGTTTTCGGTGCAGCGGGTGTGGCTTCGATCCTGCAAAAAGGGCGTCAGGATGTGGTC
GATTGTGAAGTAAAATTGACCTCTGAACGCCGCGAAGAGGCACCACGCCTGTTTACGCA
CATTAATCTGCATTTTATCGTCACCGGTCGCGACCTGAAAGACGCACCGGTTGCGCGTG
CGGTTGATCTCTCTGCCGAGAAATATTGTTCAGTGGCGTTGATGCTGGAAAAAGCGGTG
AATATTACTCACTCGTATGAAGTGGTTGCCGCGTGACTTAACTATCCCGTTGTGAAGCC
GGATAGCATTTTATCCGGCGTTAATAGCAGAGTTACTCGTTCTCTTTCCCTCCNTCAAT
CGTTGCACCAGCGGCAGCATAATTAATTCCATTGCCAAACCCATTTTTGCCGCCCGGTA
CCACTAACNTNTTGATNTTGGGAAATGAATGAACCTTGCAACATCCCCANCANCCAGGG
GAAAATTGN

COMPARISONS OF ROTATING BIO-DRUM AND SUBMERGED BIOFILTERS IN CLOSED, SEAWATER RECIRCULATING SYSTEMS WITH BLACK TIGER SHRIMP (*Penaeus monodon*) AND SEA BASS (*Lates calcarifer*)

Napaporn Kitimasak[a], Porcham Aranyakananda[b,*], Piamsak Menasveta[a,b,c]

[a]*Department of Marine Science, Chulalongkorn University, Bangkok 10330, Thailand*
[b]*Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand*
[c]*Marine Biotechnology Research Unit at Chulalongkorn University, National Center of Genetic Engineering and Biotechnology, Bangkok 10330, Thailand*

Abstract

Culture performance and system performances were compared in closed, recirculating seawater systems using two types of biofilters (bio-drum and submerged) and two culture organisms (black tiger shrimp and sea bass). Each culture trial lasted three months. With black tiger shrimp culture, ammonia and nitrite were within normal and acceptable ranges. However, low shrimp survival and biomass compromised comparisons between the two biofilters. With sea bass culture, the bio-drum biofilter gave superior performance since it maintained ammonia and nitrite at very low concentrations. The submerged biofilter maintained low ammonia but not low nitrate concentrations. With the submerged biofilter, ammonia and nitrite approached 0.8 and 1.4 mg/l respectively, while with the bio-drum these values were less than 0.5 and 0.6 mg/l respectively.

Keywords: Rotating bio-drum biofilter; Submerged biofilter; Black tiger shrimp; Sea bass

* Corresponding author.

1. Introduction

Water treatment and re-use in aquaculture is commonly used where source water quantity or quality are limited, where high health conditions are necessary, and/or where energy needs for water temperature maintenance are potentially large. Water recirculation systems typically include several essential and optional sub-components. An essential sub-component with closed or semi-closed recirculation systems includes biofilters, which convert potentially toxic ammonia and nitrite into relatively non-toxic nitrate.

Submerged biofilters have been widely used with marine shrimp culture in recirculation systems (Menasveta et al. 1989, 1991; Tseng et al. 1998), while rotating, partially submerged bio-drums have been successfully used with trout culture in Denmark (Anonymous 1980). In our present study we compared performance of a partially submerged bio-drum biofilter and a submerged biofilter using black tiger shrimp (*Penaeus monodon*) and sea bass (*Lates calcarifer*) in two closed, recirculating seawater systems.

2. Materials and Methods

2.1. Recirculation Systems

Two sets of nearly identical closed, recirculating systems were used (Fig.'s 1 & 2). Each indoor system consisted of a 38 m^3 (7-m dia. by 1 m deep), round, concrete culture tank (CT), and a 4.6x3x1 m rectangular, concrete biofilter tank (BT). The first system used a conventional, submerged-type biofilter consisting of oyster shell and PVC pipe rings (1 cm dia. and 1.5 cm long) with total surface area of 368 m^2. The second system used a partially submerged (40%) bio-drum (1 m dia.) containing hollow plastic bioballs (4 cm dia.) with total surface area of 241 m^2. The bio-drum rotated at 3 rpm and was driven by a 0.75-kw electric motor. Each system was aerated by 1.5-kw rotary blower through 10 air-diffusers in each CT and BT.

With each recirculation system, water in the CT overflowed through a 15-cm dia. central standpipe into compartment (A) of the BT, which contained either a bio-drum or a submerged biofilter (Fig.'s 1 & 2). Suspended solids settled here and were removed daily by manual siphon. Water then flowed by gravity into the second compartment (B) which contained activated charcoal and oyster shells. Lastly, water flowed by gravity into the third BT compartment (C) and was pumped to the CT at 100-l min^{-1}, which yielded water volume recirculation of 4 exchanges day^{-1}.

2.2.Black Tiger ShrimpTrials

One thousand black tiger shrimp (average 14 g) from a commercial shrimp farm in Chon Buri Province, Thailand were acclimated in a rectangular, concrete tank for two weeks prior to stocking in the recirculation system. With each system, four shrimp of total weight 50 g were stocked in each of 32 net cages in each CT. Total initial weight of these shrimp was 1.6 kg in each system. Each cage consisted of a PVC pipe frame on which nylon net was attached. The net had a 1-cm square mesh. Shrimp were fed 3 times daily, including commercial pellets at 0800 and 2100 hrs and fresh sliced squid at 1500 hrs. Feed was applied using feeding trays. Feeding rates were adjusted weekly according to feed consumption. Each shrimp in each cage was weighed and measured monthly for growth and survival.

Total ammonia, nitrite and nitrate were analyzed according to Parsons et al. (1984). Salinity was measured by refractometer (Atago Co.), while pH was measured by pH meter (Hanna Instrument). Dissolved oxygen (DO) and water temperature were measured using a Yellow Spring Instrument DO meter (model YSI-52). These water quality parameters were measured three times weekly on Monday, Wednesday and Friday throughout the 89-day trial. Freshwater was added to each system periodically to compensate for evaporative water loss. Dolomite ($CaMg(CO_3)_2$) was added to the CT to maintain pH values at 7.0 or greater.

Unless otherwise noted, means ±1 standard error (SE) are shown. Differences among the treatment means were detected by linear regression, while analysis of co-variance was used to differentiate between treatment means.

2.3 Sea bass Culture Trials

Twenty thousand, 15 days old sea bass fry were obtained from a commercial hatchery in Rayong Province, Thailand and acclimated in a 2-ton, fiberglass tank. These fry were fed *Artemia* nauplii to satiation until they were 30 days old, then fed minced fresh fish for another 2 weeks. Moist pellets (Table 1) were then fed to these fingerlings until they were stocked in the recirculation systems after another 30 days. Twelve hundred fish were stocked into each system and fed moist pellets to satiation twice daily at 0900 and 1500 hrs. Total initial fish weights in the two systems were 1.92 and 1.98 kg respectively (Table 5).

Water quality parameters, and fish growth and survival were measured and analyzed in the same manner as with tiger shrimp during the 92-day trial. Water losses were compensated for by fresh water additions.

3. Results

3.1. Black Tiger Shrimp Trials

Ammonia and nitrite in both biofilter systems fluctuated during the first month of the trial and then declined (Fig.'s 3 & 4). They were stable thereafter. Nitrate increased during the first month and then leveled off. Mean ammonia, nitrite and nitrate respectively in each biofilter system were not significantly different ($P<0.05$). Temperatures, DO's and salinities of each biofilter system were stable and not significantly different ($P<0.05$), while pH values declining with time (Table 2).

Growth and survival rates of shrimp in both systems were similar throughout the trial (Table 3). Shrimp growth in both systems was poor during the first month, normal during the second month, and poor again during the last month. Shrimp survival in both systems declined throughout the trial. Shrimp mean growth and survival in both systems were not significantly different ($P<0.05$).

3.2 Sea bass Culture Trials

Ammonia and nitrite in the bio-drum biofilter system remained stable and less than 0.5 mg l^{-1} throughout the trial (Fig. 5), while ammonia and nitrite in the submerged biofilter system fluctuated during the first two months and increased dramatically during the last month, reaching maxima of 1.4 and 0.8 mg l^{-1} respectively at trial completion (Fig. 6). Nitrate in both systems showed the same pattern of gradual increase during the first two months with a dramatic increase and maxima of 60 mg l^{-1} at trial completion. Mean ammonia and nitrite, but not nitrate of each biofilter system were significantly different ($P<0.05$). Temperatures, DO's and salinities of each biofilter system were stable, and were not significantly different ($P<0.05$), while pH declined with time (Table 4).

Sea bass growth and survival in both systems were similar throughout the trial (Table 5). There was no significant difference ($P<0.5$) in sea bass growth between the two biofilter systems. Survival in the bio-drum and submerged biofilter systems were 58% and 57% respectively. Total biomasses in the bio-drum and submerged biofilter systems were 86 kg and 77 kg respectively. Feed conversion ratio (FCR) in the bio-drum and submerged biofilter systems were 1.71 and 1.96 respectively.

4. Discussion

4.1 Black Tiger Shrimp Culture Trials

Total ammonia in both biofilter systems fluctuated within an acceptable range for aquaculture of less than 1.0 mg l^{-1} (Lawson 1995). These concentrations were also well below those recommended for black tiger shrimp culture by Chen et al. (1990) of 4.26 mg l^{-1} NH_4-N with salinity, pH and temperature of 20 ppt, 7.57 and 24.5°C, respectively. Ammonia changes in both systems during the trial were also lower than those reported by Tseng et al. (1998). Nitrites in both systems were below the limit recommended by Chen et al. (1990) of 10.6 mg l^{-1} NO_2-N with salinity, pH and temperature of 20 ppt, 7.57 and 24.5°C, respectively.

Nitrate in both systems increased with time since the final product of biofiltration is nitrate through bacterial oxidation of ammonia and nitrite respectively (Rijn 1996). With our recirculation systems, there was no provision for removing nitrate through either water exchange or denitrification. However, the greatest nitrate-N concentration of each biofilter system was well below the recommended level of 200 mg l^{-1} NO_3-N (Wickins 1976).

Seawater salinity in both systems was stable, which benefited nitrifying bacterial growth. Lawson (1995) reported that salinity change of more than 5 ppt inhibited growth of nitrifying bacteria.

The pH of both systems decreased with time, but were maintained above 7.0 by dolomite addition. Lawson (1995) reported an optimal pH range of 6-9 for biofiltration, while Boyd (1989) reported an optimal pH range of 7-9 for shrimp culture.

DO in the bio-drum system was slightly greater than that of the submerged biofilter system (Table 2). These greater DO values were presumably due to aeration caused by rotation of the partially submerged bio-drum.

Shrimp survivals in both systems after 3 months culture were poor (6% and 7%) compared survival (76-89%) reported by Tseng et al. (1998), but were more comparable to those (8-28%) reported by Panritdam (1998). Shrimp growth in both systems after 3 months culture were also poor (0.056 and 0.051 g day^{-1}) compared with 0.17 g day^{-1} reported by Tseng et al. (1998), and to 0.14 g day^{-1} reported by Panritdam (1998).

The cause of high shrimp mortality in both systems was not likely due to poor water quality, since water quality in our systems was considered high quality for shrimp culture. Excessive shrimp mortalities in our systems were most likely caused by keeping four shrimp confined in the same cage, which increased the likelihood of cannibalism during molting. In addition, shrimp sometimes also injured themselves during molting in such confinement (0.49 m^2/cage).

In conclusion, both systems maintained adequate water quality for black tiger shrimp with respect to ammonia, nitrite and nitrate at shrimp biomass and feed application rates used herein. However, slow shrimp growth and low survival resulted in low shrimp biomass and feed applications. These limitations compromised our comparisons between the two biofilters.

4.2 Sea bass Culture Trials

Ammonia concentrations in the bio-drum system were more stable compared with the submerged system which increased dramatically during the last month of the trial. However, both systems maintained total ammonia within acceptable limits of less than 1.0 mg l^{-1} (Lawson 1995). Un-ionized ammonia in the bio-drum and submerged biofilter systems were 0.010 and 0.017 mg l^{-1} NH_3-N respectively. Again, these values were well below a recommended maximum concentration of 0.0396 mg l^{-1} NH_3-N for sea bass fry (Tookwinas 1984).

Nitrite in the bio-drum system was below the recommended upper limit of 0.615 mg l^{-1} NO_2-N throughout the trial, but nitrite in the submerged biofilter system was greater than this limit during the last month.

Nitrate in both systems increased with time. However, the greatest nitrate concentration in either system was well below the recommended limit of 400 mg l^{-1} NO_3-N (Muir 1982).

DO, pH and salinity were similar to those for the black tiger shrimp trial which were also within the optimal range for fish culture.

Sea bass survival in both systems (58% and 57%) were much lower than those (72-85%) reported by Danaksumah and Ismail (1986). Lower survivals in our trials were probably due to the small size of our fish at stocking size (1.6 g) and to the wide size range of our fish (0.5-4.0 g), compared with 80 g fish at stocking used in the study of Danaksumah and Ismail (1986). Cannibalism in sea bass was reportedly greatest in the size range of 1-20 g (Khamis and Hanafi 1986; Kungvankij and Pudadera 1986).

Sea bass growth in both systems (1.228 and 1.273 g day-^{1}) were greater than that 0.272 g day-^{1} reported by Lawanyawuth (1983) who conducted a study starting with 2.5 g sea bass in a closed system with submerged biofilter for 84 days. However, growth rates in our study were lower than those of 1.33-3.58 g day-^{1} reported by Danaksumah and Ismail (1986) who conducted their study starting with 80 g sea bass in an open culture system.

Sea bass feed conversion ratios with 35% crude protein in both of our systems (1.711 and 1.963) were much better than the 4.92 FCR reported by Lawanyawuth (1983) using dry pellet, and also better than 2.93 and 3.03 FCR reported by Danaksumah and Ismail (1986) using moist pellet with 42% crude protein.

In conclusion, both biofilter systems maintained most water quality values within optimal ranges for sea bass culture. However, nitrite concentrations in the submerged biofilter system exceeded recommended limits for sea bass during the last month of our trials, while nitrites were below the limit with the bio-drum system. With either biofilter, nitrite could exceed a safe limit at high fish densities and high feed application rates, which typically occur during final growout just before reaching market size.

Acknowledgements

This investigation was supported by the Thailand Research Fund.

References

Anonymous. 1980. Fish farm in a factory basement. Fish Farming International. Sept., 16-19.

Boyd, C. E. 1989. Water quality management and aeration in shrimp farming. Fisheries and Allied Aquaculture Department series no.2. Alabama Agricultureal Experiment Station, Auburn University, Alabama, U.S.A.

Chen, J. C., Lui, P. C. and Lei, S.C. 1990. Toxicities of ammonia and nitrite to *Penaeus monodon* adolescents. Aquaculture 89, 127-137.

Danaksumah, E. and Ismail, A. 1986. Culture of seabass (*Lates calcarifer*) in earthen brackish ponds. In: Copland, J.W. and Gray, D.L. (Eds.), Management of wild and cultured sea bass/barramundi (*Lates calcarifer*). Proceeding on international workshop held at Darwin. Australia ACIAR no. 20 pp. 156-157.

Khamis, R. B. and Hanafi, H. B. 1986. Effect of stocking density on growth and survival of sea bass (Lates calcarifer). In: Copland, J.W. and Gray, D.L. (Eds.), Management of wild and cultured sea bass /barramundi (*Lates calcarifer*). Proceeding on international workshop held at Darwin. Australia ACIAR no. 20 pp. 158-160.

Kungvankij, P. and Pudadera, B. J. Jr. 1986. Biology and culture of seabass (*Lates calcarifer*). Network of aquaculture in Asia-Pacific (NACA). NACA training manual series No. 3 Bangkok Thailand.

Lawanyawuth, K. 1983. An experiment on feeding of the white seabass fingerlings (*Lates calcarifer*) with four different kinds of food in the circulation water system (closed type). M.S. Thesis. Department of Fisheries Sciences. Kasetsart University, Bangkok, Thailand.

Lawson, T. B. 1995. Fundamentals of aquaculture engineering. New York: International Thompson.

Menasveta, P., Aranyakanonda, P., Rungsupa, S., and Moree, N. 1989. Maturation and larviculture of penaeid prawn in closed recirculating seawater system. Aquac. Eng. 8, 357 - 368.

Menasveta, P., Fast, A.W., Piyatiratitivorakul, S., Rungsupa, S. 1991. An improved, closed seawater recirculating maturation system for giant tiger prawn (*Penaeus monodon* Fabricius). Aquac. Eng. 10, 173 - 181.

Muir, J.F. 1982. Recirculated water systems in aquaculture. In: Muir, J. F. and Roberts, R. J. (Eds.), Recent advances in aquaculture. Colotado: Croom Helm. pp. 359-447.

Parsons, T. R., Maita, Y., Lalli, C. M. 1984. A manual of chemical and biological methods for seawater analysis. Oxford: Pergamon.

Panritdam, T. 1998. Use a closed recirculating seawater system with a denitrification unit for the culture of black tiger shrimp (*Penaeus monodon*). M.S. Thesis. Department of Marine Science, Chulalongkorn University, Bangkok, Thailand.

Rijn, J. V. 1996. The potential for integrated biological treatment systems in recirculating fish culture-A review. Aquaculture 139, 181-201.

Tookwinas, S. 1984. Effects of nitrite and ammonia on mortality rates of black tiger shrimp larvae and sea bass fry. Technical paper No. 6/1984. National Institute of Coastal Aquaculture. Department of Fisheries.

Tseng, K. F., Su, H. M., Su, M.S. 1998. Culture of *Penaeus monodon* in a recirculating system. Aquaculture 17, 139-147.

Wickins, J. F. 1976. The tolerance of warmwater prawns to recirculated water. Aquaculture 9, 19-37.

Table 1. Composition and proximate analysis of moist pellets fed to sea bass.

Ingredient	%
Fish meal	35
Soybean meal	35
Wheat starch	14
Rice bran	10
Crude Tuna oil	4
Vitamin premix	1
Betaine	1
Total	100

Proximate analysis	%
Crude Protein	34
Fat	3
Total Carbohydrate	27
Fiber	3
Ash	10
Moisture	25

Table 2. Water quality parameters in closed, recirculating systems with bio-drum and submerged biofilter during black tiger shrimp culture trial.

Water quality parameter	Mean ± S.E. (Min.-Max.)	
	Bio-drum biofilter	Submerged biofilter
Total ammonia-N (mg l^{-1} NH_4-N)	0.019±0.025 (bd-0.149)	0.018±0.019 (0.002-0.0068)
Nitrite-N (mg l^{-1} NO_2-N)	0.019±0.011 (0.005-0.050)	0.019±0.025 (bd-0.149)
Nitrate-N (mg l^{-1} NO_3-N	7.611±2.344 (1.317-9.958)	7.607±2.510 (1.410-9.862)
Salinity (ppt)	30±0	30±0
Water temperature (^{0}C)	27.9±0.444 (27.3-28.8)	28.9±0.403 (28.2-29.7)
Dissolved oxygen (mg l^{-1})	5.8±0.158 (5.6-6.3)	5.5±0.211 (5.0-5.8)
pH	7.5±0.282 (7.0-7.9)	7.6±0.276 (7.0-7.8)

bd=below detection limit

Table 3. Growth and survival of black tiger shrimp cultured in closed, recirculating systems with bio-drum and submerged biofilter.

Time (Days) /Treatment		Mean weight±S.E. (g)	Growth rate (g/day)	Total Feed Applied (g)		Survival rate (%)
				Pellet	Fresh	
Day 0	Biodrum	14.42±2.45	-			-
	Submerged	14.51±2.68				
Day 30	Biodrum	14.93±2.88	0.017	942	1212	65.63
	Submerged	15.04±3.10	0.017	811	1238	58.89
Day 60	Biodrum	18.40±4.53	0.116	528	644	22.66
	Submerged	17.89±3.44	0.095	520	630	18.95
Day 89	Biodrum	19.40±4.15	0.033	161	245	6.25
	Submerged	19.09±3.10	0.040	152	260	7.03

Table 4. Water quality parameters in closed recirculating systems with bio-drum and submerged biofilter during sea bass culture trial.

Water quality parameter	Mean ± S.E. (Min.-Max.)	
	Bio-drum biofilter	Submerged biofilter
Total ammonia-N (mg l^{-1} NH_4-N)	0.214±0.110 (0.003-0.430)	0.283±0.179 (0.008-0.758)
Nitrite-N (mg l^{-1} NO_2-N)	0.216±0.167 (0.002-0.546)	0.372±0.441 (0.002-1.376)
Nitrate-N (mg l^{-1} NO_3-N)	18.954±16.890 (1.655-58.894)	17.780±15.127 (1.410-59.862)
Salinity (ppt)	32±0	32±0
Water temperature (^{0}C)	29.6±0.606 (28.5-30.8)	28.9±0.403 (28.9-31.0)
Dissolved oxygen (mg l^{-1})	5.4±0.253 (5.0-6.0)	5.0±0.241 (4.2-5.5)
pH	7.5±0.288 (7.1-8.0)	7.6±0.263 (7.2-8.0)

bd=below detection limit

Table 5. Growth and survival of sea bass in closed, recirculating systems with bio-drum and submerged biofilters.

Time (Days)/ Treatment		Mean weight±S.E. (g)	Growth rate (g/day)	Total Feed Applied (kg)
Day 0	Biodrum	1.60±0.76	-	
	Submerged	1.65±0.73		
Day 30	Biodrum	18.31±6.30	0.557	17.16
	Submerged	16.35±6.16	0.490	16.42
Day 60	Biodrum	55.60±22.30	1.243	43.79
	Submerged	59.11±22.86	1.426	43.58
Day 92	Biodrum	118.68±33.72	2.103	88.61
	Submerged	114.67±39.21	1.852	97.75
Average	Biodrum		1.273	
	Submerged		1.228	

Figure captions

Figure 1. Closed, recirculating system with submerged biofilter: (BT) biofilter tank; (CT) culture tank; (A) first compartment of BT; (B) second compartment of BT; (C) pump intake compartment; (P) recirculation pump.

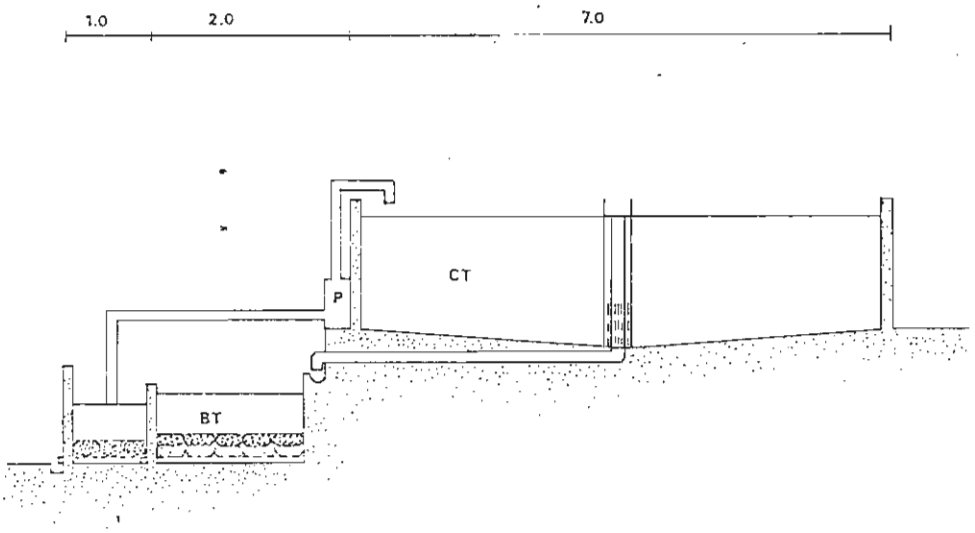
Figure 2. Closed, recirculating system with bio-drum biofilter: (BT) biofilter tank; (CT) culture tank; (A) first compartment of BT; (B) second compartment of BT; (C) pump intake compartment; (D) bio-drum; (M) bio-drum motor; (P) recirculation pump.

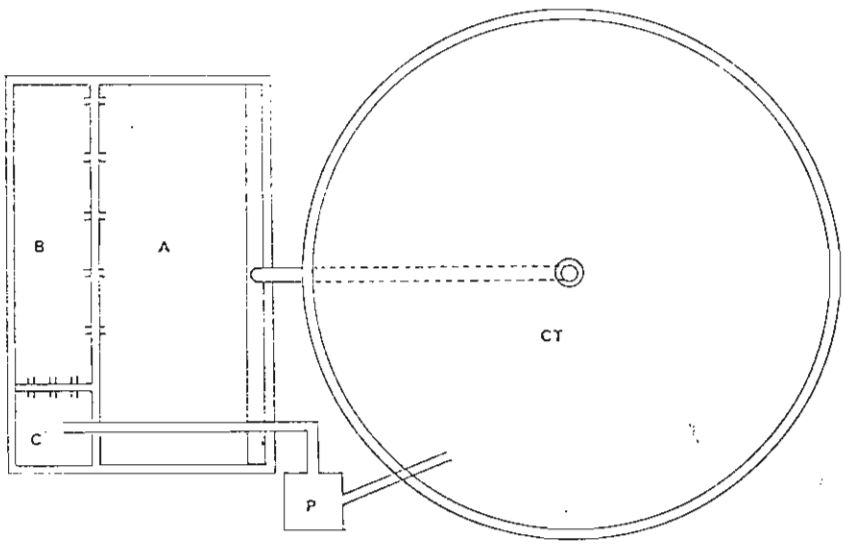
Figure 3. Total ammonia, nitrite and nitrate concentrations with the submerged biofilter and black tiger shrimp culture trial.

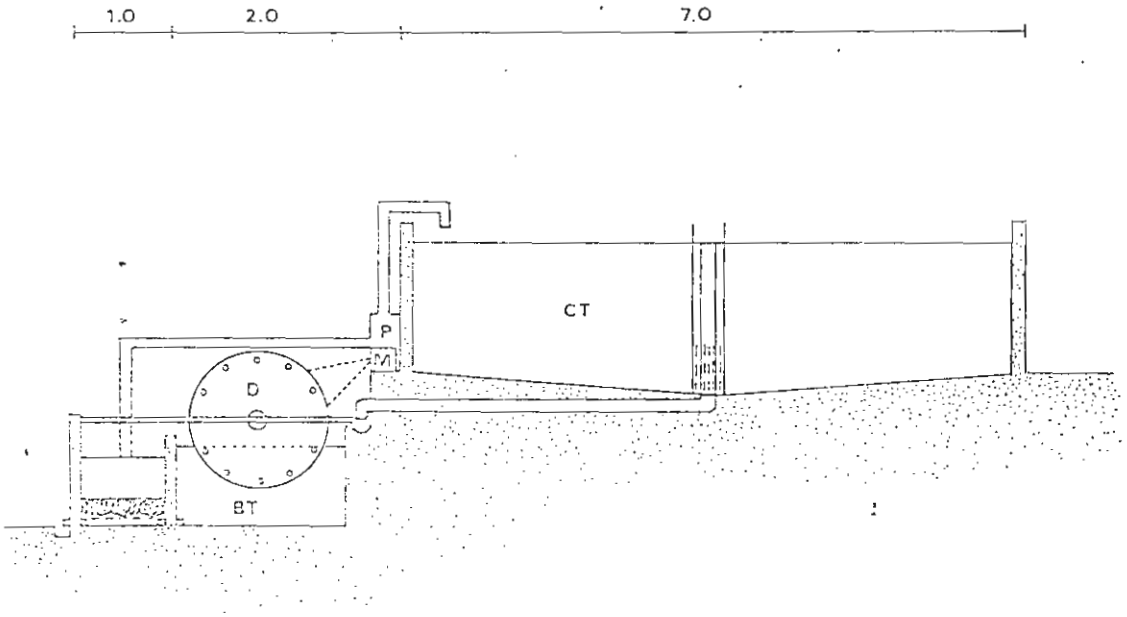
Figure 4. Total ammonia, nitrite and nitrate concentrations with the bio-drum biofilter and black tiger shrimp culture trial.

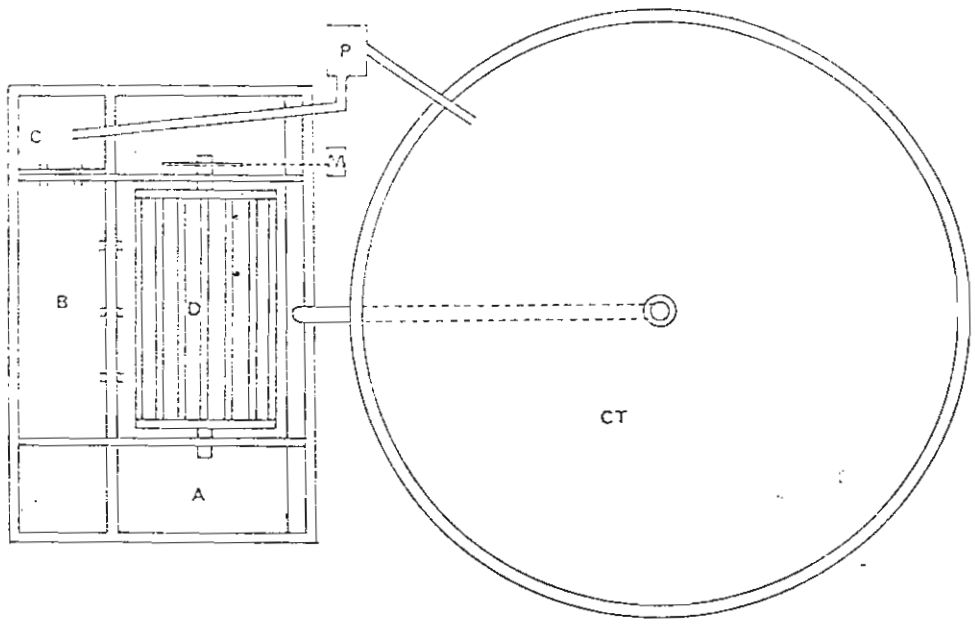
Figure 5. Total ammonia, nitrite and nitrate concentrations with the submerged biofilter and sea bass culture trial.

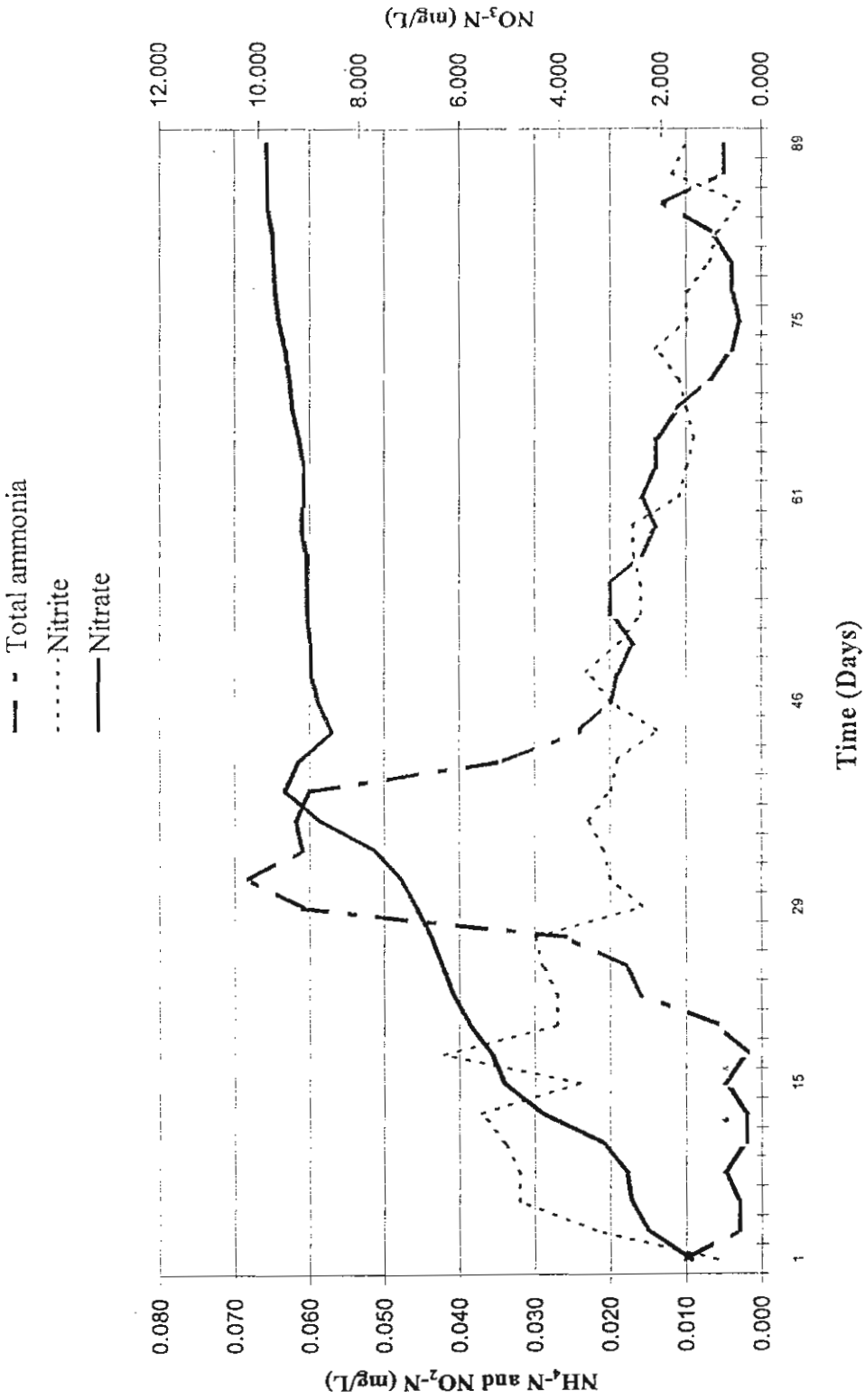
Figure 6. Total ammonia, nitrite and nitrate concentrations with the bio-drum biofilter and sea bass culture trial.

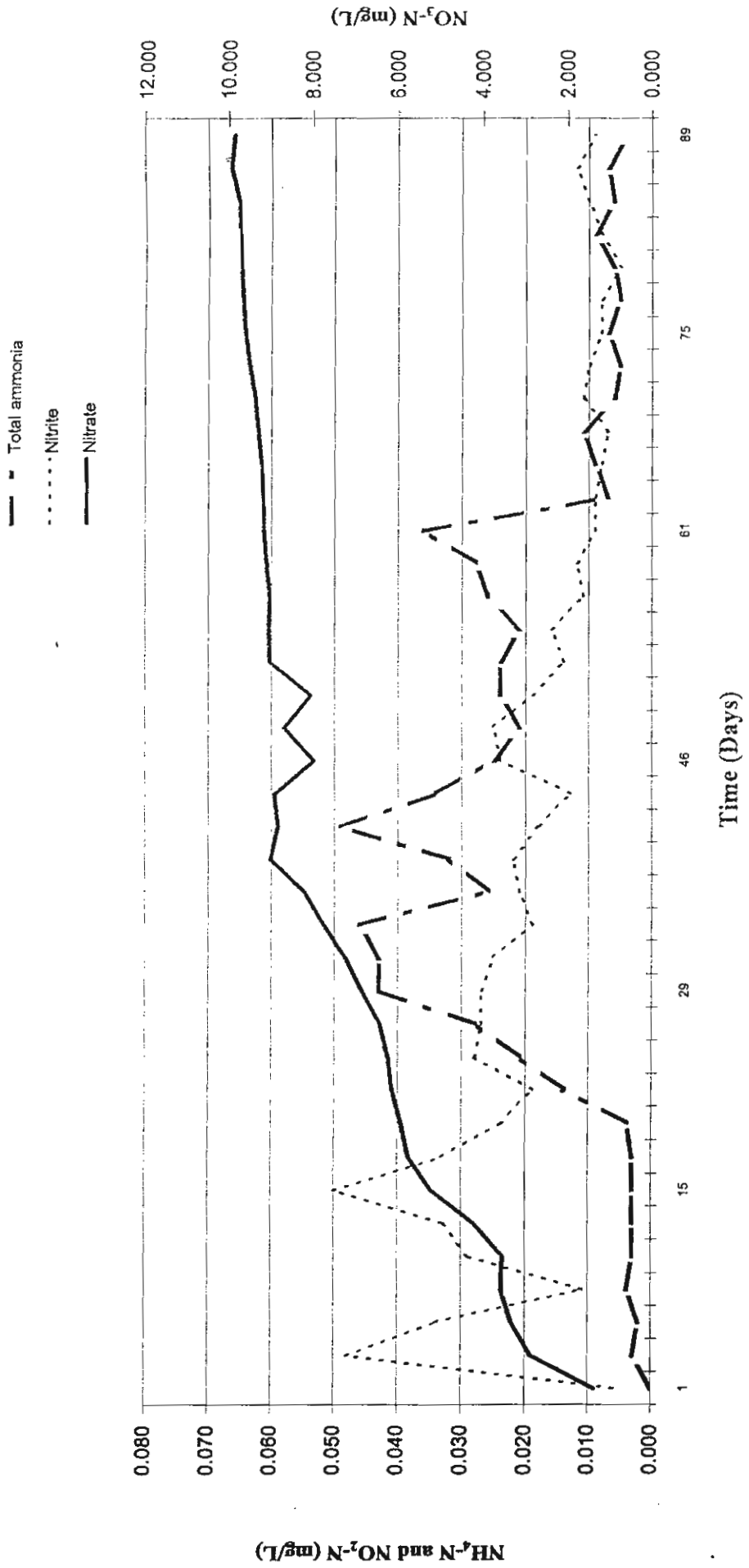

1.0
2.0
7.0
CT
P
BT

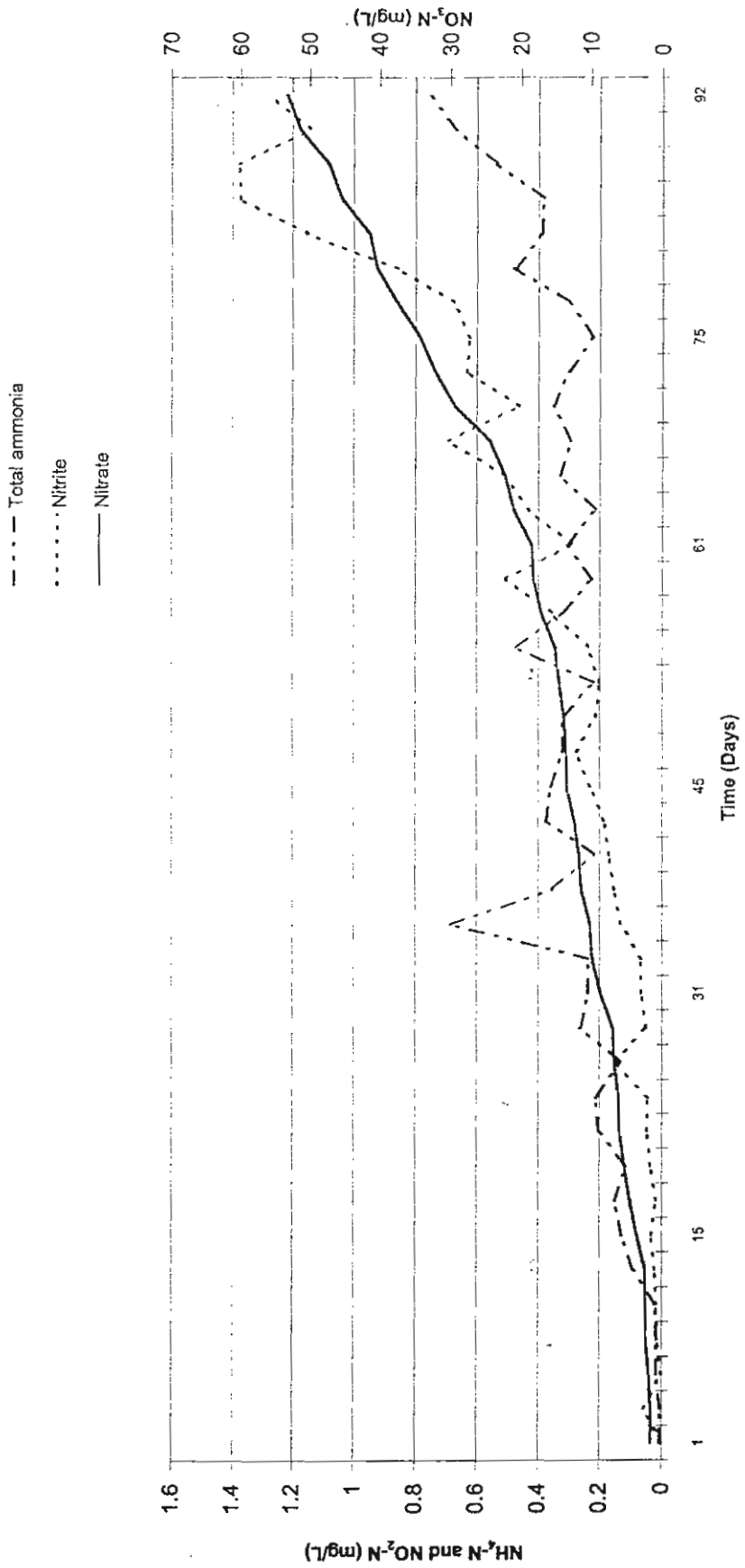

B
A
C
P
CT

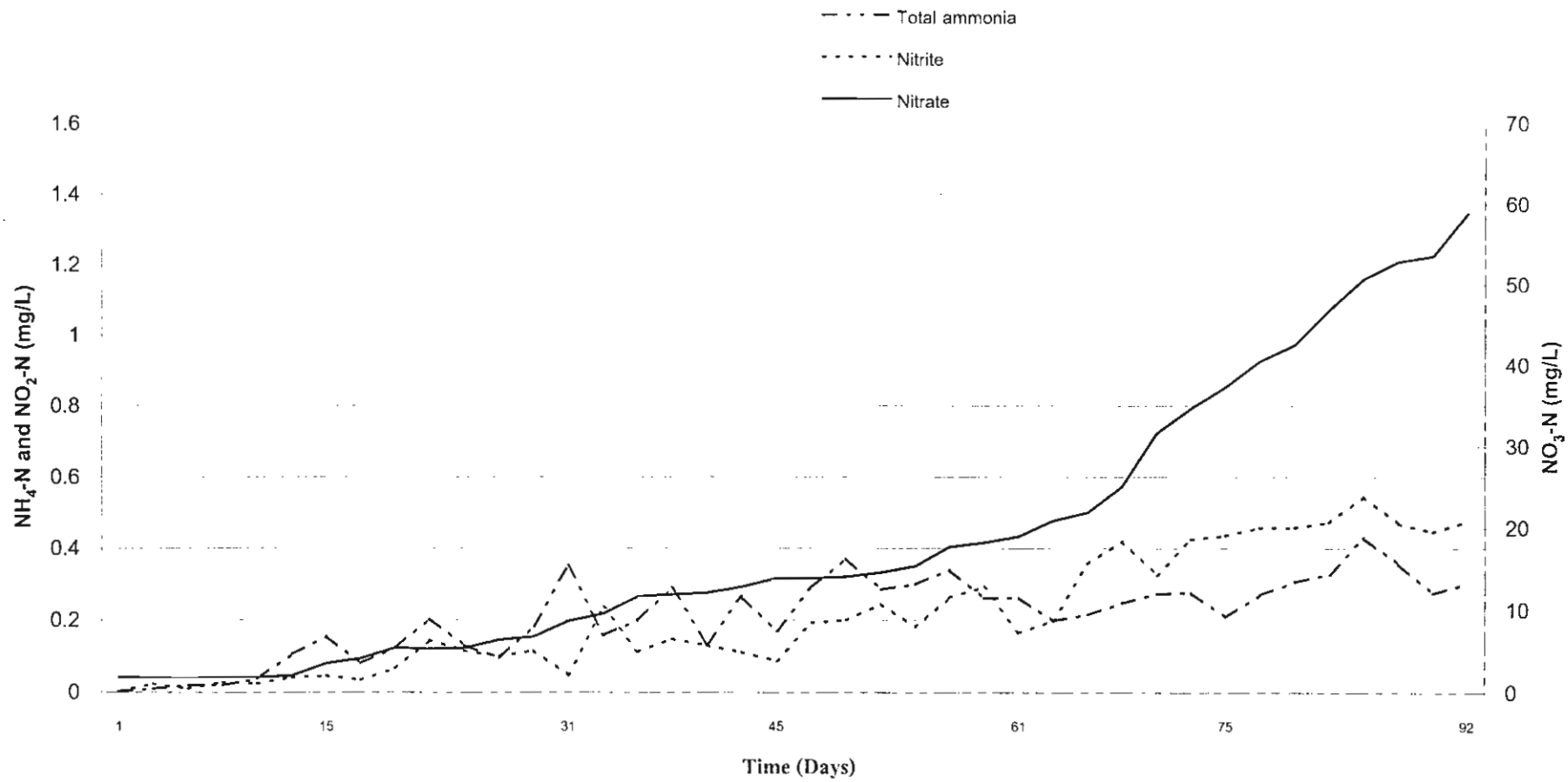

1.0
2.0
7.0
CT
P
M
D
BT


P
C
M
B
D
A
CT


Total ammonia
Nitrite
Nitrate
NH4-N and NO2-N (mg/L)
NO3-N (mg/L)
Time (Days)
0.000
0.010
0.020
0.030
0.040
0.050
0.060
0.070
0.080
0.000
2.000
4.000
6.000
8.000
10.000
12.000
1
15
29
46
61
75
89


Total ammonia
Nitrite
Nitrate
NH4-N and NO2-N (mg/L)
NO3-N (mg/L)
Time (Days)
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
12.000
10.000
8.000
6.000
4.000
2.000
0.000
1
15
29
46
61
75
89


Total ammonia
Nitrite
Nitrate
NH4-N and NO2-N (mg/L)
NO3-N (mg/L)
Time (Days)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
10
20
30
40
50
60
70
1
15
31
45
61
75
92


Total ammonia
Nitrite
Nitrate
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NH_4-N and NO_2-N (mg/L)
70
60
50
40
30
20
10
0
NO_3-N (mg/L)
1
15
31
45
61
75
92
Time (Days)

DESIGN AND FUNCTION OF CLOSED, RECIRCULATING SEAWATER SYSTEM WITH DENITRIFICATION FOR CULTURING BLACK TIGER SHRIMP BROODSTOCKS

Piamsak Menasveta[a,b,d] *, Tanya Panritdam[b], Pakitsin Sihanonth[c], Sorawit Powtongsook[d], Benjamas Chuntapa[b], Phillip Lee[e]

[a] *Aquatic Resources Research Institute, Chulalongkorn University, Bangkok, Thailand*
[b] *Department of Marine Science, Chulalongkorn University, Bangkok, Thailand*
[c] *Department of Microbiology, Chulalongkorn University, Bangkok, Thailand*
[d] *Marine Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand*
[e] *Marine Biomedical Institute, University of Texas Medical Branch, 200 University Boulevard, Galveston, Texas, USA*

Abstract

A closed, recirculating seawater system with a denitrification process was designed for a long-term culture of black tiger shrimp broodstock. The system comprised of a circular rearing tank with the loading capacity of 9 m^3. Used seawater form the rearing tank was treated by a stationary filaments biofilter "BIO-POLYMA™" situated in a tank of a dimension of 2.00 x 2.00 x 1.70 m^3. The recalculating rate was controlled at approximately 7 times per day. A denitrification process which comprised of a deoxygenated column, a bacterial substrate column, and an aerated column, was connected to the biofilter tank. The flow rate in the denitrification columns was controlled at 40 - 110 ml/min.

This closed system was run to evaluate its efficiency in controlling the water quality by comparing this system with another system of the same design but without the denitrification columns (control). The experimental period was 81 weeks, consisting of 3 consecutively periods. In the first period porous plastic balls were used in the substrate column and mangrove surface soil was used as a source of denitrifying bacteria. In the second period, crushed oyster shell was used instead of the plastic balls and a strain of laboratory cultured denitrifying bacteria was innoculated into the substrate column. Ethanol was used as a carbon source in these two periods. In the third period, methanol was used as a carbon source.

* Coresponding author. e-mail : mpiamsak@chula.ac.th

The result of the first period showed that both recirculating seawater systems could control water quality parameters namely ammonium-N, nitrite-N, and nitrate-N within the normal ranges. The nitrate-N of both systems, however, elevated to a certain point. In the second period, modification of bacterial substrates and the inoculation of denitrifying bacteria showed the ability to reduce nitrate-N in denitrification column and the rearing and bio-filter tanks ($P<0.05$), but at slow rates. In the third period, changing carbon source to methanol resulted in significant and satisfactory reduction of nitrate-N in the system.

Keywords : Closed system, Penaeus monodon, Denitrification

---

1. Introduction

Water quality is one of the important factors affecting gonad maturation success of marine shrimps. Because of this reason most shrimp matuation operators prefer to set up their facilities in the coastal areas where water is naturally clean like oceanic water. Nevertheless, such areas could not be easily found now except in the remote areas. These areas usually have much problem with logistics. Besides the areas may sometimes be influenced by the run off containing agricultural chemicals which may affect the well being of shrimp broodstocks. With respect to the above impediments, the closed, recirculating seawater system have been developed. Menasveta et al.(1989) and Menasveta et al. (1991) reported the development of the closed seawater systems for black tiger shrimp maturation. These systems, however, have some limitations. One of the limitations is the build up of nitrate-N in the systems.

The present paper describes a new design of the closed seawater system with denitrification process. The system is similar to the one reported by Whitson et al. (1993) used for culturing loliginid squids. Success of this new design will make the shrimp maturation facilities be located in any area where seawater could be transported to, i.e. inland and the seawater in such a system could be re-used for more than a year before discarding.

2. Materials and methods

2.1 *Design of the Closed, Recirculating Seawater System*

The system comprised of a circular rearing tank, a bio-filter tank, and a series of denitrification columns (Fiqure 1). The circular rearing tank had a diameter of 3.5 m and a height of 0.9 m. It was made of a plywood frame lined with black polyethylene sheet of the thickness of 0.75 mm. The plywood frame

was re-enforced by fasten three stainless steel slings around the frame. The rearing tank was central drained to a bio-filter tank though a 2.5 cm diameter PVC pipe by a magnet sealess pump. The bio-filter tank was made of steel re-enforced concrete with a size of 2.00 x 2.00 x 1.70 m^3. Stationary filament bio-filter "BIO-POLYMA™" was placed inside the bio-filter tank. Denitrification process comprised of three 30 cm diameter PVC columns. The first column was attached to the bio-filter tank for receiving a continuously partial drain from the bio-filter tank. The column was purged by nitrogen gas to reduce the dissolved oxygen to the level less than 0.5 mg/l. Deoxygenated seawater from this column was pumped to the second column at the bottom part at the rate of 40 - 110 ml/min. Bacterial substrate was packed in this column. Overflow from the second column was continuously drained to the third column situated inside the bio-filter tank. Seawater in this column was re-aerated by air before leaving through the bottom.

Another set of identical closed, recirculating system, but without denitrification process was used as the control. It was so called the control closed system.

Overflow from each bio-filter tank was drained by gravity to its circular rearing tank through a 3.75 cm diameter PVC pipe. The recirculating rate was controlled at approximately 7 times per day.

The bio-filter tank was conditioned for two weeks before starting the experiment. Ammonium chloride was added to both closed systems at the concentration of 2 mg/l and let the seawater recirculated until ammonium-N and nitrite-N reduced to 0.2 mg/l. It was taken about a week to reach this point.

2.2 *Experimental Design*

Both treatment and control closed systems were run to evaluate their efficiency in controlling water quality. Black tiger shrimps of the average size of 20 gm were stocked and maintained in the circular rearing tanks at 10 shrimps/m^2. The shrimps were fed with both fresh and compound diets at satiation everyday. Un-eaten foods and feces were siphoned out everyday.

Water quality parameters namely dissolved oxygen, pH, water temperature, and salinity were monitored using electrical probes (Metler Toledo™) connected with a computer data logging system. Ammonium-N, nitrite-N, and nitrate-N in seawater were analyzed weekly by the method described in Strickland and Parson (1972).

The experiment was run for 81 weeks and it could be divided into three consecutive periods. In the first period of 22 weeks (week 0 - 22), plastic balls with high porosity were used as substrate in the denitrification column. Besides, a small amount of mangrove soil was used as a source of denitrifying bacteria in the column. In the second period of 20 weeks (week 23 - 43), crushed oyster shells were used as the bacterial substrates in the denitrification column. Besides a strain of laboratory cultured denitrifying bacteria (Bacillus sp.) was inoculated into the column at the amount of 10.6 - 11.7 x 10" cells for several occasions. Ethanol was used as a carbon source for the first and second periods. The feeding rate was controlled by a peristaltic pump at the rate of 6.6 ml/hr.

Between the second and the third period, denitrification process was shut down for a period of 29 weeks. It was resumed again at the start of the third period.

The third period started at week 72 and the systems were run for 9 weeks. Methanol was used as a carbon source instead of ethanol, at the feeding rate of 25.8 ml/hr.

3. Results

The result of the first period of experiment showed that both closed systems could control water quality parameters namely ammonium-N, nitrite-N, and nitrate-N within the normal ranges (Figure 2, 3 and 4). Nevertheless, the closed system with denitrification process did not show the ability to control nitrate-N in the level lower than that of the control closed system.

In the second period of experiment, the inoculation of cultured denitrifying bacteria together with the change of packed column from the plastic balls to crushed oyster shells showed the ability to reduce nitrate-N to the level significantly lower than that of the control closed system ($p < 0.05$). However, bacterial inoculation had to be carried out in several occasions (Figure 4). The nitrate-N reduction rate in the treatment closed system was comparatively slow and fluctuating. Nitrate-N fluctuation in the packed column was quite noticeable (Figure 4). Nitrate-N would reduce abruptly right after the bacterial inoculation, then it started to level up again.

Changing the carbon sources from ethanol to methanol with higher feeding rate in the third period of the experiment resulted in significant and satisfactory reduction of nitrate-N (Figure 5), Nitrate-N in the treatment closed system reduced from 165 mg/l to 50 mg/l within a period of 9 weeks. Nitrate-N in the circular rearing tank, bio-filter tank, and the denitrification column of the

treatment closed system all showed similar rates of reduction (Figure 6). It should be noted however that nitrate-N concentration in the denitrification column was almost always lower than that of the circular rearing tank and the bio-filter tank (Figure 6). Besides nitrate-N always showed a vis versa concentration with nitrite-N in the denitrification column (Figure 7).

Other water quality parameters i.e. dissolved oxygen, pH, temperature, and salinity varied within the normal ranges.

4. Discussion

BIO-POLYMA^{TM}, a synthetic fiber, used in our biofilter tank could provide a good nitrification rate for most of the experimental periods. As shown in Fig 2-4, ammonium and nitrite in both experimental ponds were regulated within the safety levels. The advantage of using BIO-POLYMA^{TM} was that the bundles of thin fiber provided large surface area for bacterial attachment and the arrangement of biofilter filaments in the tank separated nitrifying area from settled sludge. This gave us an effective nitrification system compared with our previous describes (Menasveta et al., 1989; Menasveta et al., 1991). Nitrification in our system worked well so it could maintain ammonium and nitrite concentrations within the safety level even after week 72 when nitrate was accumulated to more than 200 ppm.

Accumulation of nitrate was found in most of aquaculture systems, even in a low stocking density aquarium with no significant primary productivity and low water changes. For example, nitrate in closed marine aquarium with low stocking fishes could reach 9700 μM (135.8 mg NO_3-N L^{-1}) after 5 years operation (Grguric & Coston, 1998). In intensive aquaculture ponds, accumulation of nitrate can occur more rapidly and must be reduced by changing water or by denitrification. Denitrification, however, is a complicate step to closed recirculation system. The efficiency of denitrification depended on several factors but the most important were that dissolved oxygen must be kept close to zero level and the external carbon source must be added. In contrast with industrial wastewater treatment, using denitrifying bacteria to remove nitrate from water in aquaculture system is usually a difficult process and may require close monitoring to prevent release of toxic intermediate oxides of nitrogen such as nitrite or even hydrogen sulfide. Therefore, anaerobic sludge treatment process used in conventional wastewater treatment is not recommended because nitrite or hydrogen sulfide from incomplete denitrification can possibly harm the animals. Turk et al. (1997) suggested that the parameters to be closely monitored during denitrification were dissolved oxygen (DO), oxidation-reduction potential (ORP) and pH. Those retrieved parameters from an online monitoring system incorporated with automatic

control of water flow rate and methanol (or other carbon sources) feeding rate could provided the optimized regulation of denitrification process.

In our study, denitrification process in the denitrification column during the second period of the experiment (Fig 4) occurred only in short time after additions of bacteria. In general, as suggested by Turk et al. (1997), who produced the specific pathogen free shrimps in the remote area with the an extra clean facility, denitrifying bacteria could grown naturally and worked effectively in the system without any addition of bacteria from outside. These indicated that the condition in our denitrification column during the second period was not suitable for bacteria to used nitrate as an electron acceptor in the electron transport chains although dissolved oxygen was kept low by nitrogen bubbling and ethanol was continuously added as a sole carbon source.

After the second period, both tanks were maintained by stocking shrimp for 28 weeks without denitrification so nitrate was found accumulate to approximately 200 ppm. This nitrate concentration was two times higher than that recommended limit for some species of fish but crustacea usually tolerate in greater concentration. For example, as described by Hart and O'Sullivan (1993), black tiger shrimp (*P. monodon*) could still grow in nitrate up to 200 ppm but this concentration is not recommended for normal operator and water exchange is generally needed. We were then perform the third period experiment by starting up denitrification process with methanol as a carbon source instead of ethanol. The result (Fig 5) illustrated the success of denitrification process in our system that could reduce nitrate in the culture tank down to below 50 ppm.

Our denitrification column was designed for easy maintenance. Unlike other systems such as a series of nitrification and denitrification filters described by Abeysinghe et al. (1996), our system connected a denitrification unit to nitrification biofilter tank as a separate loop. Therefore, the system could still run uninterruptedly even when the denitrification unit did not worked properly. However, as suggested by Lee (1995), methanol addition rate must be controlled not to exceed the demand of denitrifying bacteria. In this experiment, we did control the methanol addition manually by adjusting the peristatic pump at the carbon to nitrogen ratio of approximately 1:1. Bacterial activity in the column produced the correlated peaks of nitrite, which is the intermediate substance of denitrification (Fig 7). As no accumulation of nitrite was found, it could be assumed that the denitrification process occurred completely. However, the fluctuating of nitrate concentrations had occurred several times because of the methanol pump failure especially in week 76 and 77. It could be noted that if there was no failure of the methanol pump, nitrate concentrations would have been reduced from 110 ppm to less than 20 ppm within two weeks.

The only disadvantage of denitrification unit described here is that the running cost was considerably high for normal aquaculture operators. Liquid

nitrogen used for nitrogen purging in the oxygen reduction column seemed to be the most expensive operating cost. We believed that an automatic control for optimizing denitrification in the column, if there was, might have improved the efficiency but it must also be carefully thought about the expense. Therefore, a low cost, simple automatic methanol pump controller, and the improvement of oxygen reducing column without using nitrogen gas should be a further step of a more economical system development.

5. References

Abeysinghe, D.H., Shanableh, A. and Rigden, B. (1996). Biofilters for water reuse in aquaculture. *Water Science and Technology* 34(11): 253-260.

Grguric, G. & Coston, C.J. (1998). Modeling of nitrate and bromate in a seawater aquarium. *Water Research* 32(6): 1759-1768.

Hart, P. & O'Sullivan, D. (1993). Recirculation systems: design construction and management. *An Aquaculture Sourcebook publication*, University of Tasmania, p.14.

Lee, P.G. (1995). A review of automated control systems for aquaculture and design criteria for their implementation. *Aquacultural Engineering* 14(3): 205-227.

Menasveta, P., Aranyakanonda, P., Rungsupa, S., and Moree, N. (1989), Maturation and larviculture of peneid prawns in closed recirculating seawater systems. Aquacultural Engineering 8(5) : 357-368.

Menasveta, P., Fast, A.W., Piyatiratitivorakul, S. and Rungsupa, S. (1991). An improved closed recirculating seawater system for maturation process of giant tiger prawn (Penaeus monodon Fabricius). Aquacultural Engineering 10 : 173-181.

Strickland, J.D.H. & Parson, T.R. (1972). *A practical handbook of seawater analysis*. (2nd ed) Fisheries Research Board of Canada Bulletin No.167.

Turk, P.E., Zuercher, C., Lawrence, A.L. and Lee, P.G. (1997). Design and operation of an environmentally isolated, marine shrimp broodstock culture system using closed, recirculating water filtration. In Timmons M.B. and Losordo, T. (eds) *Advances in Aquacultural Engineering*. Proceedings from the Aquacultural Engineering Society (AES) Technical Sessions at the Fourth International Symposium on Tilapia in Aquaculture, Florida, November 9-12, 1997, Northeast Regional Agricultural Engineering Service, pp.209-218.

Whitson, J., Turk, P.E. & Lee, P.G. (1993). Biological denitrification in a closed recirculating marine culture system. *Techniques for Modern Aquaculture*, American Society of Agricultural Engineering, St. Joseph, Michigan., June 21, 1993, pp.458-466.

Figure captions

Figure 1 Diagrams illustrate the closed, recirculating seawater systems used in this experiment.

1 circular rearing tank (treatment)	2 circular rearing tank (control)
3 bio-filter tank (treatment)	4 bio-filter tank (control)
5 oxygen reduction column	6 denitrification column
7 re-aerated column	8 bio-filter (BIO-POLYMA™)

Figure 2 Ammonium-N concentration in control (◆) and treatment (□) rearing tanks.

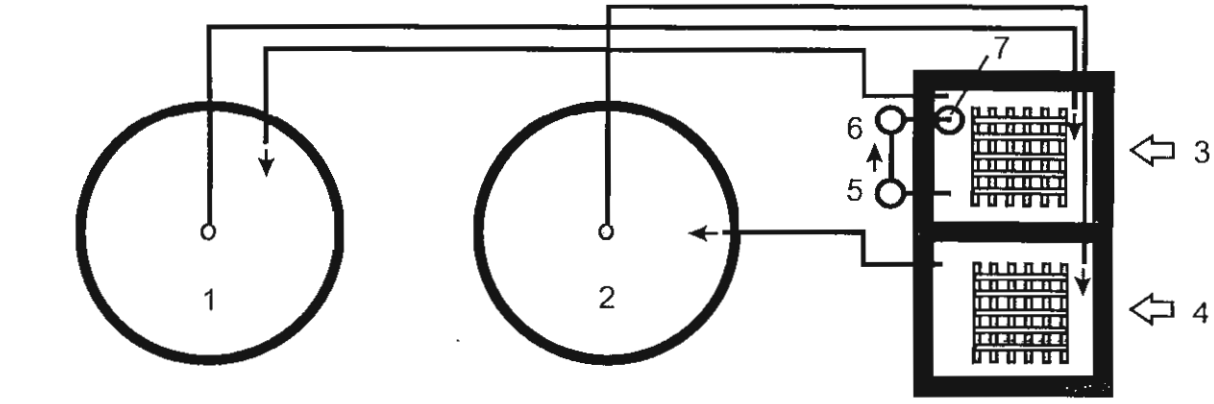
Figure 3 Nitrite-N concentration in control (◆) and treatment (□) rearing tanks.

Figure 4 Nitrate-N concentration in control rearing tank (◆), treatment rearing tank (□) and denitrification column (▲). Arrows indicate innoculation of *Bacilus* sp. (10.6 - 11.7x10^{11} cells) into the denitrification column.

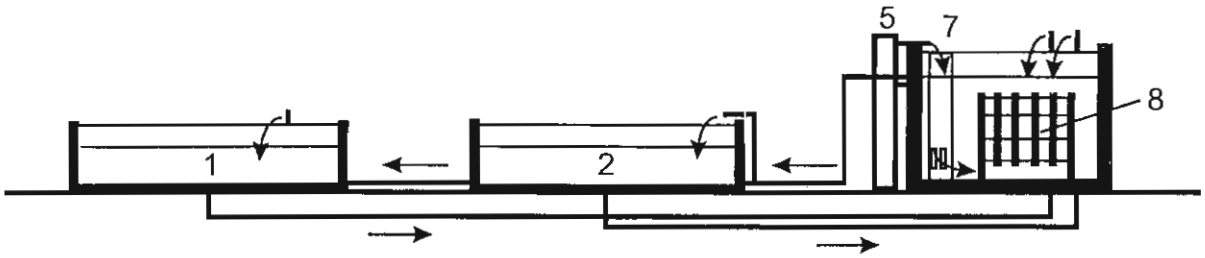
Figure 5 Nitrate-N concentration in control rearing tank (◆) and treatment rearing tank (□), during the third period of experiment.

Figure 6 Nitrate-N in rearing tank (◆), biofilter tank (□) and denitrification column ( ) of the treatment (top) and control (bottom) closed systems during the third period of experiment.

Figure 7 Nitrate-N (◆) and nitrite-N (□) concentration in the denitrification column during the third period of experiment.



Top view



Side view

Figure 1

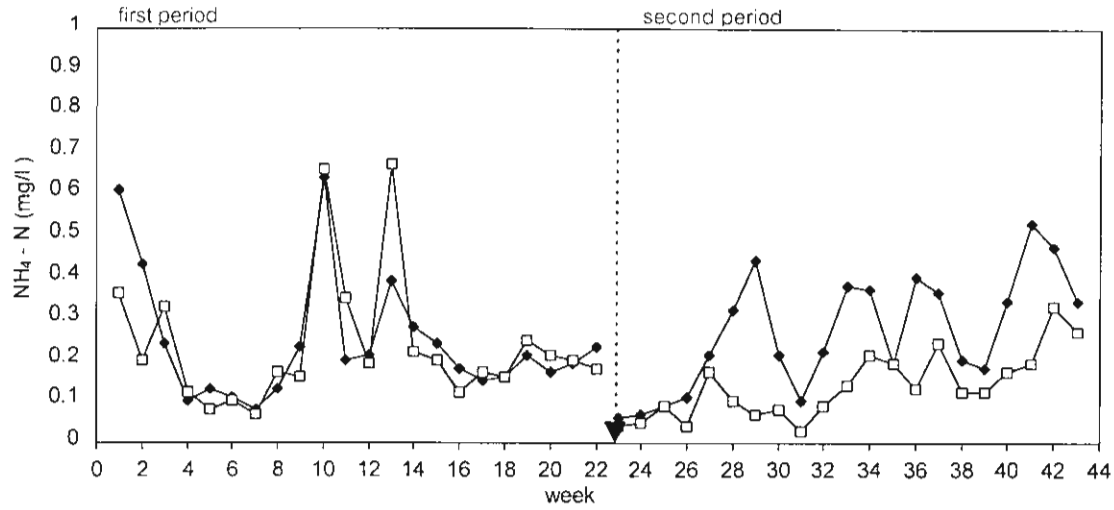


Figure 2

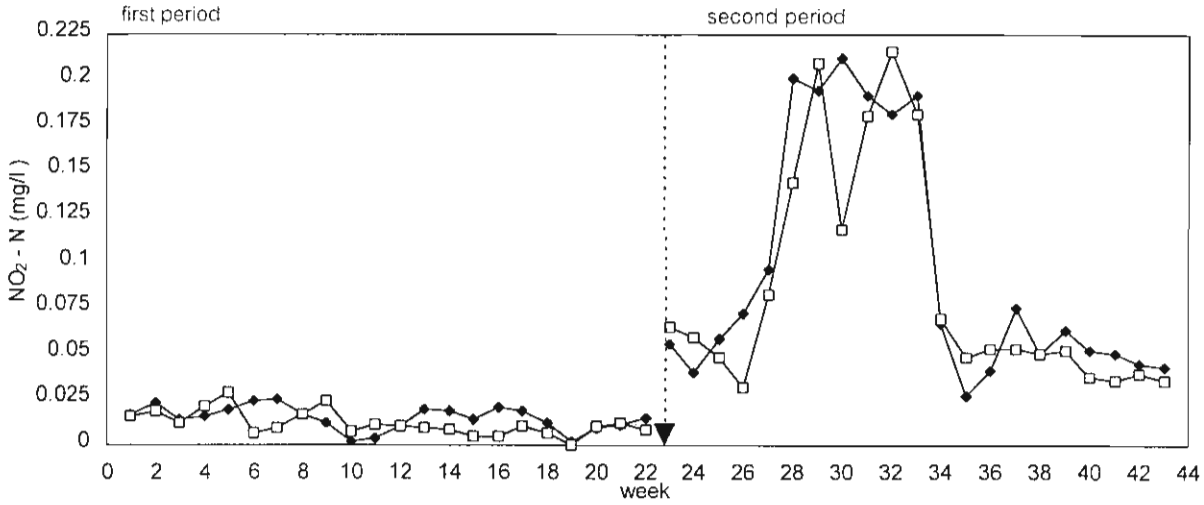


Figure 3

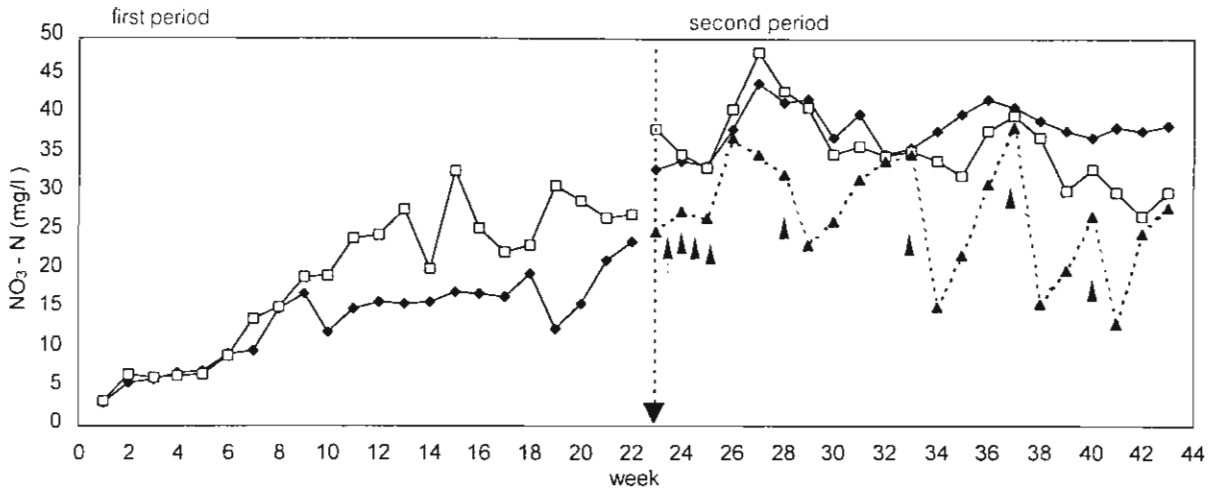


Figure 4

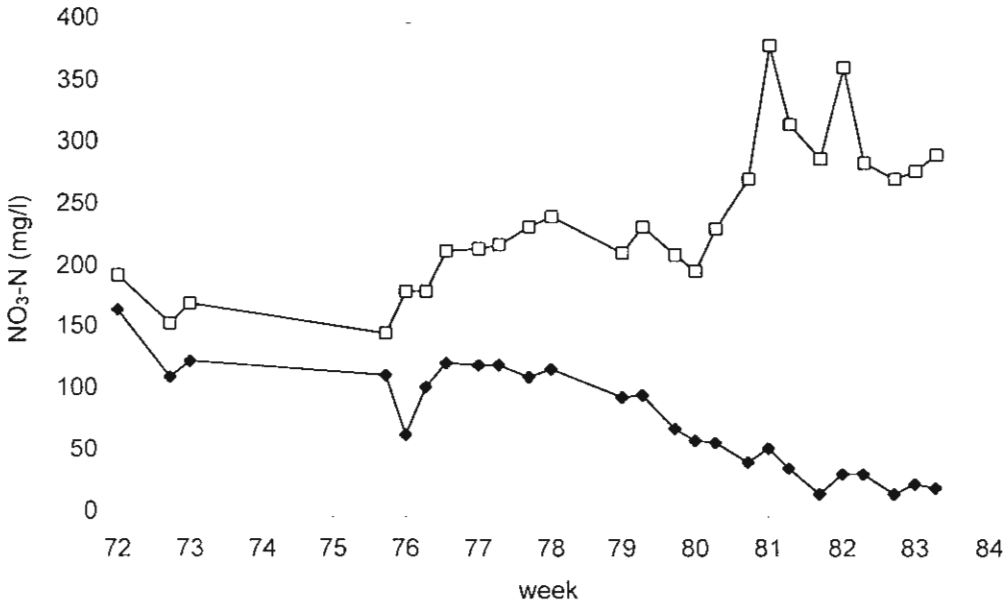


Figure 5

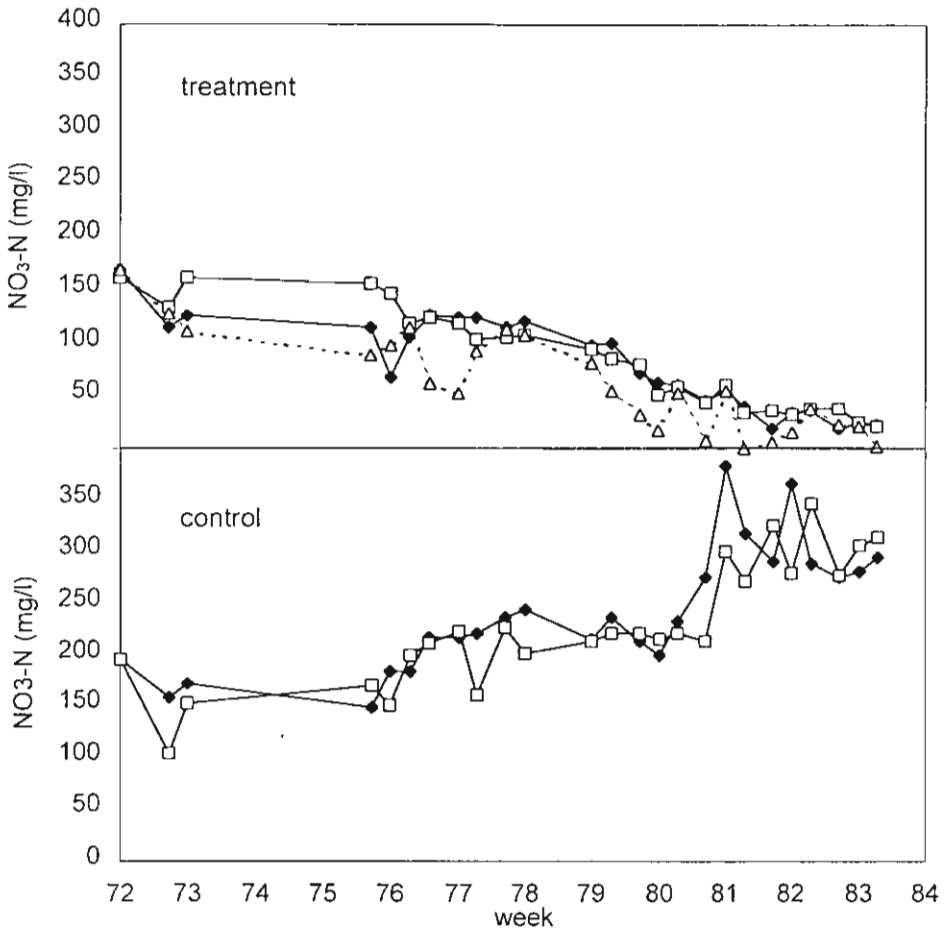

treatment
control
NO3-N (mg/l)
NO3-N (mg/l)
400
350
300
250
200
150
100
50
350
300
250
200
150
100
50
0
72
73
74
75
76
77
78
79
80
81
82
83
84
week

Figure 6

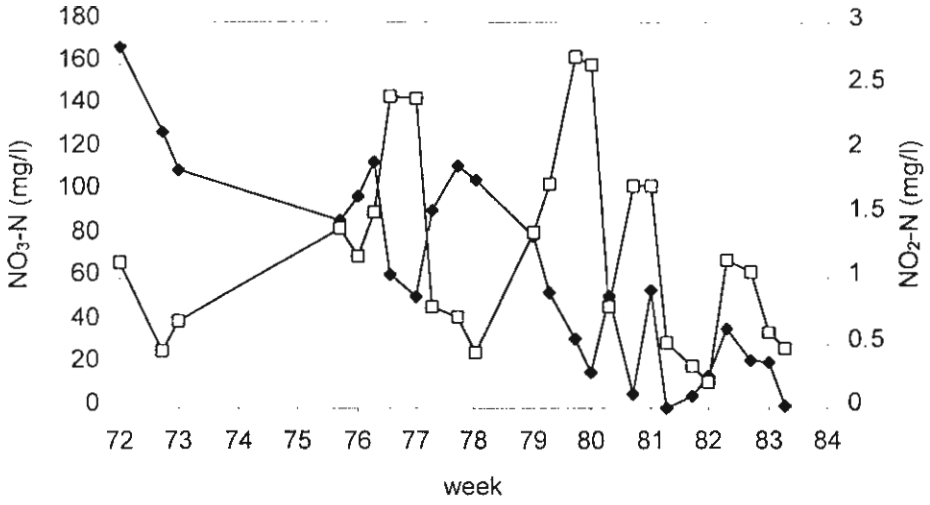

180
160
140
120
100
80
60
40
20
0
NO_3-N (mg/l)
3
2.5
2
1.5
1
0.5
0
NO_2-N (mg/l)
72 73 74 75 76 77 78 79 80 81 82 83 84
week

Figure 7

Optimal Photosynthetic Activities of two Macrophytes used in Recirculating Seawater Systems in Thailand

Sorawit Powtongsook[1], Alisa Chokwiwattanawanit[2] and Piamsak Menasveta[3]

[1]Marine Biotechnology Research Unit, National Centre for Genetic Engineering and Biotechnology (BIOTEC), Ministry of Science, Technology and Environment
[2]Interdepartment of Environmental Science, Chulalongkorn University
[3]Aquatic Resources Research Institute, Chulalongkorn University

ABSTRACT

Caulerpa lentillifera and *Acanthophora* sp. are two aquatic macrophytes often used in shrimp wastewater treatment ponds in Thailand. These algae rapidly assimilate ammonia and nitrate during growth. As part of our research, we evaluated these algae for use with small, recirculating seawater systems. A primary consideration for this application is light intensity at which photosynthetic activity is maximum (P_{max}). We measured photosynthesis by oxygen evolution method and found P_{max} at light intensities of 15,000-20,000 lux (200-270 μ mol m^{-2} s^{-1} photon flux density) with both algae. We did not observe photoinhabition at light intensities up to 60,000 lux. With small, indoor recirculating seawater systems, 4 to 5 fluorescent lights provide sufficient illumination for P_{max}.

INTRODUCTION

In Thailand, two macrophytes, a green alga (*Caulerpa*) and a red alga (*Acanthophora*) have been used recently for nitrogen waste removal with commercial shrimp culture in ponds, using recirculating seawater systems. These algae were grown in outdoor ponds of about 0.5-ha with 50-cm water depth. Algal ponds were part of the water treatment systems, following sedimentation ponds. These algae removed nutrients and iron from seawater. *Caulerpa* has also been used for water treatment with indoor aquariums, such as the invertebrate aquarium at Bangsaen Institute of Marine Science, Chonburi, Thailand. Algae reduced nitrate, which can harm invertebrates such as corals and sea anemones, even at low concentration (V. Muthuwan, personal communication, 1999). Algae growing in indoor aquariums received much lower light intensity than in outdoor ponds, but still showed high nutrient uptake.

There are many published research articles describing wastewater treatment with algae in shrimp culture systems. For example, Chaiyakam and Tunvilai (1992) used the red macrophyte *Gracilaria* sp. in combination with green mussel for biological wastewater treatment in shrimp culture ponds, and Tunsutapanich *et al.* (1998) integrated macrophytes species with fishes as the biological filter in reservoir and treatment ponds. However, physiological optimization studies with *Caulerpa* and *Acanthophora* have not been conducted.

Algae take up nitrogen and phosphorus for cellular metabolism and during growth. Maximum nutrient uptake rate is achieved by optimizing growth, and therefore by optimizing photosynthetic rate.

Photosynthesis is usually measured by two techniques, oxygen evolution or chlorophyll fluorescence. Although a modulated chlorophyll fluorescence technique provides quick response and accurate evaluation of algal photosynthesis, it also requires sophisticate and expensive instrument (Rodrigues *et al.*, 1993). Oxygen evolution, on the other hand, is a classical but less sophisticated technique. Since oxygen is produced during algal photosynthesis, changes in dissolved oxygen are used to measure photosynthetic activity of algae. During our study, we evaluated optimum light intensities for two algae, *Acanthophora* sp. and *Caulerpa lentillifera* using light saturation curves determined by photosynthetic oxygen evolution.

MATERIALS AND METHODS

Algae and stock culture condition

Caulerpa lentillifera and *Acanthophora* sp. were collected from seawater treatment ponds of Bunchong Farm, Chachoengsao Province, Thailand (Figure 1). Algae were kept in 50-l fiberglass tanks with 25-l of 30-ppt seawater supplemented with nutrients (F/2 medium) under ambient light and temperatures (27-34°C).

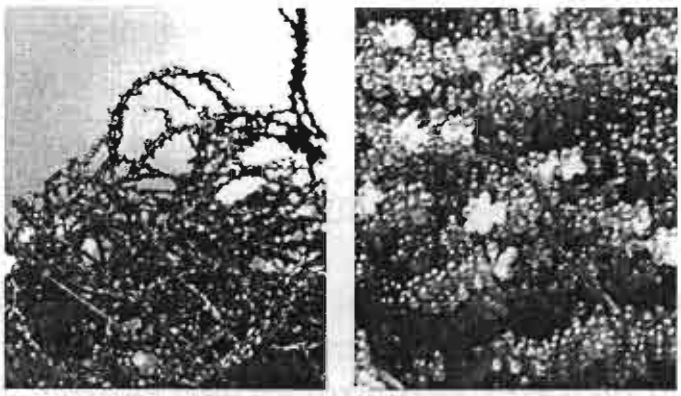


Figure 1. Photographs of the red alga *Acanthophora* sp. (left) and the green alga *Caulerpa lentillifera* (right). Both algae are used for wastewater treatment of shrimp farm effluents in Thailand.

Photosynthesis oxygen measurements in laboratory

Oxygen evolution was measured in a custom built Plexiglas chamber. The chamber was 10-cm diameter by 14 cm high, and contained 20-g of algal sample in 700-ml of sterile seawater. Water movement in the chamber was achieved using a magnetic stirrer to prevent oxygen depletion on the oxygen probe's membrane surface. Illumination was provided by 500 W or 1000 W halogen lamps in conjunction with heat reduction using a water jacket. Sodium bicarbonate (0.5 g/l) was added to the chamber to prevent carbon deficiency. Before each measurement, algae were kept in the chamber, in darkness for 10 min to equilibrate. Oxygen concentrations were measured using a Hanna 964400 dissolved oxygen (DO) meter with automatic temperature compensation, which was connected to a computer for data acquisition. Light intensity was measured inside the chamber using a Digicon LX-50 lux meter. Temperature was controlled at 29±1°C. Light intensity was adjusted by changing light sources (500 W or 1000 W halogen lamps), and using neutral density filters.

Oxygen evolution in ambient, outdoor light condition

Algae (100-g) were placed in a 10-l transparent jar with 4-l of seawater under ambient, outdoor light. Nitrogen gas was bubbling into the water to reduce DO to 1 mg/l. Nitrogen gas injection was terminated and measurements began. Oxygen evolution was monitored continuously for 1-hr, together with light intensity. Oxygen evolution from algae was always measured in parallel with controls (seawater in jars without algae). Net oxygen evolution rate was calculated by subtracting oxygen increase in the control from evolution rate

with algae. Finally, net oxygen evolution rate was plotted against average light intensity for each data set.

RESULTS

Figure 2 illustrates the effect of light intensity on oxygen evolution rate with *Acanthophora* sp. during five independent trials. Oxygen evolution rate increased with light intensity until approximately 15,000 lux (photo flux density (PFD) = 200 μ mol photon m^2 s^{-1}). No further increase in oxygen evolution rate occurred after 15,000 lux.

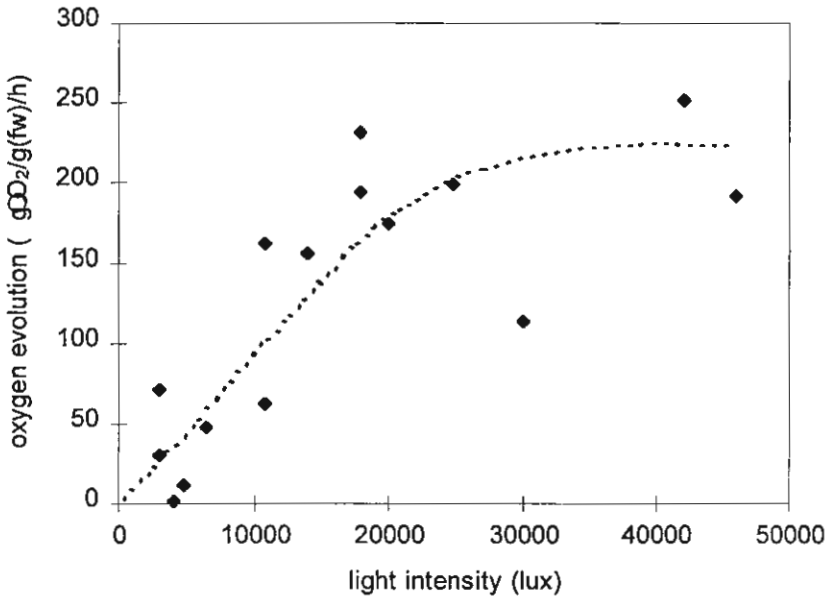


Figure 2. Photosynthetic oxygen evolution by *Acanthophora* sp. in laboratory conditions. Data were combined from five independent trials.

Caulerpa lentillifera had the same oxygen evolution pattern as *Acanthophora*, but was lower magnitude. Light saturation also occurred at about 15,000 lux (Fig. 3). However, at the highest light intensity of 45,000 lux (600 μ mol photon m^2 s^{-1} PFD), oxygen evolution appeared to decline. Since we could not provide light intensities greater than 45,000 lux with our indoor equipment, we measure oxygen evolution outdoors with natural light at higher intensities. The results are shown in Figure 4.

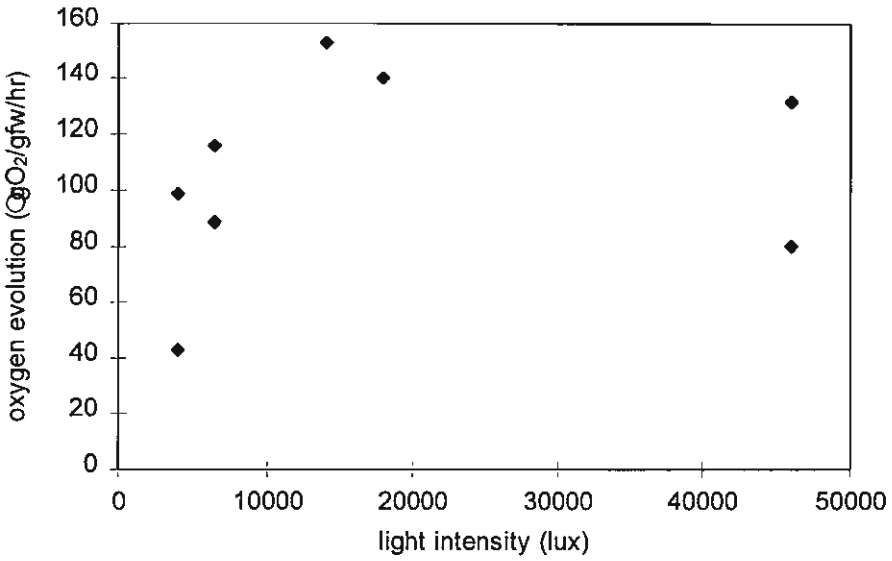


Figure 3. Photosynthetic oxygen evolution by *Caulerpa lentillifera* in laboratory conditions. Data were from three independent trials.

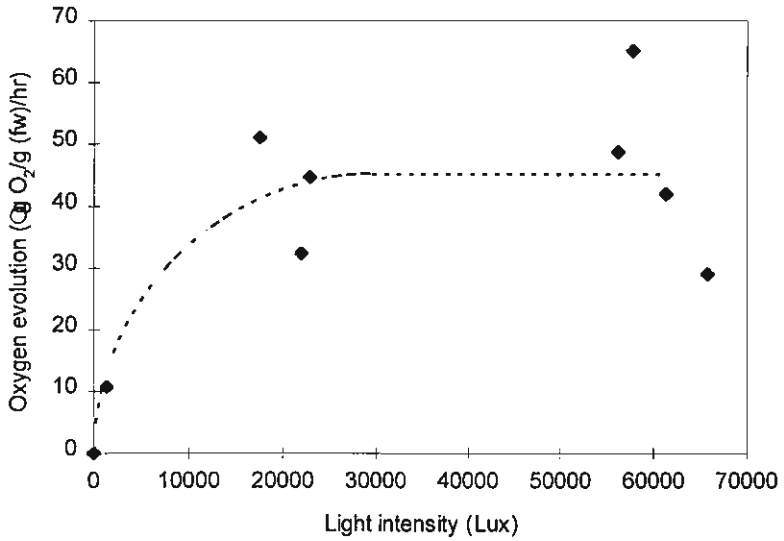


Figure 4. Photosynthetic oxygen evolution from *Caulerpa lentillifera* in outdoor conditions. Data were from nine independent trials.

Caulerpa photosynthetic DO production patterns were the same under both outdoors and laboratory conditions. There was no difference between oxygen evolution rates at 20,000 lux (270 μ mol m^{-2} s^{-1} PFD) and 65,000 lux (870 μ mol m^{-2} s^{-1} PFD). However, oxygen evolution was lower with the outdoor trials. This was probably due to atmospheric oxygen losses since the outdoor containers were open to the atmosphere.

DISCUSSION

With both laboratory and outdoor trials, *Acanthophora* sp. and *Caularpa lentillifera* had the same photosynthetic (P_{max}) light saturation intensities of 15,000-20,000 lux (200-270 μ mol photon m^2 s^{-1} PFD). This was similar to reported P_{max} values for other algae, such as brown algae *Feldmannia* spp. (Robledo *et al.*, 1994) and *Laminaria abyssalis* (Rodrigues *et al.*, 1993). Normally, P_{max} values correlate with an algae's preferred habitat. For example, P_{max} for intertidal species are in the range of 400-600 μ mol photon m^{-2} s^{-1}, while upper and mid-sublittoral species saturate at 150-250 μ mol photon m^{-2} s^{-1} (Lobban & Harrison, 1994). In Thailand, *Caulerpa* spp. is usually found attached to rocks or sand in shallow water, below low-tide level, close to coral reef (Lewmanomont and Ogawa, 1995). Light saturation of 200 μ mol photon m^2 s^{-1} PFD is expected. However, *Acanthophora* spp., especially on the Eastern Coast of the Gulf of Thailand, is normally found in the intertidal zone, 30-50 m from shore (Supowkit *el al.*, 1991). In our trials, *Acanthophora* sp. exhibited lower light saturation than other common, intertidal algae. One explanation for this is that *Acanthophora* used in our experiment were obtained from shrimp wastewater treatment ponds. Algae in these ponds were submerged at all times. Einav *et al.* (1995) found that *Acanthophora najadiformis* had much higher photosynthetic rates in air (during desiccation) than in water. This is an advantage for species growing in the mid-intertidal zone, since they are often exposed to air during low tides. In our case, *Caulerpa* may have adapted to lower light intensities while submerged in the ponds, and therefore had higher P_{max} values.

Very high light intensity can cause non-permanent damage to a plant's photosynthesis mechanism. This is called photoinhibition. Photoinhibition is the main factor affecting algal growth and metabolism when algae are exposed to strong light. With a blue-green alga (*Spirulina*), photoinhibition after midday caused a 30% decrease in growth (Richmond *et al.*, 1990). Normally, photoinhibition occurs with intertidal seaweed during desiccation stress during low tide (Herbert, 1990). In our trials, photoinhibition in *Caulerpa* was not clearly observed since our maximum light intensity was only 65,000 lux. However, photoinhibition can occur in seaweeds. For example, photoinhibition in Mediterranean species of *Caulerpa* (*C. prolifera*) was observed in the field (Häder *et al.*, 1997), and with a brown alga (*Fucus serratus*) when submerge (Huppertz *et al.*, 1990).

Algal photosynthesis and growth are highly correlated since energy and organic compounds used for cellular metabolism are all derived from photosynthesis. Light intensities that maximize photosynthesis therefore maximize growth rate. This was shown with *Laminaria* gametophyte cell

culture in a photobioreactor, where specific growth rate remained unchanged at light intensities higher than 100 μ mol photon m^{-2} s^{-1} (Qi & Rorrer, 1995). Normally, with optimum photosynthesis and maximum growth rate, maximum nutrient uptake occurs. Nutrient uptake in algae is an active process involving transport proteins and specific enzymes, such as nitrate reductase and nitrite reductase. Nitrate transport and assimilation processes require energy. Oxygen evolution by blue-green alga (*Phormidium laminosum*) declined when nitrate starvation occurred. When nitrate was later be added, algal photosynthesis increased in response to nitrate uptake (Ochoa de Alda *et al.*, 1996). Energy requirements for nutrient uptake also depend on nutrient types. Ammonium-nitrogen uptake occurs at lower light intensity (requires less energy) than nitrate-nitrogen uptake, because nitrate needs eight additional reductant molecules to reduce nitrate (NO_3^-) to ammonium (NH_4^+) during assimilation. This was confirmed by Lomas *et al.* (1996).

In summary, optimum light intensity for growing *Acanthophora* and *Caulerpa* is 15,000 to 20,000 lux. Direct outdoor light is not necessary for photosynthesis and can cause lethal water temperatures. Temperatures greater than 40°C sometimes occur in the treatment ponds and cause massive algal deaths. Plastic shade netting (30-40%) over algal wastewater treatment ponds could solve this problem. With indoor aquariums, 4 to 5 white daylight fluorescence lamps are enough to provide maximum photosynthesis efficiency for both *Caulerpa lentillifera* and *Acanthophora* sp.

ACKNOWEDGEMENTS

The authors thank Thailand Research Fund (TRF) for financial support to Prof. Piamsak Menasveta as part of the Sustainable Shrimp Culture Project. We also thank Mr. Bunchong Nisapawanit (Bunchong Farm) and Dr. Somkiat Piyateratitiworakul for supplying algae for the experiments, and Dr. Arlo W. Fast for editing assistance.

LITERATURE CITED

Chaiyakam, K. & Tunvilai, D. (1992). Experiments on green mussel, *Mytilus* sp. and seaweed, *Gracilaria* sp. for biological wastewater discharged treatment from intensive shrimp ponds. *In Proceedings of the 1992 Annual Seminar, Department of Fisheries, Royal Thai Government*, pp. 413-424. (in Thai)

Einav, R., Breckle, S. & Beer, S. (1995) Ecophysiological adaptation strategies of some intertidal marine macrophytes of the Israeli Mediterranean coast. *Marine Ecology Progress Series* 125, 219-228.

Häder, D-P., Porst, M., Herrmann, H., Schäfer, J. & Santas, R. (1997) Photosynthesis of the mediterranean green alga *Caulerpa prolifera* measured in the field under solar irradiation. *Journal of Photochemistry and Photobiology B: Biology* 37, 66-73.

Herbert, S.K. (1990). Photoinhibition resistance in the red alga *Porphyra perforata*. The role of photoinhibition repair. *Plant Physiology* 92, 514-519.

Huppertz, K., Hanelt, D. & Nultsch, W. (1990). Photoinhibition of photosynthesis in the marine brown alga *Fucus serratus* as studied in field experiments. *Marine Ecology Progress Series* 66, 175-182.

Lewmanomont, K. & Ogawa, H. (1995). *Common seaweeds and seagrasses of Thailand*. Faculty of Fisheries, Kasetsart University, Thailand, 164 p.

Lobban, C.S. & Harrison, P.J. (1994). Light and photosynthesis. In Lobban, C.S. & Harrison (eds) *Seaweed ecology and physiology*. Cambridge University Press, p. 137.

Lomas, M.W., Glibert, P.M., Berg, G.M. & Burford, M. (1996). Characterization of nitrogen uptake by natural populations of *Aureococcus anophagefferens* (Chrysophyceae) as a function of incubation duration, substrate concentration, light, and temperature. *Journal of Phycology* 32, 907-916.

Ocha de Alda, J.A.G., Tapia, M.I., Llama, M.J. & Serra, J.L. (1996) Bioenergetic processes are modified during nitrogen starvation and recovery in *Phormidium laminosum* (Cyanophyceae). *Journal of Phycology* 32, 258-265.

Qi, H. & Rorrer, G.L. (1995) Photolithotrophic cultivation of *Laminaria saccharina* Gametophyte cells in a stirred-tank bioreactor. *Biotechnology and Bioengineering*, 45, 251-260.

Richmond, A., Lichtenberg, E., Stahl, B. & Vonshak, A. (1990). Quantitative assesment of the major limitations on productivity of *Spirulina platensis* in open raceways. *Journal of Applied Phycology* 2, 195-206.

Robledo, D.R., Sosa, P.A., Garcia-Reina, G. & Müller, D.G. (1994). Photosynthetic performance of healthy and virus-infected *Feldmannia irregularis* and *F. simplex* (Phaeophyceae). *European Journal of Phycology* 29, 247-251.

Rodrigues, M.A., Yoneshigue-Valentin, Y. & Dos Santos, C.P. (1993). A comparative analysis of wild and laboratory grown *Laminaria abyssalis* (Phaeophyta) using modulated fluorescence. *Hydrobiologia* 260/261, 463-469.

Supowkit, N., Hormchong, T. & Pedyod, P. (1991). Distribution of the red algae on the Eastern Coast of the Gulf of Thailand. Research Report no. 45/2534, Institute of Marine Science, Burapha University, Thailand, 34 p. (in Thai)

Tunsutapanich, A., Sanggontangit, T., Permngarm, T. & Omanee, C. (1998). Study on elimination of pollutants in closed culture system of Giant Tiger Shrimp (*Penaeus monodon*). *Thai Fisheries Gazette* 51, 203-209. (in Thai)

OZONE WATER TREATMENT IN MARINE SHRIMP CULTURE

Oraporn Meunpol[1], Kanyajit Lopinyosiri[2] and Piamsak Menasaveta[3]

[1] Marine Biotechnology Research Units, National Center for Genetic Engineering and Biotechnology, Bangkok

[2] Interdepartment of Environmental Science, Faculty of Science, Chulalongkorn University, Bangkok.

[3] Aquatic Resources Research Institute, Chulalongkorn University, Bangkok.

Abstract

Impacts of ozone on bacterial growth, shrimp (*Penaeus monodon*) postlarvae (PL) survival, and water quality were investigated in laboratory systems. Maximum safe ozone concentration (when continuously administered) for shrimp PL was 97.49 mg O_3/l soluble ozone (0.42 mg O_3/l residual ozone) produced from a 100 mg O_3/l/hr Ozoniser, and 154.27 mg O_3/l soluble ozone (0.28 mg O_3/l residual ozone) produced from a 2 g O_3/l/hr Ozone Generator. There were no negative effects on PL respiratory rate, but histological gill damage occurred after 8-hrs exposure (2 g O_3/l/hr, Ozone Generator). At 25.6 mg O_3/l soluble ozone (0.34 residual ozone), 3 log units of *Vibrio harveyi* D331 were inactivated, however, most of the bacteria recovered within 9 hrs. Greater bacterial reductions (24-hr bacteria inactivation) were achieved at 128 mg O_3/l soluble ozone (2.5 mg O_3/l residual ozone). Ozone inhibition of *Bacillus* growth was slightly less. Higher ozone concentration (420-mg O_3/l soluble ozone or 0.09 mg O_3/l residual ozone) with shrimp pond wastewater improved water quality better than aeration, although the differences were not statistically different. Ozone can be used at high concentrations to successfully eliminate bacteria and improve water quality prior to stocking shrimp.

Introduction

Disease is the major cause of cultured shrimp losses in Thailand. Efforts have been made to control or lessen this problem, including use of recirculation closed-water systems to prevent disease spread, and use of disinfectants to eliminate pathogens. Well-known

disinfectants like formalin or chlorine can suppress disease outbreak (Majumdar and Sproul, 1974; Rosenthal, 1980), but their residuals create potential environmental problem (Rosenthal, 1980; Matsumura et al., 1998; Strong et al, nd). Ozone has been used routinely for water treatment for human consumption (Tate, 1991). Comparing the effects of chlorination and ozonation on *E. coli*, ozone destroyed this bacteria 600-3000 more quickly (Majumdar and Sproul, 1974). Ozone possess strong oxidising properties, but it is unstable (Rosenthal, 1980). Ozone's strong oxidising capacity destroys organic and inorganic compounds in water (Rosenthal, 1980). Ozone is therefore considered a good candidate for shrimp culture to solve disease problems, and at the same time improve water quality (Rosenthal, 1980; Menasveta, 1980; Honn and Chavin, 1976; Colberg and Lingg, 1978; Matsumura et al, 1998). Despite ozone's potentials, few shrimp culturists embrace its use (Matsumura et al, 1998), perhaps because practical details are often lacking, and because of inconsistent results from prior investigations.

The aims of our study included measurements of; total ozone production, ozone toxicity to shrimp, effects of ozone on water quality, and ozone's bactericidal properties. The results of our experiments will be used to design effective dosages and appropriate applications for killing pathogens, reducing harmful water quality conditions; while at the same time not injuring shrimp. The possibility of using ozone in growout ponds is also discussed.

Material and Methods

Experiment 1. Ozone measurement

We tested two ozone generator models: Ozoniser (100 mg O_3/l/hr), and Ozone Generator (2 g O_3/l/hr). Their respective ozone concentrations when ozone outputs were injected into seawater was measured. This information was used for subsequent experiments.

Ozone gas produced from either the Ozoniser (100 mg O_3/l/hr) or the Ozone Generator (model OZ 3050, EBASE CORP, Ltd.; 2 g O_3/l/hr) was trapped in 200 ml of 20% potassium iodide solution for 1, 5, 10, 20, 30, 40, 50 and 60 min. The potassium bi-iodate compound was titrated against 0.005 N sodium thiosulfate until end point. The data were analyzed using regression analysis.

Experiment 2. Ozone toxicity to shrimp postlarvae

2.1 Effect of residual ozone on shrimp postlarvae

Two thousand *Penaeus monodon* postlarvae (PL_{15}) from Chachoengsao Province were stocked and acclimatised in 6 ppt seawater for one week prior to the trials. PL were fed twice daily with postlarval feed pellets. Water was changed as needed.

Five litres of seawater (6 ppt) were ozonated respectively with the Ozoniser (100-mg O_3/l/hr) and the Ozone Generator (2-g O_3/l/hr) for 1, 5, 10, 30 min and 1 hr. Fifty PL were placed in each container. During the experiments, PL were fed live Artemia nauplii. Container water was static with no air added. Water temperature was 25°C, and pH was 7.4-7.6. Observations were made every 2 hrs for 24 hrs. Death was assumed when PL were immobile and showed no response when touched with a glass rod. Dead PL were removed immediately to prevent water pollution.

2.2 Effects of continuous ozone exposure on shrimp postlarvae

A 22-l glass container was divided into the sections (represented three replications), with each receiving 100 shrimp PL. Each ozonator was operated separately at maximum capacity (100 mg O_3/hr or 2 g O_3/l/hr) for 24 hr. Shrimp survival and ozone concentrations were checked every 2 hrs. Residual ozone concentrations were measured using a Spectroquant test kit (Merck).

2.3 Physiological aspects of ozone toxicity to shrimp postlarvae

Shrimp PL (PL_{15}-PL_{20}) were exposed to direct ozonation for 4, 6, 8, 10, 12, 14 and 16 hrs. At designated times, each PL was transferred into a 5 ml respiratory chamber with 25 ppt sterilised seawater, which was already acclimatised at 25°C for 1 hr. Shrimp were allowed to acclimate in these chambers for one hr, after which oxygen consumption rates were recorded every 10-min for 1 hr. Upon termination of the trials, PL were fixed in Davidson's fixative, transferred into wax, and 5-μm sections were cut and stained with haematoxylin and eosin. Histology examination of shrimp gill tissue, at different ozone exposure times was conducted using light microscopy at Srinakarintaravirot University.

Experiment 3. Bacteria inhibition by ozone

3.1 Inactivation of *Vibrio harveyi* D331

Seawater with 25-ppt salinity was autoclaved, and each separate 500-ml container of seawater was exposed to ozone for 0, 1, 5, 10, and 20 min at standard output (2 g/h/l Ozone Generator). Ten ml of *Vibrio harveyi* D331 culture at approximately 10^6 CFU/ml were immediately placed in the ozonated seawater to achieve a final concentration of approximately 10^5 CFU/ml, then exposed for 0, 30 sec, 15 min, 30 min and 1, 2, 4, 6,9 12, 24, 36 and 48 hr. Samples were withdrawn and placed into sterile 10 ml test tubes containing 0.1 ml 10% $Na_2S_2O_3$ to neutralize any remaining ozone and to stop bactericidal action of residual oxidant during sample transit (APHA, 1985)

Seawater samples were serially diluted with 0.85% NaCl and inoculated to obtain CFU for *V. harveyi*. The spread plate method on TCBS was used for viable bacteria count prior to, and during exposure to the disinfectants. Numbers of *V. harveyi* were enumerated using two tubes.

3.2 The inactivation of *Bacillus* S11

Bacillus S11 were cultured in BHI media at 37°C for 18 hrs, after which about 10^5 CFU/ml were inoculated into 500 ml of 25 ppt sterilized seawater and exposed to ozone for 0, 1, 5, 10, and 20 min at a standard output (2 g/h/l Ozone Generator). *Bacillus* was sampled at 0, 0.5, 15, and 30 min, and at 1, 2, 4, 6, 9, 12, 24, 36 and 48 hrs in the same manner as with *Vibrio*.

Experiment 4. Water quality treatment by ozone

Shrimp pond waste waters were collected and analysed for total ammonia-nitrogen, nitrate-nitrogen, nitrite-nitrogen, BOC, total suspended solids, total-phosphate and alkalinity (APHA, 1985). A 1-l water sample was ozonated for 1-hr while the control was aerated with compressed air. All parameters were measured after the designated time, except BOD, which was analysed after five days incubation.

Results

Experiment 1. Ozone production

Ozone concentrations in water increased linearly with increased ozonation time for each ozonator; with [y = 2.2341x] for the Ozoniser (100 mg O_3/l/hr; Fig. 1), and [y = 7.0707x] for the Ozone Generator (2g O_3/l/hr; Fig. 2). With pure oxygen gas, ozone production from the Ozone Generator increased greatly [y = 25.616x] (Fig. 3). Not all of the ozone gas produced by the ozonation machines dissolved in water. Ozone which did dissolve in water dissipated quickly, and after 24 hrs residual ozone was almost non-detectable.

Experiment 2. Toxicity of ozone to shrimp

2.1 Effect of residual ozone on shrimp postlarvae

No shrimp mortality occurred with residual ozone concentrations of 26.8 mg total soluble O_3/l (0.45mg/l residual ozone, from 100 mg O_3/l/hr running for one hr); nor with 84.8 mg total soluble O_3/l (0.06 mg/l residual ozone, from 2 g O3/hr/l running for one hr), compared with controls. Shrimp losses were mostly from cannibalism.

2.2 Effect of continuous ozone exposure on shrimp postlarvae

With the Ozoniser (100 mg/h/l) operating up to 12 hrs, all shrimp showed normal behaviour until 14 hrs (Table 1; total soluble ozone concentration = 97.49 mg O_3/l, 0.42 mg O_3/l residual ozone). Initial signs of shrimp weakness appeared from 14 hrs onward. Most shrimp PL were badly damaged after 24hrs exposure to direct ozonation (total soluble ozone concentration = 146.2 mg O_3/l, 0.16 mg O_3/l residual ozone).

With the Ozone Generator (2 g/h/l), effect of continuous ozonation on shrimp PL were more prominent (Table 2). At 8 hrs, signs of shrimp weakness were evident (total soluble ozone concentration = 154.27 mg O_3/l, 0.28 mg O_3/l residual ozone). After that, 50% of the shrimp lost balance, became immobile, and were carried around by water currents. They exhibited occasional, feeble and spasmodic movements of pleopods and swimmerets. Eventually, some shrimp recovered their equilibrium, but remained lethargic.

We concluded that if direct ozone is used in the water system with shrimp, total ozone concentrations should not exceed 97.49 mg total soluble O_3/l.

2.3 Physiological aspects of ozone toxicity to shrimp postlarvae

There were no significantly differences in oxygen consumption rates ($p<0.05$) of shrimp exposed to ozonation (100 mg O_3/l/hr) from 4 to16 hrs (residual ozone between 0.12 to 2.00 mg O_3/l), compared with control. However, histology studies revealed increased gill degeneration of ozone treated shrimp with time, although swelling of gill lamellae were also observed in all treatments including controls (Fig. 4). Gills had increased hypertrophy at 10-hrs exposure. At advance stages, pycnotic nuclei of gill nucleus occurred. Finally, after 16 hrs of ozonation, the gills were severely deteriorated (Fig. 5).

Experiment 3. Bacteria inactivation

Figure 6 shows the logarithmic number of colony forming units (CFU) versus contact time for *Vibrio harveyi* D331 when microbial suspensions were treated with residual ozone produced from pure dry oxygen with the same flow rates. A 3-fold, log reduction within 60 sec occurred with all four treatments. The rate of CFU reduction was fastest during the first 60 sec, but then leveled off. This suggests reduced bactericidal activity after initial ozone exposure. Residual ozone might have been too low to damage bacterial cells. This was confirmed by a decline in residual ozone concentrations in the waters, which dropped from 0.34 to 0.1 mg O_3/l during 1 hr.

Comparing all treatments, 1 min ozonation (25.6 mg total soluble O_3/l; 0.34 mg residual O_3/l) was the least effective. Within 9 hrs, *Vibrio* recovered to its original values. Other treatments (5, 10, and 20 min ozonation) also strongly affected *Vibrio* concentrations. However, there were no significant difference between 5 min treatments (128 mg total soluble O_3/l; 2.5015 mg O_3/l residual ozone) and 10 or 20 min ozonation. Therefore, we included that 5 min ozonation was sufficient to inactivate 4 log units of the *Vibrio* compared to control after 24 hrs. It is worth noting that although ozone exposed *Vibrio* recovered after being initially inhibited, their colony sizes were reduced.

In a separate study, where the source of oxygen changed from pure oxygen gas to compressed air, ozone production capacity from the same machine was reduced greatly. The Ozone Generator coupled with pure oxygen gas gave 25.6 mg total soluble O_3/l/min,

compared with only 7.0 mg total soluble O_3/l/min with compressed air. It was inconsistent that 2 min ozonation (4.47 mg O_3/l total soluble ozone; 0.14 mg O_3/l residual ozone) was more effective in *Vibrio* inactivation than 5 (11.17 ppm) and 10 min ozonation (22.3 ppm; Fig. 7). A 1.75 log units reduction was achieved from 2 min ozonation. *Vibrio* recovered faster than in the first experiment. CFU values returned to the initial values within 6 hrs. Oxygen source was the main factor effecting ozone concentrations.

Bacillus tolerated ozone slightly better than *Vibrio*. A maximum of 2.2 log units reduction was achieved at 30 sec with 25.6 mg O_3/l (Fig. 8), while a 3-log unit reduction occurred with *Vibrio* at the same ozone concentration. *Bacillus* also recovered after first inactivation, but at a slower rate than *Vibrio*. This may be due to a slower growth rate of *Bacillus* compared to *Vibrio*. However, the effects of each treatment did not differ greatly. A reduction in measured residual ozone concentration was observed in all four treatments during bacterial exposure. The reduction in ozone ranged from 0.26 to 0.04 mg/l in 24hr.

Experiment 4. Water quality

At an ozone concentration of 424.2 mg O_3/l (2 g O_3/hr/l running for 1 hr; residual ozone 0.09 ppm) NH_4-N was reduced 12%, NO_2-N increased 933%, and BOD was reduced 18% (Table 4). Aeration reduced NH_4-N by 6%, increased NO_2-N by 422%, and reduced BOD by 11%. Ozonation improved water quality more than aeration, although the data were not statistically different.

Discussion

Ozone (O_3) is produced from oxygen (O_2) with energy input either from an electric discharge or by ultraviolet radiation (Yanco Industries, 1999). Different commercial, ozone generation machines are available in the market place. Their ozone production capacities, as stated by the manufacturer is based on measurements of ozone gas generated by each machine, which is much less than ozone concentrations dissolved in water. Seawater contains various minerals and impurities, which can reduce ozone potency (Liltved et al., 1995). Effective ozone concentrations measured in one situation may not apply to another

situation because of differences in respective water qualities. Accordingly, actual ozone concentrations produced from an ozone generator must be measured during each trial.

From our results based on seawater of 25-ppt salinity, which is common for shrimp culture, resultant ozone in water was much less than might be expected. Ozone solubility depends on various factors such as the salinity, hardness, pH, temperature, source of oxygen, and the ozone application technique (Colberg and Lingg, 1978; Liltved et al., 1995; Wongchrinda, 1994). Ozone is lost from water by three main processes (Liltved et al., 1995); reaction with water impurities, decomposition to O_2, and ozone loss to the atmosphere. These losses are rapid and make ozone determinations in water problematic (Rosenthal, 1980). Residual ozone concentrations are not always related to ozoniser operating time, and therefore the term "residual ozone" is inappropriate. We therefore considered ozone dosage as the amount of O_3 added to water per unit time, which agrees with conventions used by other researchers (Liltved et al., 1995).

Although ozone has widely proven benefits for potable and waste water treatments (Majumdar and Spoul, 1974; Colberg and Lingg, 1978; Rosenthal, 1980), caution is necessary with aquaculture applications due to its potential harmful effects on cultured animals. Ozone toxicity in seawater is due in part to hypobromous acid formed when ozone gas combines with bromine, similar to its reaction with chlorine in seawater (Blogoslawski et al., 1976). The oxidation residual of ozone gradually decreases with time (Majumdar and Sproul, 1974). Ozone concentrations for aquaculture applications should be sufficient to inhibit pathogens of concern, and/or otherwise improving water quality with minimum risk to animal safety. Optimum ozone dosages vary according to species and age of animals. For example, concentrations of more than 0.5 mg/l residual ozone can damage oyster larvae (Maclean et al., 1973; Blogoslawski et al., 1978). With fish, lethal thresholds are lower than those for crustaceans. The 96-hr LC50, using 10-13 cm rainbow trout (*Salmo gairdneri*) was 9.3 µg/l residual ozone. Ozone causes massive destruction of the gill lamellae epithelium together with severe hydro-mineral imbalance in juvenile rainbow trout. At lower ozone concentrations, but longer exposure times (2 µg O_3/l, 96hr), hyperplasia of the lamella epithelium was noticed (Wedemeyer et al., 1979).

Our findings suggest that shrimp PL had higher tolerance to residual ozone exposure than oyster larvae or rainbow trout. Shrimp PL were able to live normally in direct ozonation with ozone concentrations of up to 97.49 mg total soluble O_3/l (0.42 mg/l residual ozone).

Physiological disturbance of animals can be evaluated by calculating their oxygen consumption rate. Often, detrimental treatments cause increased oxygen consumption, as determined by a Gilson differential respirometer (Cebrian et al., 1990). This method is also capable of verifying metabolic rate changes of eggs, such as with egg pore respiration of *Callosobruchus maculatus* (Daniel and Smith, 1994). With our experiments, ozone had no effect on oxygen consumption of *P. monodon* PL at the maximum total soluble ozone concentration of 154.27 mg/l.

We observed that at 25.2 mg/l total soluble ozone (0.34 mg/l residual ozone), 3 log units of *Vibrio harveyi* D331 were suppressed for 9 hrs, and 128 mg/l of total soluble ozone (2.5 mg/l residual ozone) inhibited the same amount of *Vibrio* for longer time (24 hrs). Therefore, for disinfecting purposes, ozone at 128 ppm should be used in order to achieve greater disinfecting power. With shrimp present, however, lower ozone concentrations (i.e. 97.49 mg total soluble O_3/l) should be used. Bacteria inactivation can be achieved with both proper time of contact and ozone concentration (Majumdar and Sproul, 1974). Ozone can inactivate both *Vibrio* and *Bacillus,* although at slightly different rates. Therefore, it is important to apply ozone well before stocking shrimp, or adding probiotics.

Biocide action of ozone is the result of membrane component disruption leading to loss of their barrier function (Trukhacheva et al., 1993). Bactericidal effects of ozone after an appropriate dosage is reached are sudden and total, and no further inactivation is achieved (Yang and Chen, 1979). This might be because of a reduction of residual ozone (Yang and Chen, 1979). which occurs from oxidation of the most reactive organic groups on the cells' surfaces, and on decreased O_3 absorbed after ozone interacts with these organic compounds (Trukhacheva et al., 1993).

Sodium chloride concentrations affect microorganism destruction by ozone. At lower salinity (NaCl below 2.5%), the bactericidal effect of ozone was enhanced, while 5% NaCl

showed a slight protective effect (Yang and Chen, 1978). These results were similar to Wongchrinda's (1994) studies, which showed higher solubility of ozone in lower salinity. However, Liltved et al. (1995) stated that salinity differences did not cause any substantial differences in bactericidal activity of ozone.

A number of researchers have reported on the efficacy of ozone for water treatment. For example, ozone can reduce total suspended solids (Rueter and Johnson, 1995), reduce colour (Otte et al., 1977), reduce odor ((Millamena, 1992), improve nitrification in hatcheries (Colberg and Lingg, 1978; Menasveta, 1980), and improve water quality in growout ponds (Mutsumura et al., 1998; Honn and Chavin, 1976). However, ozone's effectiveness depends on various factors, including raw water hardness, initial suspended solids concentration (Rueter and Johnson, 1995), temperature, and pH (Colberg and Lingg, 1978)

Millamena (1992) reported that low ozone concentrations (0.11 g/hr) did not effectively remove most organic matter in slaughterhouse wastewater. Higher ozone concentrations were recommended. This agrees with our findings where ozone concentration of 424.2 mg total soluble O_3/l resulted in only a 12% reduction in total ammonia-nitrogen. Nevertheless, when compared to controls (air only), water quality was improved more with ozone. In particular, ozonation enhanced conversion of ammonia-nitrogen to nitrite-nitrogen, although the final step of nitrification to NO_3 was not observed. Millamena (1992) explained that the highly polluted slaughterhouse waste prevented complete oxidation of organic matter by ozone. However, with wastewater pretreatment, overall ozonation performances was improved with 42% reduction in BOD, total suspended solids reduction of 34%, and reduced COD by 57.5%. Majumdar and Sproul (1974) noted a similar range of water quality improvements when a high residual ozone concentration (2.17 mg/l) was used with secondary wastewater (34% reduction of total suspended solids and 54% reduction of COD).

In conclusion, we found that with shrimp PL culture, ozone is most applicable for disinfecting purposes, especially in hatchery, recirculating water systems (Menasveta, 1980; Colberg and Lingg, 1978; Rueter and Johnson, 1995). When PL are present, ozonation should be combined with carbon filtration to remove offending substances, or to ensure their dissipation (Colberg and Lingg, 1978). Ozone can be routinely used to disinfect water

supplies and hatchery effluents to prevent spread of diseases, and to reduce virus or bacteria infection of fertilized eggs (Arimoto et al., 1996). Moreover, ozone application in shrimp growout ponds has not been ruled out (Matsumura et al., 1998; Strong et al., nd). Ozone generator capability of 15 kg/hr is recommended with a water recirculation system on a 550 ha farm to prevent outbreaks of infectious disease, blooms of blue-green algae, to reduce inorganic nutrient concentrations, and to improve effluent water quality (Strong et al., nd.).

Acknowledgement

This research was funded by the Thailand Research Fund (TRF) through Prof. Dr. Piamsak Menasveta. Sincere thank to Dr. Arlo W. Fast for revising the manuscript; the Thai Union Aqua Products (Thailand) Co. LTD. for the use of Ozone generator OZ-5405; Assoc. Dr. Sirirut Rengpipat for *Vibrio harveyi* D331, *Bacillus* S11 stock and technical suggestions; Assoc. Dr. Paisarn Sitthigornkul for histology facilities; Dr.Pikul Jirawanichpaisarn for shrimp histology analysis.

Literature Cited

American Public Health Association (APHRA). 1985. Standard Methods for the examination of water and wastewater. 16th ed .Washington, DC.: APHRA/AWW/WPCF; 426-429,856.

Arimoto, M., J. Sato, K. Maruyama and G. Mimura. 1996. Effect of chemical and physical treatments on the inactivation of striped jack nervous necrosis virus (SJNNV). Aquaculture 143: 15-22.

Austin, B. 1983. Effectiveness of ozone for disinfection of laboratory effluent. Fed. Eur. Microbiol. Soc. 19: 211-214. Cited after Liltved, H., H. Hektoen and H. Efraimsen. 1995. Inactivation of bacterial and viral fish pathogens by ozonation or irradiation in water of different salinity. Aquacutural Engineering 14: 107-122.

Blogolawski, W.J. and M.E. Stewart, 1977. Marine application of ozone water treatment. In Fochtman, E.G., R.G. Rice and M.R. Browning (eds) 1977. Forum on ozone disinfection. International Ozone Institute, Syracuse, New York. Pp 266-276.

Blogoslawski, W.,L., Farwell, R. Garceau and P. Derrig. 1976. Production of oxidants in ozonized seawater. Proc. Second. Inter. Symp. Ozone Tech. Montreal, Canada. May 11-14, 1975. Pp 671-681.

Blogoslawski, W.J., M.E. Stewart and E.W. Rhodes. 1978. Bacterial Disinfection in Shellfish Hatchery Disease Control. Proceeding of Ninth Annual Meeting World Mariculture Society, Atlanta, Georgia, Jan 3-6, 1978. Pp 589-602.

Cebrian, C., E. Andreu-Moliner, A.F. Casalderry and M.D. Ferrando. 1990. Changes of oxygen consumption on *Procambarus clarkii* exposed to methidathion: effect on isolated gills. J. Environ. Sci. Healthr. Part B B25(6): 767-775. Abstract

Chen, H.C., S.H. Huang, M.W. Moody and S.T. Jiang. 1992. Bacteriocidal and mutagenic effects of ozone on shrimp (*Penaeus monodon*) meat. J. Food Sci. 57(4): 923-927.

Daniel, S.H. and R.H. Smith. 1994. Functional anatomy of the egg pore in *Callosobruchus maculatus*: a trade-off between gas-exchange and protective functions?. Physiol. Entomol. 19(1):30-38. abstract

Honn, K.V. and W. Chavin. 1976. Utility of ozone treatment in the maintenance of water quality in closed marine system. Mar. Biol. 34: 210-219.

Liltved, H., H. Hektoen and H. Efraimsen. 1995. Inactivation of bacterial and viral fish pathogens by ozonation or irradiation in water of different salinity. Aquacultural Engineering 14: 107-122.

Maclean, S.A., A.C. Longwell and W. J. Blogoslawski. 1973. Effects of ozone- treated seawater, fertilized, meiotic, and cleaving eggs of the commercial American oyster. Mutation research. 21: 283-285.

Majumdar, S.B. and O.J. Sproul. 1974. Technical and economical aspects of wastewater and wastewater ozonation: a critical review. Water Research 8: 253-260.

Matsumura, M., V.P. Migo, D. Balobalo, H.K. Young and J.D. Albaladejo. 1998. Preservation of water quality in shrimp ponds by ozone. In Flegel, T.W. (ed) Advances in Shrimp Biotechnology. National Center for Genertic Engineering and Biotechnology, Bangkok. Pp 93-99.

Menasveta, P. 1980. Effect of ozone treatment on the survival of shrimp larvae reared in closed recirculating water systems. Thai Fish. Gaz. 33(6): 677-83.

Millamena, O.M. 1992. Ozone treatment of slaughter house and laboratory wastewater. Aquaculture Engineering 11: 23-31.

Rosenthal, HR. 1980. Ozonation and Sterilization. Symposium on New Developments in Utilization of Heated Effluents and of Recirculation Systems for Intensive Aquaculture. Eur. Inland Fish. Advs. Comm. FAO EIFAC/80/Symp.:R/8/May 1980. Pp 75.

Rueter, J. and R. Johnson. 1995. The use of ozone to improve solids removal during disinfection. Aquacultural Engineering 14: 123-141.

Strong, C., W.J. Blogolawski, C.E. Perez, M.L. Hunter and A.M. Schuur. nd. Achieving Sustainable High Yields in Shrimp Pond Culture Using Water Recirculating and Ozone Treatment. Handout.

Tate, C.H. 1991. Survey of ozone installations in North America. J. American Water Works 83(5): 40-47.

Trukhacheva, T.V., V.B. Gavrilov, G.A. Malama and V.A. Astakhov. 1993. Kinetic patterns of the death of microorganism under the action of ozone. Microbiology 61(4): 467-471.

Wedemeyer, G.A. and N.C. Nelson. 1977. Survival of two bacterial pathogens (*Aeromonas salmonicida* and the Enteric Redmouth Bacterium) in ozonated, chlorinated and untreated waters. J. Fish. Res. Board. Can. 34: 429-432.

Wedemeyer, G.A., N.C. Nelson and W.T. Yasutake. 1979. Physiology and biochemical aspects of ozone to rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 36(6): 605-614.

Wongchrinda, N. 1994. Ozone destruction of *Vibrio vulnificus*. Annual Report of Fishery Technological Development Institutes 1994, Fisheries Dept. Pp 168-177.

Yanco Industries LTD. 1999. Ozone Services; What is Ozone? hrttp://www.O3zone.com/ozoneser/articles/001hrtm

Yang, P.P.W. and T.C. Chen. 1979. Stability of ozone and its germicidal properties on poultry meat microorganisms in liquid phrase. J. Food Sci. 44(2): 501-504.

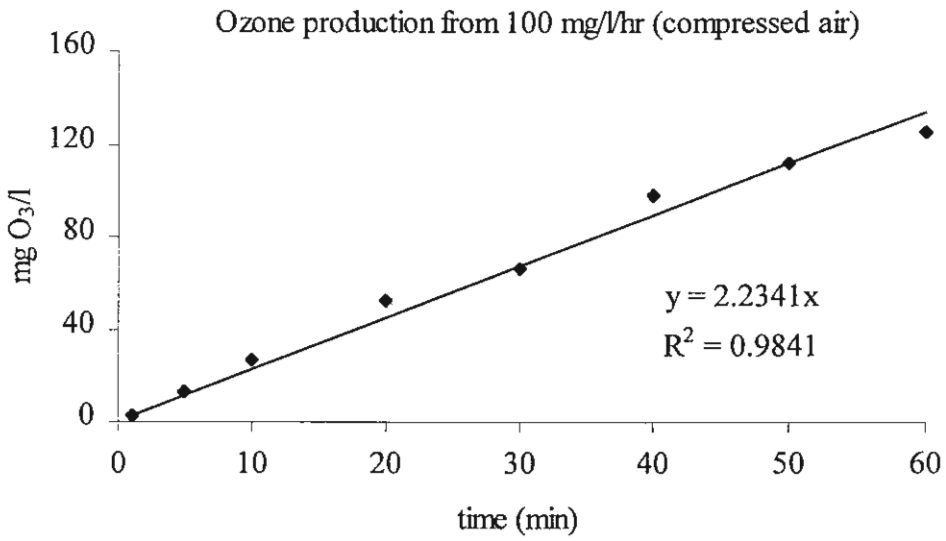


Figure 1 Regression of ozone concentrations in water produced from operating a 100 mg O_3/l/hr ozone generator up to one hour using compressed air.

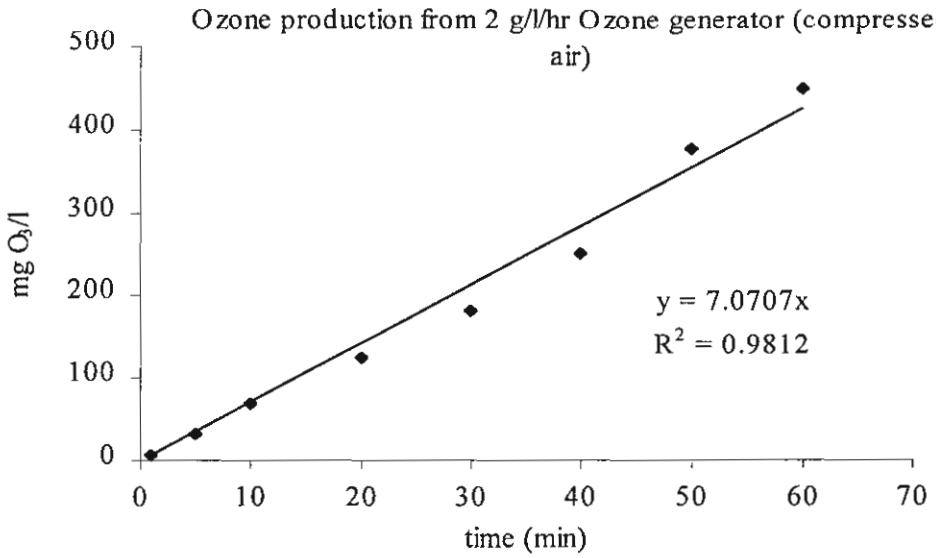


Figure 2.Regression of ozone concentrations in water produced from operating a 2 g O_3/l/hr ozone generator up to one hour using compressed air.

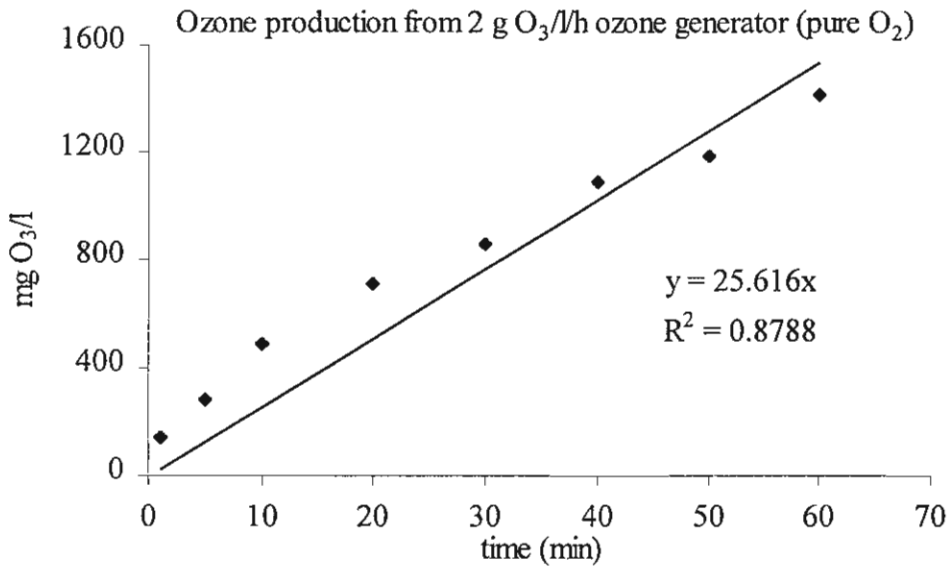


Figure 3. Regression of ozone concentrations in water produced from operating a 2 g O_3/l/hr ozone generator up to one hour using pure oxygen gas.

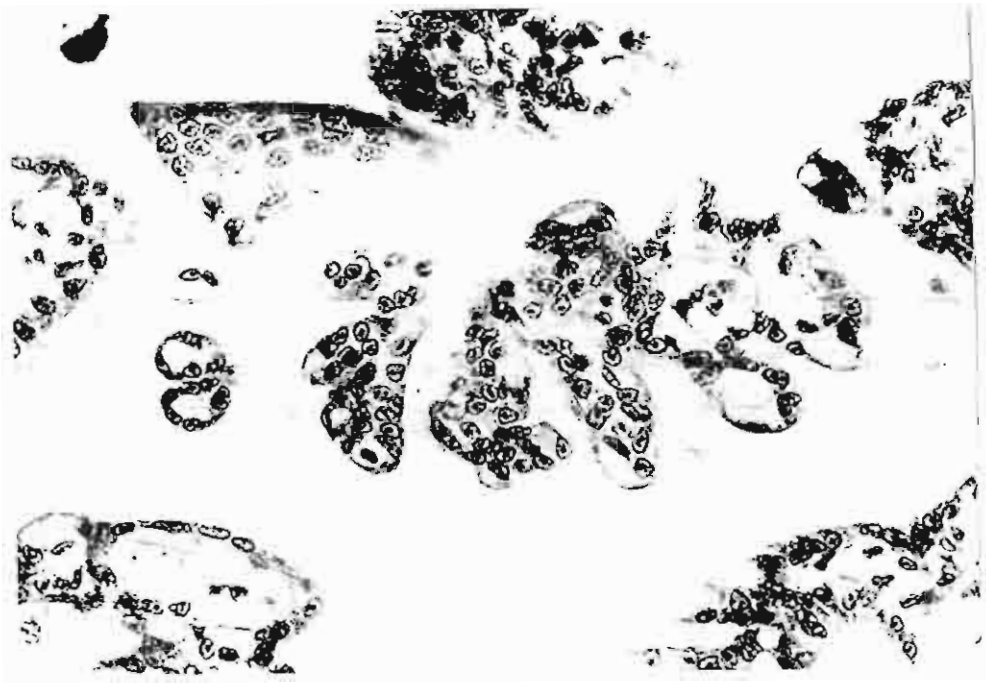


Figure 4. Gill histology of unexposed, control shrimp in ozone. Haematoxylin and eosin stain. Magnification at x1700.

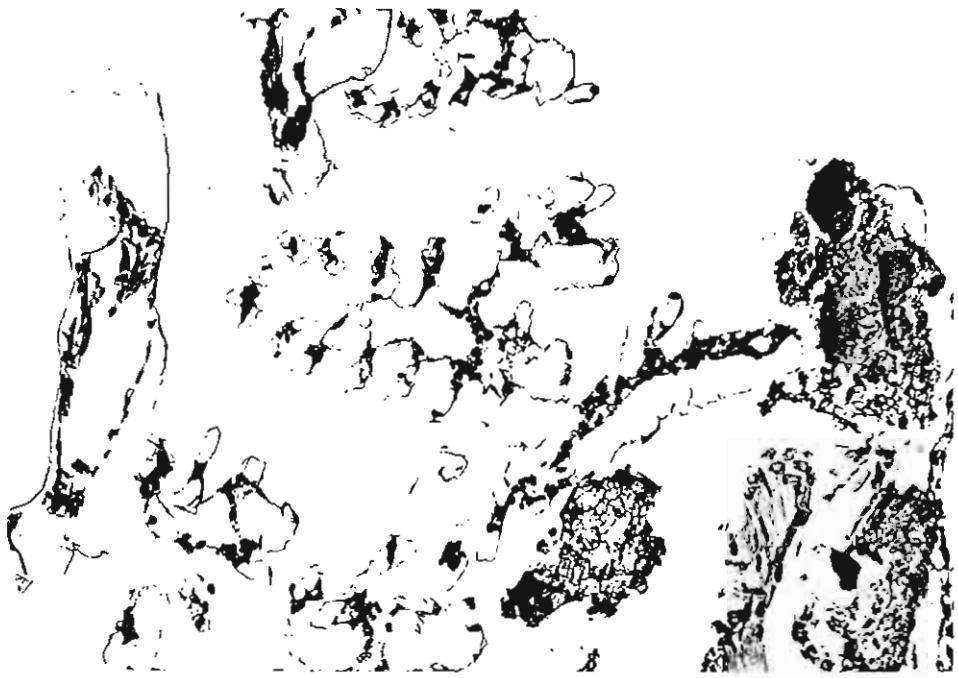


Figure 5. Histopathological changes of shrimp gills after 16 hrs exposure to ozone. Notice the pycnotic nucleoi and gill deterioration. Magnification at x1700.

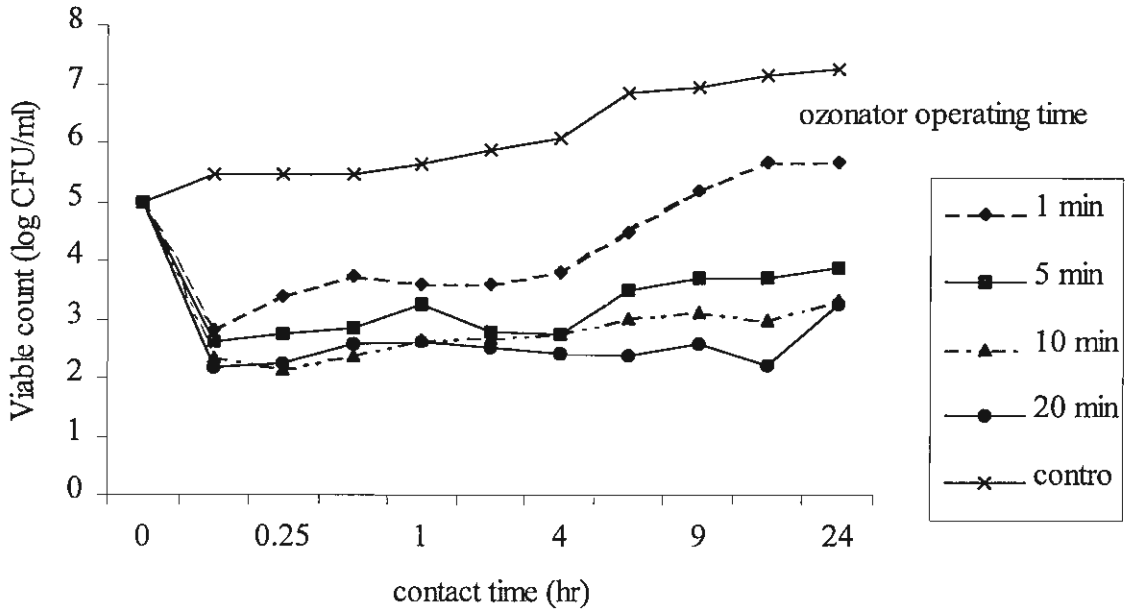


Figure 6. *Vibrio* activity after ozone exposure of up to 24 hours. Ozone was generated by using a 2 g O_3/l/hr Ozone Generator with pure oxygen gas. The Ozone Generator was operated from 0 to 20 min before contact time = 0.

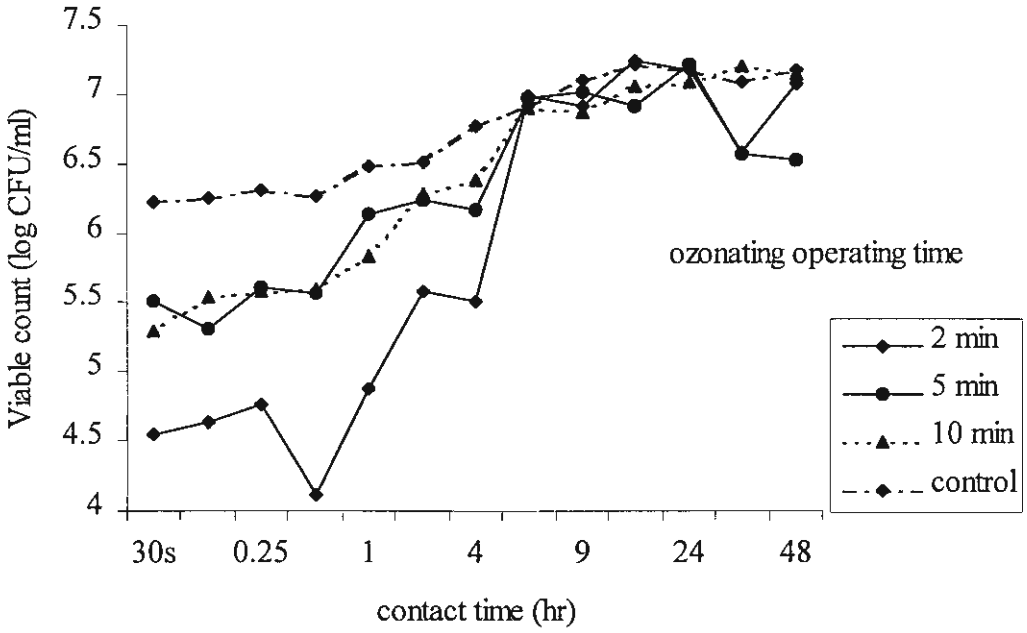


Figure 7 *Vibrio* activity after ozone exposure of up to 24 hours. Ozone was generated by using a 2 g O_3/l/hr Ozone Generator with compressed air. The Ozone Generator was operated from 0 to 20 min before contact time = 0.

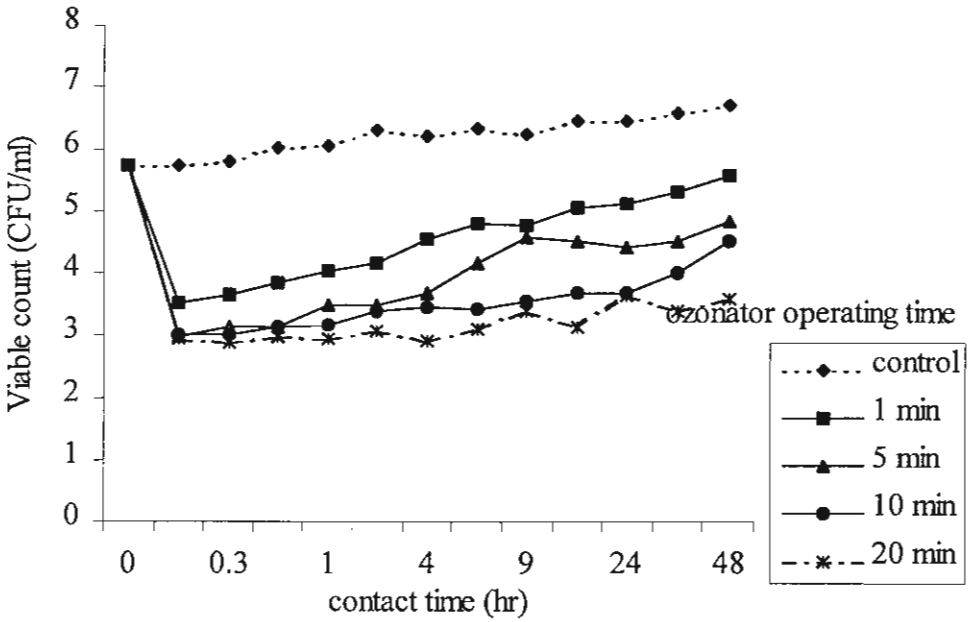


Figure 8 *Bacillus* S11 activity after ozone exposure of up to 24 hours. Ozone was generated by using a 2 g O_3/l/hr Ozone Generator with pure oxygen gas. The Ozone Generator was operated from 0 to 20 min before contact time = 0.

Table 1. Number of shrimp PL affected by ozonation from 100 mg O_3/l/hr Ozone Generator using compressed air. Number of dead PL observed, and total residual ozone (TRO) for different exposure times are shown.

TRO (ppm)	0.154	0.408	0.454	0.408	0.388	0.392	0.413	0.417	0.354	0.4	0.167	0.158
Rep	2 hr	4 hr	6 hr	8 hr	10 hr	12 hr	14 hr	16 hr	18 hr	20 hr	22 hr	24 hr
1	0	0	0	0	0	0	0	0	0	2	4	24
2	0	0	0	0	0	0	0	0	0	3	4	72
3	0	0	0	0	0	0	0	1	0	2	4	73

Table 2. Number of shrimp PL affected by ozonation from 2 g O_3/l/hr Ozone Generator using compressed air. Number of dead PL observed, and total residual ozone (TRO) for different exposure times are shown.

TRO (ppm)	0.173	0.382	0.556	0.278	0.382	0.573	0.382	0.486	0.434	0.503	0.503	0.313
Rep	2 hr	4 hr	6 hr	8 hr	10 hr	12 hr	14 hr	16 hr	18 hr	20 hr	22 hr	24 hr
1	0	0	0	1	26	11	6	1	6	-	-	-
2	0	0	0	1	20	5	4	4	11	1	2	-
3	0	0	0	1	24	0	5	1	6	2	3	-

Table 3. Total residual ozone (TRO) in respiratory rate trials with 2 g O_3/l/hr Ozone Generator using compressed air compressor.

Hours	TRO (ppm)
4	0.354
6	0.265
8	0.375
10	0.386
12	0.306

Table 4. Shrimp pond waste water treatment by ozonation (2 g/h/l running for one hour) compared to aeration. Data are average value of three trials.

	NH4	NO2	PO4	BOD
before	59.5	0.27	1.3	4.1
ozone	52.2	2.79	1.3	3.3
air	56.0	1.41	1.3	3.7
	%difference from control			
ozone	-12.2	+932	+1.2	-18.6
air	-5.86	+422	+5.6	-10.5

Table 5. Review of effective ozone dosages with different pathogens.

Pathogens	**TRO (ppm)**	**Time**	**Amount reduction**	**Reference**
V. harveyi	1	30 s	1.5 log units	Matsumura et al, 1998
V. Cholera	0.95	17 min	Total	Chen et al, 1992
V. parahaemolyticus	0.81	13	Total	Chen et al, 1992
V. vulnificus	1.0	2 min	Total	Wongchinda, 1994
	0.98	> 2 min	Total	Wongchinda, 1994
Bacteria	0.56		2-3 log units	
4 bacteria: *Aeromonas salmonicida* *V. anguillarum* *V. salmonicida* *Yersini ruckeri*	0.15-02	180 s	4 log units	Liltved et al, 1995
Pseudomonas *Flavobacterium* *Achomobacter*	0.56		2-3 log units	Blogoslawski et al, 1978
A salmonicida	1	1 min	Total	Colberg and Lingg, 1978
3 bacteria: *Aeromonas salmonicida* *Renibacterium salmoninarum* *V. anguillarum*	0.1	4 min		Austin, 1983 (cited after Liltved et al, 1995)
Marine bacteria Marine phytoplankton Arthropods Fish	0.56 0.08-1		Total	Blogolawski and Stewart, 1977
SJNNV	0.1 ug/ml	2.5 min		Arimoto et al, 1996
IPNV	0.1-0.2	60 s	Total	Liltved et al, 1995
IPNV IHNV	0.01-0.02 0.01	60 s 30 s	Total	Wedemeyer and Nelson, 1977
V. harveyi *Bacillus*	0.34 2.5 0.14 0.26	30 s	3 log for 9 hr 3 log for 24 hr 1.75 log for 6 hr 2.2 log for 2 .hr	This study

SOME RECENT INNOVATIONS IN MARINE SHRIMP POND CULTURE

Arlo W. Fast[1] and Piamsak Menasveta[2]

[1]Hawaii Institute of Marine Biology, University of Hawaii at Manoa, P.O. Box 1346, Kaneohe, Hawaii 96744, U.S.A.
<Email: arlo@hawaii.edu>

[2]Aquatic Resources Research Institute and Department of Marine Science, Chulalongkorn University, Bangkok 10330, Thailand
<Email: mpiamsak@chula.ac.th>

ABSTRACT

World cultured shrimp production increased from 0.4 million MT in 1990 to about 0.8 million MT in 1999, or about 25% of total shrimp supply. Increased production was well below 1.2 million MT predicted 10 years earlier. The primary reason for this shortfall was shrimp disease, which effected shrimp yields worldwide. The most serious diseases were viral, for which there are still few solutions. As a result of shrimp disease problems, pond culture practices changed to reduce disease incidence. These changes included: use of specific pathogen free (SPF) and specific pathogen resistant (SPR) shrimp seed; reduced or zero water exchange during pond growout; shrimp culture at inland locations away from coastal influences; use of water recycling and reuse growout systems; development of biosecure systems to prevent disease access during shrimp's entire culture cycle; development of probiotics to reduce disease susceptibility; and genetic selection and improvements through closed, life-cycle culture. In addition, environmental awareness and concerns about shrimp culture sustainability became increasingly important with the informed public during the 1990s. This included concerns about habitat degradation and destruction, reduced biodiversity, and exotic shrimp introductions. We review herein developments with these culture innovations and environmental issues that have occurred during the past 10 years.

Key words : marine shrimp aquaculture, ponds, genetics, probiotics, environmental protection, best management plan, re-circulation aquaculture

I. INTRODUCTION

Before abundant marine shrimp seed availability from hatcheries and from wild caught seed, shrimp pond growout techniques changed very little over the centuries. Shrimp were mostly cultured incidentally with fish and other crustaceans in large, extensive pond systems characterized by low productivity (Table 1; Fast 1991, 1992a). These polyculture systems were basically catch, hold and harvest systems with little or no energy (other than tidal), feed, or material inputs. Shrimp yields were at best perhaps a few hundred kg/ha/yr. Growout was low-tech by any standards.

After breakthroughs in large-scale, shrimp larviculture technologies during the 1970's, all aspects of shrimp culture underwent rapid and innovative changes. These changes were motivated by abundant shrimp seed available for pond culture, static supplies of ocean caught shrimp, and high profits from pond cultured shrimp. Abundant, low-cost seed availability resulted in numerous growout innovations and intensification. Pond yields with the more intensive systems increased to >10,000 kg/ha/yr during the mid-1980s.

These innovations in all aspects of marine shrimp culture led to dramatic and substantial increases in pond cultured shrimp production during the 1980s. During 1980, only 2% of the world's shrimp production came from ponds, but by 1989 pond cultured shrimp accounted for 26% of total world production (Rosenberry 1990). Marine shrimp harvest from the world's oceans increased only slightly from 1.3 million metric tons (MT) during 1975 to 1.9 million MT in 1985 (Csavas 1988). Since 1985, ocean harvest of shrimp has been relatively stable at about 2 million MT, and has most likely reached or exceeded maximum sustainable yield (National Research Council 1992). Exponential increases in cultured shrimp production between 1970 and 1990 led to speculations and projections that by the year 2000 perhaps 45% or more of the world shrimp harvest would be from ponds (Fast and Lester 1992; Fig. 1). Estimated total culture shrimp production by the year 2000 was almost 1.2 million (MT). However realistic these estimates seemed 10 years ago, they were soon proven inaccurate. A combination of disease and pollution, mostly the former led to a series of cultured shrimp industry collapses in leading shrimp culture countries, including Taiwan, China, Thailand and Ecuador. During 1997 and 1998, total culture shrimp production was 0.66 and 0.75 million MT respectively (Jory 1998, 1999; Table 2), up from 0.4 million MT in 1990,

but well below the 1.2 million MT forecast earlier. During 1997, cultured shrimp were about 25% of total world shrimp supplies (New 1999).

The single most important factor limiting continued expansion of the cultured shrimp industry during the 1990's was shrimp diseases (Lightner et al. 1997). While shrimp diseases have always been present, they became epidemic during large-scale, intensive shrimp monoculture. Viral diseases are the most devastating since they are often difficult to detect and impossible to treat in ponds (Brock et al. 1997). Although more than 20 known penaeid viruses, five viral species have caused greatest economic losses. These five are; yellow head virus (YHV), white spot syndrome virus (WSSV) and monodon baculovirus (MB) with black tiger shrimp (*Penaeus monodon*) in Asia, and infectious hypodermal and hematopoietic necrosis virus (IHHNV) and taura syndrome virus (TSV) with Mexican white shrimp (*P. vannamei*) and Pacific blue shrimp (*P. stylirostris*) in the Americas. In most cases, the only viable solution to viral diseases is to keep diseases from entering the culture system, and if infected, to clean up and disinfect.

Shrimp diseases can easily enter a shrimp pond by one or more means, including:

a. *Seed.* Both wild-caught and hatchery produced seed are primary sources of infection (Lightner et al. 1997). High density, intensive culture and stress amplify viral infections and can lead to infection of all shrimp within a short time (Lightner 1999). Hatchery produced seed are especially susceptible to infection since viruses can be introduced through infected broodstock, and cross contaminated within the hatchery. Seed from a given infected hatchery may be shipped to many farms with further spread of disease between farms.

b. *Source Waters.* Shrimp disease can be introduced to a farm through source water inflows, especially if these waters are from surface sources (Browdy and Bratvold 1998). Diseases may be carried in with source waters on organic and/or inorganic particles, in shrimp larvae or PL, or with other crustaceans. Diseases are often spread throughout a farm, and between farms with effluent and influent waters.

c. *Pond Intruders.* Pond intruders such as crabs and small shrimp species, or insects such as water boatmen are possible sources of viral disease introductions into shrimp ponds. Perhaps 40 or more crustacean species can harbor WSSV (Jory 1999a). Crabs come in through influent waters or by crawling over the dikes, while insects enter with water or by

flying. Small shrimp species such as *Acetes* sp. And *Palaemon styliferus* can easily enter the pond with surface, source waters (Flegel et al. 1995).

d. *Birds and Mammals.* Garza et al. (1997) have shown that viable Taura syndrome virus (TSV) can pass through the gastrointestinal tract of seagulls, thus providing a probable transport vector between ponds and between farms. In Thailand, shrimp farmers are often first aware of disease problems in a pond by the presence of large numbers of seagulls and other birds feeding on dead and dying shrimp. These gulls could easily fly to other ponds and spread disease. Although not verified, it is likely that mammals such as rodents or mongoose could also serve as similar vectors.

e. *Feeds.* Fresh feeds probably pose a potentially greater threat than prepared, pelletized feeds. Fresh feed, especially frozen or fresh "trash fish", bi-catch could contain or be contaminated by shrimp. Ogle and Lotz (1998) caution that, "Feed that is devoid of shrimp meal will be less likely to carry contamination than feeds that employ shrimp meal." The dangers of shrimp viral contamination from pelleted feeds has not been confirmed, and Chamberlain (1998) doubts that virus would survive shrimp feed manufacturing processes.

f. *People and Equipment.* People and equipment, especially those that move between farms and/or between farms and shrimp processing plants could spread disease.

g. *Frozen and Fresh Shrimp.* Imported, frozen shrimp can contain shrimp viruses (Nunam et al., unpublished). If these are used as bait by sport fisher-persons or others, exotic shrimp diseases could be introduced locally. Food processors that import contaminated, raw shrimp and then reprocess the shrimp are another source of disease transmittal (Jory 1999a). Likewise, infected live shrimp used as bait can spread diseases.

All of the above sources undoubtedly contributed to disease problems, magnified by widespread distributions of broodstock and seed, and by the tendency to site farms in clusters. Clustered farms often recycle each other's waste effluents, including disease organisms.

Shrimp disease problems are far from over. Many of the most recent innovations in shrimp pond culture, and many of those innovations still in progress are direct responses to this threat. We will attempt to draw this to your attention during our review as we discuss different pond culture innovations.

In our present paper, we will describe some innovations that mostly occurred during the past 10 years. Fast (1991, 1992b,c,d) described many innovations in pond growout techniques that occurred during the 1980s, 1970s, and earlier. Additionally, we will also speculate on certain other technologies that have not yet been widely recognized, but which we feel hold special promise and potential for further improvements in pond culture of marine shrimp.

II. ENVIRONMENTAL CONCERNS AND SUSTAINABILITY

Before the 1990's, the main concern or emphasis of shrimp culture was on technological advancements, production increases, and profitability. There was relatively little awareness or concern by the industry of environmental issues and sustainability. That all changed during the 1990s. What had formerly been a murmur of concern about environmental issues became a roar as environmental and other non-government organizations (NGOs) became increasingly vocal about environmental and social impacts of aquaculture in general, and marine shrimp culture in particular. These growing concerns were highlighted in what is known as the *Choluteca Declaration* by 21 NGOs in Choluteca, Honduras during October 1996 (Accion Ecologica et al. 1997).

The Choluteca Declaration clearly stated the NGOs' concerns and listed 18 demands. Their concerns centered on what the NGOs perceived of as increasing environmental destruction by unsustainable shrimp farming. This destruction included: mangrove forest and wetland losses; eutrophication and sedimentation of receiving waters; salination of soils and aquifers; disease transfers to wild stocks; exotic species introductions; discharge of toxic and/or bioreactive substances; reduced biodiversity in shrimp cultured areas; and creation of social inequities and problems. They called for, "...a global moratorium on any further expansion of shrimp aquaculture in coastal areas until the criteria for sustainable shrimp aquaculture are put into practice." They also demanded, "...the formation of an independent body of national, regional and international organizations, including non-government organizations, to monitor the implementation of this process at the global level."

The Choluteca Declaration and what followed awakened deep and searching interests in the shrimp culture industry about all aspects of shrimp culture (Hargreaves 1997). This interest was further stimulated by treats of

consumer boycotts by NGOs. Lockwood (1997) cautioned the industry to take these threats seriously since, "Historically, actions by environmental groups directed at consumers have been successfully employed in environmental disputes. In Europe, the consumer boycott led by Greenpeace against Shell over the Brent-Spar issue realized the desired objective. In the U.S., boycotts and protests at retail stores against irradiated foods have succeeded. Laws regulating seafood harvests now protect turtle and mammal populations. Organized environmental protests are a proven tool for change." Lookwood also correctly stated that a successful marine shrimp boycott in the U.S., Europe and Japan would have devastating economic effects on the lives of perhaps millions of people around the world, and, "...would result in great social and economic harm to some of the most impoverished people in the world, causing more poverty, more pollution, and increased pressure on the harvest of wild shrimp from the ocean, a resource which has its own set of environmental problems."

A centerpiece of the NGOs' goals was to develop a certification program aimed at identifying farmed shrimp which complied with their ideals of environmentally safe and socially just shrimp culture. This was presumably modeled on earlier successes with "dolphin-safe" and "turtle-safe" tuna certifications. However well meaning, such a certification program for cultured marine shrimp is essentially unworkable. The first hurdle is distinguishing ocean-trawler caught shrimp (75% of world production), from farmed shrimp (25%). It should be kept in mind that trawler catches are perhaps even more ecologically destructive than farms in terms of by-catch wastes and under-utilization (Fast et al. 1995). Secondly, and more importantly is rating of individual farms as either "safe" or "unsafe" and tracking production from each farm through processing and world wide distribution. Presumably, certification would not distinguish farm production that was only partially safe. It would require safe or unsafe rating. In the vast majority of farms, there will be no clear-cut distinction between safe and unsafe. Who would fund this certification program? Who would do the rating and certification? What criteria would be used? Most recent estimates are that almost 200,000 shrimp farms exist worldwide (Table 2). Will each farm be visited and rated on some regular basis? Who would check to see if a given farm had upgraded or decreased its rating? Who will trace farm harvests from farm to consumer? Will there be an appeals process? This thicket of certification problems essentially renders any fair certification process

unworkable. However, unfair certification could happen and is the most likely outcome if NGOs or others attempt to institute such a program.

The concerns raised by NGOs have not gone unheeded. One result has been the formation of another international NGO supported by aquaculture businesses and organizations. This NGO, the Global Aquaculture Alliance (GAA) has a stated mission of furthering, "...environmentally responsible aquaculture to meet world food needs", (Boyd 1999a). The GAA has drafted a *Code of Practice for Responsible Shrimp Farming*. This code of practice is founded on an earlier *Code of Conduct for Responsible Fisheries* adapted by the 28th Session of FAO during October 1995 (FAO 1995a, Anonymous 1997). GAA's and FAO's codes of practice clearly addresses most, if not all of the concerns listed in the Choluteca Declaration.

Implementation of any code, best management plan, or other set of rules for the world aquaculture industry is problematic. Almost certainly, most large shrimp farms will adhere to these practices. It makes good business sense in almost all cases. However, the industry is mostly composed of relatively small farms, especially in Asia with 73% of total shrimp farm, culture area (Table 2). Average farm size in Asia was 3.8 ha. In Thailand, the world leading producer of farmed shrimp, 70% to 80% of all intensive farms had one or two ponds ranging from 0.16 to 1.6 ha (Lin 1995, Anonymous 1996d). Small farms and farmers often lack sufficient land and/or capital to adopt all of the most desirable culture practices.

II.1. <u>The mangrove issue.</u> Mangrove forest destruction is a core issue with environmental NGOs. There is deep concern about widespread mangrove forest destruction. Shrimp farms are often blamed as the major culprit for this destruction.

There is no question about the value of mangroves as important sources of: biodiversity; nursery grounds for a wide variety of aquatic species; lumber, charcoal, tannin, dyes, food and income for artisanal communities; and as protection against storm damage (Macintosh and Phillips 1992, Hambrey 1996). There is also no question about significant mangrove losses, especially during the past 50 years. The question then is how much of these losses are due to shrimp farm construction, and what if anything can be done.

Shrimp farms have been built on mangrove areas. In some cases, these shrimp farms directly converted mangroves to their use. In other cases, perhaps the majority in recent times, shrimp farms were converted from existing fish ponds, salt evaporation operations, or from agricultural farms that were themselves built many years ago on former mangrove or other lands. In some cases such as Indonesia and the Philippines, these original conversions occurred many years or even centuries ago, mostly for extensive fish culture operations (Ling 1977).

Mangrove lands are some of the poorest sites for shrimp farms for a number of important reasons, including:

a. Mangrove soils typically have high organic content, which is not suitable for shrimp well being. Ideal soils for shrimp farming are mineral with at most 5-10% organic matter (Boyd 1995). Highly organic soils also provide poor dike construction.
b. Mangrove soils often contain jelocite (sulfur containing substances), which oxidizes in ponds when exposed to oxygen and forms strongly acid conditions. This is a major cause of new shrimp farm failures in acid soil locations.
c. Mangrove lands near the ocean often do not have adequate elevation to allow complete and rapid draining of shrimp ponds. This greatly hampers shrimp harvest and water exchange.
d. In mangrove areas, numerous fish and crustaceans enter shrimp ponds with influent waters, or by crawling over dikes. This leads to shrimp crop losses due to disease introductions, competition for food and other pond resources, and predation on shrimp.
e. In mangrove areas, shrimp pond source waters and pond effluents often co-mingle due to inefficient water circulation. This results in wastewater recycling and deteriorated water quality in ponds.

For the above and other reasons, including social-economic, shrimp farms are better sited outside the inter-tidal mangrove area. If they are sited close to, but not in mangroves, both the shrimp farms and the mangroves can benefit from farm effluent discharges into the mangroves. These discharges provide nutrients and settleable solids, which benefit mangroves (Robertson and Phillips 1995).

The contention that shrimp farms are the main cause of mangrove forest destruction is unsupported (Hambrey 1996). While shrimp farms have destroyed a high percentage of mangroves in some areas, worldwide less

than 6% of total mangrove resources have been converted to shrimp farms (Macintosh and Phillips 1992).

Mangrove conversion was documented in Thailand, the world's leading cultured shrimp producer for the past seven years, by a joint working committee of the Thai government departments of forestry, fishing, land development, and the National Research Council. They found that 47% of total mangrove land existing before 1961 was destroyed between 1961 and 1986, before large increases occurred in marine shrimp production in Thailand (Menasveta 1997). From 1986 to 1993, Thai cultured shrimp production increased from 17,886 MT to 225,514 MT, while total mangrove reserves decreased by only another 7% during this period. Only a portion of this 7% decrease was due to shrimp farm construction. Overall, shrimp farms in 1993 accounted for 17.5% of total mangrove areas that existed before 1961 (Table 3). This means that about one-third the mangrove areas converted to other uses since 1961 were eventually used for shrimp farm construction by 1993, while two-thirds of the converted mangroves were used for other purposes.

Most mangrove destruction in Thailand occurred before 1986, and that destruction attributed to shrimp farms during this time was mostly for extensive farm types. These farms typically have large ponds, tidal water exchange, and very low yields (Table 1). During 1986, average yields were <400 kg/ha/yr (Menasveta 1997). Most new farms constructed after 1986 were intensive types with much greater yields. Average shrimp farm production increased to >3,100 kg/ha/yr by 1993. Intensive culture, shrimp farms in Thailand now account for about 85% of total shrimp culture area. The implications of this statistic are important. Since much greater yields are achieved on relatively small land areas with intensive culture, total land area devoted to shrimp culture can be reduced by conversion to intensive culture systems. Proper farm siting should reduce pressures on land use and reduce environmental impacts. With proper farm management, water and effluent use, and other environmentally acceptable practices, impacts of these farms should be greatly reduced compared with more extensive farm operations. Conversion to intensive farms may be not only desirable but necessary if we wish to reduce environmental impacts. Marsh (1998) cautioned that, "If farming on the existing area does not become more intensive, environmental degradation will be unstoppable."

Thai farmers soon learned that intensive farms sited in mangrove areas produced poor results, and most of these new farms were thus sited on higher ground, or in areas without mangroves (Menasveta and Fast 1998). Many older, unprofitable farms in mangrove areas have even reverted to mangroves, a trend, which could continue as marine shrimp culture intensifies and culture techniques improve.

Governments, farmers, and the general public in most shrimp growing areas are now aware of the mangrove destruction issue. There is strong public concern about further mangrove forest destruction, regardless of the reason. The shrimp culture industry is on record as opposing any further use of mangroves for shrimp farms (Boyd 1999a).

II.2. Sustainability. Current concepts of sustainable shrimp culture were not widely held 10 years ago. Although most of the elements associated with sustainability were understood and accepted by the industry, there was not as much interest focused on trying to understand all the ramifications and meanings of the term as there is now.

Shrimp culture sustainability is perhaps an outgrowth of the even larger issue of human society sustainability. Sustainability has at least partial origins in the environmental movement which gathered considerable momentum about 30 years ago. Bardach (1997) describes formulation of sustainable development concepts by a special United Nations commission beginning in 1983 and concluded that what sustainability really means depends, "...on whether one sees the world through ecological or economic glasses. As long as populations grow and economic conditions improve for many, the most sustainable development will be one that attains the best possible relationship of the forces active in local and regional dynamic cultural and economic systems as well as in larger dynamic, but normally slower-changing, ecological systems. To be sustainable these systems must allow (a) human life to continue indefinately, (b) human individuals to flourish, and (c) human cultures to develop; at the same time the effects of human activities must remain within bounds so as not to destroy the diversity, complexity, and function of the ecological life support system", (Costanza 1991, Bardach 1997). FAO (1991) further defined sustainable development as, "...the management and conservation of the natural resource base, and the orientation of technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. Such sustainable

development conserves land, water, plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable."

These definitions of sustainable, with appropriate modifications could be adopted to marine shrimp culture sustainability. In particular, sustainability must encompass, "...the broad, overlapping dimensions or systems associated with sustainability: economic, environmental and social. Each of these broad domains provides unique perspective, or partial "truth", regarding sustainable aquaculture", (Hargreaves 1997).

However, if you are looking for a concise, completely unambiguous and encompassing definition of shrimp culture sustainability, you could be disappointed. "Sustainability is a worthless word in the environmental context for no one knows what it means. We should work hard to replace it with the term environmental management. What we need for aquaculture is sound systems of environmental management to prevent or reduce negative environmental effects", (Boyd 1999b). "It is currently popular to talk about sustainability of agricultural, industrial, and other activities that utilize the world's resources. In fact, sustainability is used so often and in such varied contexts that it is essentially meaningless unless defined in relation to a particular activity. In the most literal sense, nothing is sustainable forever, but through wise use of resources, most activities can be sustained for a long time and until an alternative activity or resource is found to be appropriate", (Boyd 1999a).

Shrimp culture sustainability perhaps does not need a precise definition. Rather, it should include the concepts of: best available, non-polluting shrimp culture practices; financial profitability; and social justice. These concepts and ideals have been discussed more fully by signers of the *Choluteca Declaration* (Accion Ecologica et al. 1997), the Global Aquaculture Alliance (Boyd 199a), and FAO (1995a, Anonymous 1997).

It is also relevant to mention the role of government in all of this. Declarations and statements by environmental and industry NGOs, and others are appropriate and necessary, but in the end it is government's role to sort out a clear legal path that protects the public's best interests, and which includes providing acceptable means of food production (New 1999). "There are so many diverse opinions and special interests and so much greed that without government intervention anarchy would exist. There are

those who want to use all the resources without regard to environmental effects, and there are those who want to limit resource use beyond the point of reason or necessity. Governments can, if they will, encourage a middle ground so that human needs can be supplied, producers can profit, and the environment can be protected', (Boyd 1999b).

II.3. Human population pressures. Food demand and production, whether by aquaculture or terrestrial agriculture are driven by the world's human population size. The world's population recently exceeded 6 billion people, doubling from 3 billion less than 40 years ago. Population expansion continues with at least 12 billion predicted before the end of the 21st century (Bailey 1997). These human population increases put demands on food production systems, and on the environment. So far, humanity has been able to meet food demands through increased production, as a result of increased yields per land area and through culture area increases. These increases were possible only through continued improvements in culture stocks, culture techniques, and through continued reliance on fossil fuels and other items produced with fossil fuels. Within about 40 years, the world's supply of petroleum fossil fuel will be exhausted. It is now unclear what alternative energies will take the place of petroleum, and at what economic, environmental and social cost.

Human population increases have put extra-ordinary demands on aquatic animals for food. The world's fishery production has increased from 4 million MT (MMT) in 1900 (Borgstrom 1962) to about 100 MMT in 1995 (FAO 1995b) or more than 20 times increase. At the same time, human population increased from 1.6 to about 6 billion people, a 4 times increase. Capture fishery products from the world's oceans and freshwaters have reached, or in some cases exceeded sustainable supplies (FAO 1995b,c). Any further net increase in fisheries products must therefore come from aquaculture production. Indeed, aquaculture production has increased in response to this need. Between 1987 and 1996, capture fisheries production increased from 85 MMT to 95 MMT, while aquaculture production increased from 10.6 MMT to 26.4 MMT (New 1999). This represents a 249% increase in total aquaculture production, and an increase in aquaculture production from 11.1% to 21.8% of total fishery production. Aquaculture production is likely to continue its increase, while captive fisheries should at best remain nearly static.

Along with increased aquaculture production, production costs of some high valued species have also increased relative to agricultural products such as poultry and pork. This trend is likely to continue since many aquaculture feeds require higher levels of animal protein, mostly from fishmeal. By 1992, about 15% of the worlds fishmeal was used for aquaculture feed (Tacon 1994); including feeds for salmonids, marine shrimp and most other fishes that will accept pelleted feed. Fishmeal and fish oil consumption for aqua-feeds continues to increase along with aquaculture production, but at a greater rate than production. Fishmeal and fish oil consumption for aqua-feeds is are projected to be 25% and 80% of world production respectively for these commodities by the year 2010 (Pike 1997/8). Tacon (1997/8) forecasts even greater usage, and predicts 25% of the worlds fishmeal will be used in aqua-feeds by the year 2000. Aquaculture must compete against other agricultural animal crops for fishmeal and fish oil. This competition, plus periodic collapses of the forage fish stocks (Glantz and Thompson 1981) could not only reduce aquaculture growth, but it could also drastically alter economics and sustainability of high valued crops such as marine shrimp that rely on fishmeal and oil (Bailey 1997). Unless alternatives are developed to these high cost protein ingredients, aquaculture is more and more likely to provide food mostly for middle and upper income people. Aquaculture products are likely to become less and less a source of protein for the poor. Marine shrimp exemplify this trend.

II.4. Summary: Substantial, rapid and visible aquaculture expansion in many areas of the world during the past 10 to 20 years resulted in certain real and perceived deleterious environmental impacts. This has drawn the attention of a number of environmental NGOs, which pose threats to the aquaculture industry. The threats include forced certification programs and other restrictive legislation. The industry has responded by developing sets of best management plans, codes of conduct, and other guidelines intended to foster environmentally and socially conscientious aquaculture production methods, while at the same time promoting profitability. The marine shrimp culture industry was a primary target of thee environmental groups, and one that has responded most forcefully to the threats. So far, this forced scrutiny has been intense but rewarding. While the process is still on going, thee most likely outcome at this time seems to be a more environmentally and socially aware industry, especially by large corporate entities. It should also result in social and regulatory pressures towards more cost efficient and sustainable production systems. Intensive culture systems could benefit

from this scrutiny since they require relatively small land areas, and their environmental impacts are more easily managed and mitigated by proper siting and operation. Ultimately, thee source of these problems relate to human world population growth which continues largely unabated. Planet earth's human population has increased from 1.6 to 6 billion people during thee last century, and is forecast to exceed 12 billion sometime during the 21st century. This population increase will put major stress on all aspects of food production, environmental quality, and on other societal functions.

III. NO WATER EXCHANGE

Intensive monoculture of marine shrimp is potentially unstable and risky. It requires large applications of organic feed and mechanical energy per unit water volume. These applications focus productivity from much larger land and oceanic areas within a smaller area of shrimp growout pond (Folke and Kautsky 1992). The pond becomes the "tip of the funnel." As a result, large amounts of uneaten feed, feces and metabolic wastes accumulate in pond waters and pond soils. These wastes are further degraded through microbial and other decomposition processes to produce among other things; ammonia, nitrite, nitrate and phosphate. These mineralized nutrients stimulate algal growth and lead to dense blooms in the pond. In addition to toxicity from some of these degradation products, algal population collapses can also cause shrimp stress and mortality through disease, oxygen depletion and increased metabolic toxicities. A conventional solution to this situation has been increased water exchange. Excess metabolites, suspended solids and algae are thus removed from the pond and replaced with water of lesser metabolite and algal concentrations and greater oxygen content. This water exchange or flushing solution is, however, not without potential perils of its own. In many cases, source waters for flushing contain disease organisms which infect the shrimp crop and cause massive mortalities. Industrial, domestic and agricultural pollution of source waters can likewise cause massive shrimp mortalities. In addition, source waters often contain high concentrations of suspended sediments which settle in the pond and cause shoaling of pond water depths which requires time consuming removal and disposal between crops. An alternative to high rates of water exchange includes systems with minimal, or no water exchange during crop culture.

Ten years ago water exchange rates of 10-20% or more per day were common with semi-intensive and intensive shrimp pond culture (Clifford

1985, Colvin 1985, Fast 1991). Semi-intensive culture ponds in Latin America typically pumped large volumes of estuarine water through very large ponds on a daily basis as a means of maintaining adequate dissolved oxygen (DO) concentrations in the ponds. Much smaller, intensive culture ponds in Asia combined high water exchange rates with electric paddlewheel aerators to achieve desirable DO and water quality (Fast et al. 1989). Water exchange and aeration also kept settleable solids in suspension where these materials could be flushed from the pond rather than accumulate on pond bottoms and contribute to toxic sediment conditions.

We now know that high water exchange rates through shrimp ponds are not always environmentally friendly, and do not always benefit shrimp culture. Water intakes can entrain and/or impinge biota, which are then lost. Pond influent/effluent waters also carry shrimp diseases into ponds, and discharge diseases from ponds into the environment. Nutrients and suspended solids in effluents can cause eutrophication and sedimentation in receiving waters. High sediment loads in source waters can lead to pond depth shoaling, increased operating costs, and lost culture time to remove sediments. Exotic shrimp species can also escape to the environment with waste waters and potentially become established, and/or cause disease transmittals. When genetic improvements occur in shrimp culture stocks and these stocks are more widely used, "improved" shrimp stocks could jeopardize population genetics of wild stocks through escapement and inter-breeding.

Water discharges from ponds occur for a variety of reasons. The most common reason is for water exchange as noted above, followed by drain harvest at the end of each shrimp crop cycle. Drain harvest typically contribute the highest concentrations of solid materials to receiving waters, especially in the last portion of the drain when sediments are resuspended and carried out with drain waters. The last 10-20% of pond drain waters can contain >60% of total settleable solids and >40% of suspended solids (Teichert-Coddington et al. 1999). Other discharges result from heavy rainfall, which overflows the outlet weir. In some cases in arid areas or during dry seasons, water must be exchanged to maintain pond salinity within acceptable ranges for shrimp (Hopkins et al. 1995). With extensive pond culture, water is often exchanged to provide seed, and to provide nutrients to stimulate in-pond productivity.

Ten years ago, common knowledge was that high rates of water exchange were necessary with intensive shrimp culture to remove nitrogenous and other potentially toxic metabolic waste products, and to prevent accumulations of potentially toxic organic sediments. With intensive culture, these wastes were thought to be one of the main limiting factors for shrimp production. Waste concentrations are related to feed input rates (Brune and Drapcho 1991); or more specifically to feed quantity and protein content (Westerman et al. 1993).

Before 1990, there was little of no systematic research on the relationships between water exchange, water quality, and shrimp yields from ponds. One of the few analyses of water exchange effects showed a dramatic increase in black tiger shrimp production with increased water exchange. Production increased from 10 MT/ha/yr with 20% maximum daily water exchange to 25 MT/ha/yr with 100% exchange (Hirasawa 1985, Fig. 2). More recent research, however, now casts doubts on the need for, or value of such high rates of water exchange. Indeed, there is now solid evidence that with proper pond management water exchange can be reduced to zero in many cases.

III.1. <u>Waddell Mariculture Center.</u> Shrimp pond and tank experiments with reduced water exchange began at the Waddell Mariculture Center, South Carolina during 1985-87 (Sandifer et al. 1988). During 1985, they intensively cultured *P. vannamei* using stocking densities, aeration and water exchange rates reported from Asia with *P. monodon* (Liu and Mancebo 1983, Chiang and Liao 1985). This included stocking 0.1 ha ponds with 40 PL/m^2, water exchange of 16-17%/day, and paddlewheel aeration. After five months culture, yields ranged from 6,010 to 7,503 kg/ha, which agreed with reported *P. monodon* results. The following year (1986), eight ponds were stocked with *P. vannamei* postlarvae ranging from 40 to 60 PL/m^2, water exchange was reduced to 8.3-8.5%/day, and aeration was reduced by 60%. Despite intense drought conditions during 1986, yields from these trials ranged form 4,390 to 6,881 kg/ha, with 77.3% average survival, and 2.5 average feed conversion ratio (FCR). They continued these trials during 1987 with a wider range of stocking densities (20-100 PL/m^2), and again with 8%/day water exchange. Yields ranged from a low of 2,487 kg/ha with 20 PL/m^2 to 12,680 kg/ha with 100 PL/m^2. Survival was 90%.

Following these successful pond trials with reduced water exchange of 8-9%/day during 1985-87, Hopkins et al. (1993) further evaluated water exchange and shrimp pond yields at the Waddell Mariculture Center during 1990. With these trials, they stocked 0.25 and 0.5 ha ponds with *P. vannamei* PL at 76/m^2 and with water exchange of either 14% or 4%/day. They observed no effects of reduced water exchange on yield, with average yields of 7,565 kg/ha in the 4%/day water exchange treatment, and 7,462 kg/ha with 14%/day water exchange.

Success with water exchange reduction to 4% with very high yields prompted Hopkins et al. (1993) to further reduce water exchange to zero during 1991. During 1991 they stocked five ponds with northern white shrimp (*P. setiferus*) PL at 22, 44 and 60 PL/m^2, water exchange of 25%, 2.5% and 0%/day, and aeration of 10-20 hp/ha. After 89 and 125 days culture, two ponds with zero water exchange and 44 and 60 PL/m^2 respectively, experienced mass shrimp mortality. Ponds with 2.5% and 25%/day water exchange yielded 6,375 and 5,718 kg/ha crops respectively, with corresponding 79.5% and 81.9% shrimp survival (Table 4). Only one pond with 0%/day water exchange yielded a credible crop. This pond was stocked at 22 PL/m^2 and yielded 3,219 kg/ha. Hopkins et al. (1993) could not relate mass mortality to any water quality parameters. They concluded that these shrimp mortalities were probably caused by gill fouling, since microscopic gill inspections revealed, "...some epicommensal bacteria and large amounts of trapped debris, similar to the filamentous gill disease described by Lightner et al. (1975) and Lightner (1983)." They associated this gill fouling in the 66 PL/m^2 and zero water exchange pond with abnormally high concentrations of suspended and organic solids in pond waters, although in the 44 Pl/m^2 and zero water exchange pond that had mass mortality, these solids were not excessive. Dissolved oxygen was well above critical levels at all times in all ponds. Hopkins et al. (1993) concluded that water exchange could be reduced to zero with *P. setiferus* provided that shrimp stocking densities were 44 PL/m^2 or less, and peak feed applications were 70-140 kg/ha/day. Further increases in feed applications would risk idiopathic mass mortality of shrimp due to gill fouling.

In a related set of trials during 1991, Hopkins et al. (1994) compared water quality and shrimp yields from three ponds with different sediment treatments. All three ponds had zero water exchange, *P. setiferus* stocked at 44 PL/m^2, and average feed rates of 97 kg/ha/day. All three ponds were

aerated with one paddlewheel aerator (10 hp/ha) until day 55, when a second aerator was added (20 hp/ha). Pond sediments were handled very differently in each pond. In the REMAIN pond, sediments were allowed to settle and remain in place undisturbed (Fig. 3). In the REMOVE pond, sediments were removed using a pump, while in the RESUSPEND pond, the aerator was shifted 30° each day to resuspend bottom sediments and to keep these sediments from settling. As noted above, mass shrimp mortality occurred in the REMAIN pond on day 125 due to gill fouling, while survivals were not particularly high in the other two ponds with 32.8% and 54.1% (Table 5). Significant water quality and nutrient cycling differences existed between the three ponds. Nutrients in the REMPVE pond were lower and DO higher compared with the RESUSPEND pond. It also appeared that denitrification was inhibited in the RESUSPEND pond.

Undaunted by these mass shrimp mortalities during 1991, Hopkins et al. (1995) stocked four 0.25 ha ponds during 1993 with *P. vannamei* at 39 or 78 PL/m^2, fed prepared feeds with either 20% or 40% protein (2x2 factorial design). All ponds were fed at a constant rate of either 68 or 136 kg/ha/day during the entire 131-day trials, with the high and low feed rates corresponding to high and low shrimp stocking densities. All ponds had zero water exchange, and either 20 or 40 kWh/hr/ha paddlewheel aeration. The two high feed and high shrimp density ponds had aeration failures during the night on one occasion each, which resulted in observed DO of 0.8 and 0.5 mg/l respectively. Survival in these ponds correspondingly decreased to 57.5% and 63.3% (Table 5). Yet, yields were still very respectable, averaging 6,001 kg/ha for the low-feed/density ponds to 6,863 kg/ha for the high-feed/density ponds. Water quality reflects these differences in feed applications and shrimp densities. Average dissolved oxygen, turbidity, total suspended solids, organic suspended solids, total phosphorus and nitrate were all much higher in the high-feed/density ponds, and there was no apparent difference in shrimp survival, growth or yield between feeds with either 20% or 40% protein. FCR at high-feed/density do not reflect actual feed conversions due to DO induced mortalities. With intensive culture of *P. vannamei*, FCR typically average >1.8, and more typically >2.0 (Sandifer et al. 1988, Reid and Arnold 1992, Wyban et al. 1988). Almost as importantly, there were no incidents of mass shrimp mortality with *P. vannamei* due to gill fouling in the higher stocking and feed application ponds. Zero water exchange and high aeration rates, taken together with low FCR, high shrimp yields and the observed water quality parameters suggested that the microbial food web played an important role

with successful, intensive culture of *P. vannamei* under these culture conditions.

Hopkins et al. (1997) and Browdy and Bratvold (1997) conducted further studies on water exchange at the Waddell Mariculture Center during 1995-96. Six 0.1 ha ponds were stocked each year with *P. vannamei* at 38 and 78 PL/m^2 each year respectively. Three of the six ponds had a constant 15%/day water exchange, while the other three had no water exchange. Feed rates were constant at 57 kg/ha/day during 1995, and 116 kg/ha/day during 1996, corresponding to different shrimp stocking densities each year. There were no significant differences between water exchange treatments either year. During 1995, survival and yields averaged 93.4% and 5,890 kg/ha for ponds with water exchange, and 91.2% and 5,443 kg/ha without water exchange (Table 6). Shrimp growth rates were much reduced during 1997 compared with 1996, but again survival and yields were similar for both water exchange treatments. Bacterial abundance and oxygen consumption were much higher in the ponds without water exchange (integrated ponds), during 1996 (Fig. 4).

III.2. **Australia.** Allan and Maquire (1993) cultured school prawn (*Metapenaeus macleayi*) in small pools in Australia with water exchanges ranging from 0-40%/day, and at stocking densities of 20 to 40 shrimp/m^2. They found no difference in shrimp survival, growth or FCR related to water exchange. High water exchange did reduce phosphorus and phytoplankton pigments, but had no effect on pH or nitrogen. They concluded that, "...simply increasing daily water exchange rates may not necessarily increase shrimp growth or survival. Water exchange can reduce nutrient concentrations and phytoplankton densities but most of the reduction occurs at water exchange rates of 0-5%/d."

III.3. **Belize shrimp farm.** Proof of zero water exchange benefits was clearly demonstrated on a commercial shrimp farm in Belize, Central America during 1997-present. The farm had sixteen 0.065 ha and eight 0.37 ha growout ponds, plus two 0.7 ha settling ponds and one 0.5 ha reservoir pond (McIntosh et al. 1999). Production ponds were all lined with HDPE plastic without substrate, while pond water depths were 1.4 and 2.3 m at shallow and deep ends respectively. The smaller ponds had 60 hp/ha of aeration with paddlewheel and aspirating-impeller aerators, while the larger ponds had 28 hp/ha. Aeration was provided to maintain adequate DO and to keep settleable solids suspended in the water column. Source waters

were from the ocean (high salinity) and from a creek (low salinity). Creek water was used to lower salinity during the dry season. Pond drain waters were re-cycled through the settling basins and re-used in growout ponds. Sediments were removed by pumping every three weeks from pond areas where sediments collected. Sediments were drained into the settling basin. There were nearly zero water and sediment discharges from this farm.

McIntosh el al. (1999) cultured SPF *P. vannamei* (Mexican strain), SPF&R *P. vannamei* (resistant to TSV), and SPF *P. stylirostris* (Ecuadorian strain). Stocking densities ranged from 63 to 121 PL/m^2. Shrimp were fed a mixture of prepared feed (30% protein, complete diet), and a pelleted, organic mixture of soy meal, ground wheat and corn. The purpose of the organic mix was to stimulate growth of heterotrophic microbes in pond waters. During early crop cycles, feeds were applied at 70%/30% apportionment of organic mix and prepared diets respectively. This ratio was adjusted during growout such that by harvest the proportion was then 20%/80%. Peak feed applications often exceeded 350 kg/ha/day by harvest.

During the first year, 65,941 kg of shrimp were produced from 26 pond harvests, with average yields of 11,233 kg/ha/crop (McIntosh el al. 1999). Highest crop yield was 19,600 kg/ha. Average survival during the first two years of operation ranged from 56% for the TSV resistant *P. vannamei* to 82% for *P. vannamei* Mexican strain (Table 7). *Penaeus stylirostris* had intermediate survivals of 60%. Average yields were also greater with the Mexican strain, averaging 14,190 kg/ha/crop, compared with 10,340 kg/ha/crop for the TSV resistant strain and 7,450 kg/ha/crop for *P. stylirostris*. McIntosh and his colleagues felt that the Mexican strain of *P. vannamei* was much better suited for their high density and highly heterotrophic culture conditions, and that its superior performance was related to its having been bred in captivity for more than eight generations. This closed life-cycle culture over many genertions in intensive culture conditions undoubtedly resulted in selection for characteristics most suited for high-density culture. Small ponds produced higher yields than large ponds mostly because they were stocked at higher densities. There was also seasonal effects since the winters are cooler with higher salinity, and the summers have higher temperatures and more rain. Weekly average, high and low temperatures were 23°C and 32.5°C.

McIntosh et al. (1999) also observed profound changes in pond water quality and appearance during a growout cycle, "Ponds changed from a

predominately autotrophic phytoplankton based pond ecology to a heterotrophic bacterial based pond ecology. Pond water coloration often changes from a green to a dark brown/black coloration with visible bacterial flocs present in suspension." These physical changes were accompanied by large changes in certain chemical parameters. Alkalinity, pH, DO, and transparency all decreased during culture, while carbon dioxide, nitrogen and phosphorus increased substantially (Table 8).

Substantial amounts of sludge were produced during zero water exchange shrimp culture. This sludge was pumped or drained to settling basins. For every kg of shrimp produced, 0.72 kg of dry sludge was produced (McIntosh et al. 1999). Overall water use was greatly reduced compared with conventional shrimp culture systems since only 2 m^3 of water were used to produce 1 kg of shrimp, and this water was re-used for subsequent crops. Semi-intensive shrimp culture in Central and South America typically used >100 m^3 of water per 1 kg shrimp (Clifford 1992). Energy consumption was increased with zero water exchange, however, since considerably more energy was consumed for aeration. With typical, high water exchange shrimp culture practices and 15%/day water exchange in semi-intensive culture (1,000-1,500 kg/ha), energy consumption for water pumps is 2.0 to 2.5 kWh/kg of shrimp produced. With zero water exchange, 3.47 and 4.62 kWh/kg of shrimp were used during the wet and dry seasons respectively. Added energy cost of zero water exchange in this case was thus $0.21/kg of shrimp at $0.12/kWh. This increased operating cost for aeration could be partially offset by lower capital and maintenance costs for the water pumping systems, and by more consistent production since reduced water exchange also decreases disease risks and chances of significant crop losses. As noted, this added aeration was essential to maintain adequate DO, to keep settleable solids in suspension, and to maintain healthy heterotrophic pond ecology.

McIntosh et al. (1999) had some disease problems, especially with the TSV resistant strain during high salinity (38‰) and temperatures (31.5°C). The other shrimp strains and species were relatively unaffected. Overall, though, their zero water exchange was highly successful. They attributed their success to four primary factors:

1. Use of virus free, SPF shrimp, which were adapted to high-density culture with no water exchange.
2. Pond management practices which promoted healthy, heterotrophic environments in the ponds.

3. Feeds, which promoted healthy, heterotrophic pond conditions.
4. Use of deep, lined ponds.

It is also worth noting that Belize is currently isolated from other shrimp farming areas. There are currently no shrimp farms in the Yucatan of Mexico, or on the Caribbean side of Guatemala or Honduras. Visits to their farm are discouraged to avoid disease imports. Belize is also currently free of TSV and most other serious shrimp viruses.

III.4. <u>Summary and conclusions:</u> Although our understanding of shrimp pond dynamics relative to water exchange is much less than perfect, it is now clear that shrimp yields in the range of 5,000 to 15,000 kg/ha/crop or more are possible without water exchange. This would have seemed unthinkable perhaps even 10 years ago.

Although intensive and semi-intensive shrimp culture has historically relied on high water exchange rates, some intensive fish culture has developed using little or no water exchange. In the U.S., channel catfish (*Ictalurus punctatus*) are commonly cultured in freshwater, earthen ponds with no water exchange and at yields greater than 7,500 kg/ha/yr (Tucker and Robinson 1990). These ponds use paddlewheel aerators and have prepared feed (30% protein) applications of 100 to 150 kg/ha/day, with average peak feed applications of about 112 kg/ha/day (Schwedler and English 1991).

Pond fish and shrimp yields in tropical areas, based solely on autotrophic production with solar radiation as the energy source and inorganic nutrients as the materials source, are limited to perhaps not more than 130 kg/ha/month (1,560 kg/ha/yr) of shrimp and/or fish crop biomass (Moriarty 1997). With a net 4.6 g C fixed/m^2/day by plants with primary production as the only organic carbon source, about 1% of net primary production is thus converted into shrimp and/or fish through a food web consisting of meiofauna, protozoa, zooplankton and macrofauna. Increased crop production above this level requires energy and material inputs from outside the pond. At high crop yields, the pond becomes a net consumer of energy and materials rather than a net producer. Usually, these inputs are animal manures, prepared feeds, and/or some other organic materials. Direct consumption of animal manures usually does not produce high crop yields, but manure fed ponds, which allow for manure digestion by a wide variety of microbes can produce very high yields of fish and other crops

species. Microbes digest manures, thus converting this organic matter into more digestible and injestable forms, which are then consumed by fish and crustaceans. Fish yields of >7,000 kg/ha/yr in manure fed ponds with little or no water exchange are not uncommon (Tang 1970, Moav et al. 1977). Schroeder (1978) reported yields of >7,000 kg/ha during 220 days culture (>11,000 kg/ha/yr) with polyculture ponds in Israel (Fig. 5). Gut analyses of fishes from these manure fed ponds revealed predominance of detritus-like organic particles. Along with these particles were the same decomposer microorganisms found on organic detritus in the ponds. After passing through the fishes' guts, these particles were re-colonized by more decomposers and again re-cycled providing more food for fish.

Nutritional benefits of detritus fed systems to larger fish and crustaceans are widely recognized for other aquatic systems, such as freshwater streams and rivers (Cummins et al. 1995), where leaf litter and other organic debris from the watersheds are repeatedly passed through a wide variety of animals. With this mineralization process, energy and organic compounds benefit the decomposers and detritivores, who in turn provide food for fish and larger crustaceans.

Like certain pond fishes, marine shrimp have wide ranging food habits. In natural systems, shrimp consume detritus aggregates including bacteria, meiofauna including protozoa, micro-algae, zooplankton, macrobenthos and other items (Dall 1968, Gleason and Wellington 1988, Chong and Sasekumar 1997, Moriarty 1997). The importance of each food group or item in shrimp diets is unclear, but what is clear is that shrimp readily inject and presumably benefit nutritionally from the detritus food items.

Until recently, conventional thought was that with intensive shrimp culture, shrimp derived almost all of their nutrition from applied feeds, which were considered nutritionally complete. However, recent microcosm investigations at the Oceanic Institute in Hawaii using waters from intensive shrimp culture ponds clearly revealed that shrimp derive significant benefits from small suspended and settleable solids in these pond waters (Leber and Pruder 1988, Moss et al. 1992, Moss 1995). These microcosm trials included using unfiltered pond waters taken from a 337 m^2 round pond, which was stocked at high shrimp densities, fed a high quality prepared feed, and with peak water exchanges of 30%/day or more (Wyban and Sweeney 1989). In addition, clear well water, and pond water filtered using different filter mesh sizes were used in the microcosm trials. Shrimp

(*P. vannamei*) in each microcosm treatment were fed high quality, nutritionally complete, prepared feed. Trial results indicated that shrimp reared in clean well water and fed prepared feed only had the lowest growth rates (Fig. 6, Moss et al. 1992). Shrimp reared in pond waters filtered through 0.5 μm mesh with or without granulated activated charcoal (GAC) had growth rates statistically similar to the well water treatment. Shrimp in microcosms with pond water filtered through 5.0 μm mesh grew 53% larger than shrimp in the well water treatment, while shrimp receiving unfiltered pond water grew 89% larger than shrimp in the well water treatment. These results demonstrated that suspended pond water solids greater than 0.5 μm were making significant contributions to shrimp growth even in the presence of high quality shrimp diet. Pond water solids in the 0.5 to 5.0 μm were mostly small diatoms and microbial-detritus fragments, while solids >5.0 μm were mostly larger diatoms and large detritus aggregates. The diatoms were produced in the culture pond, while the detritus and other microbial materials were all produced from plankton, uneaten feed, and shrimp wastes through the detrital food web. Again, conventional wisdom has been that most penaeid shrimp obtained their food by probing the bottom with their chelated periopods and by transferring food items to their mouths (Hindley and Alexander 1978, Hill 1990). It is now clear that *P. vannamei* and presumably other penaeids can capture significant amounts of small, suspended particles from the water column and meet a substantial portion of their nutritional needs from these items. This work on detrital materials in pond waters helps explain improved feed conversions, successful use of low protein feeds, and other benefits of heterotrophic, aerobic pond culture experienced at Waddell Mariculture Center and in Belize.

Successful application of zero water exchange requires several conditions, or changes in normal pond management strategies. First and foremost, when water exchange is reduced to zero, then aeration and water turbulence must be increased to some suitable level to achieve continuous suspension, and/or resuspension of organic detritus and wastes, and to provide additional oxygen to compensate for increased BOD (Fig. 7). If aeration/turbulence are insufficient, suspended and organic solids will settle to the bottom in low turbulence areas of the pond and create anaerobic sediment accumulations. To prevent this occurrence, these sediment accumulations should either be removed from the pond, or resuspended before they produce substantial amounts of anaerobic decomposition products. Anaerobic conditions are generally considered undesirable,

particularly when toxic hydrogen sulfide is produced in quantity. Large amounts of anaerobic sediment deposits may be most dangerous when disturbed since they could release substantial quantities of toxicants at one time. If adequate aeration/turbulence are provided, this will create an aerobic floc suspension with associated heterotrophic decomposers. This floc, and its associated microbes will provide valuable nutrition for the shrimp crop, increase shrimp growth rates, reduce protein requirements for the feed, reduce total feed requirements, and increase nitrogen conversion efficiencies from feed to shrimp. Low protein, prepared feeds are lower cost and use less fishmeal than high protein feeds. These cost savings will help offset higher energy costs for aeration.

There are still many important aspects of zero water exchange culture that need clarification. However, progress during the past 10 years has provided not only valuable insight into pond dynamic processes, but has also led to successful commercial applications using zero water exchange.

VI. BRINE BASED AND OTHER INLAND POND CULTURE

IV.1. Inland shrimp farming Thai-style. Intensive culture of *P. monodon* became increasingly popular in coastal areas of Thailand more than 10 years ago. Cultured shrimp production increased very rapidly as a result of existing hatchery, farm and feed infrastructure, and highly trained personnel for culture of freshwater prawn (*Macrobrachium rosenbergii*). Thailand has been the world's leading marine shrimp producer since 1991, and in 1998 produced 210,000 MT (Table 2). The Thai shrimp culture industry employs about 200,000 people in farms, hatcheries, processing plants, exporters, feed mills, equipment providers, and other aspects of the industry. Inland shrimp farming has now become a sizeable portion of total marine shrimp production in Thailand.

Brackishwater shrimp culture initially developed along some of the main rivers and estuaries in the upper Gulf of Thailand. During the dry season, with low volumes of river water outflows, seawater intruded up-river and provided adequate salinity for shrimp farms along the rivers. During the wet season, however, high volume outflows from rivers eliminated this source of saltwater. Some of these farmers soon discovered that they could truck concentrated brines from salt production works to their farms, mix brines with freshwater, and cultures *P. monodon* at low salinity (Flaherty and Vandergeest 1998, Miller et al. 1999). These successes,

combined with serious disease problems in many coastal areas led to rapid expansion of shrimp culture into many inland areas, some hundred of kilometers from the coast. Traditional rice culture areas were often used for shrimp culture due to low land prices and availability. "In lieu of government regulation, the growth of inland shrimp culture is limited only by the availability of freshwater supplies and adequate road infra-structure to support saline water and post-larvae deliveries," (Miller et al. 1999).

A 1997 survey revealed 11,504 ha of inland shrimp farms in 12 central Thailand provinces (Musig and Boonnom 1998), while total estimated marine shrimp culture area in Thailand during 1997/98 was 70,000 ha (Table 2, Jory 1999, 1999). Some estimates indicate that as much as 27% of Thailand's total marine shrimp production came from these inland farms during 1998 (Pongthanapanich 1999).

In many ways, inland shrimp culture is similar to coastal shrimp culture, but there are some notable exceptions. With inland culture, shrimp ponds are prepared in the traditional way and filled to 30-50% of their volume with freshwater, usually from irrigation canals built for rice and other traditional crops. Concentrated brines (150-200‰) are trucked to the farm and added to this water, increasing pond salinity to 5-9‰ in most cases (Musig and Boonnom 1999), but ranging from 4-10‰ (Miller et al. 1999). Tanker trucks of 12-m^3 water capacity are used to transport brine to shrimp farms at $80 to $200/load depending on distance. *Penaeus monodon* PL are stocked at 30-65/m^2 or more, and pond water volumes are increased by freshwater additions, with further salinity decreases. Additional salinity decreases may occur during growout. Water may be added during the dry season to compensate for evaporation and seepage, although seepage is very low in most areas with thick clay soils. Rainfall can dilute salinity during the wet season. Typically, salinity decreases during crop growout to between 1 and 5% by harvest. Water is not normally discharged from ponds until harvest.

Most small farms, which constitute perhaps 80% of inland shrimp farms discharge pond waters and sediments into irrigation canals during harvest. Small farms typically have one hectare of ponds, or less. Large farms are much more likely to recycle their pond waters using dedicated reservoir ponds or by pumping between ponds. Many of these larger farms do not discharge any effluents, but use "closed-cycle" culture practices. This not only reduced environmental impacts, but it also saves money on salt

purchase, reduces salt usage, and reduces overall disease risks not only to that farm, but to surrounding farms as well.

Especially with closed-cycle culture and use of SPF seed, disease risks are greatly reduced at inland shrimp farms. There is less risk of disease introductions through water exchange, and many of the common disease vectors such as seagulls and marine crustaceans are not present. There have been, however, incidents of yellow head and white spot virus outbreaks in these farms. The main source of infection appeared to be infected shrimp seed (PL), and freshwaters contaminated by discharges from other farms.

Inland farms have other problems not normally experienced by coastal shrimp farms. Two of the most common problems are off-flavor shrimp, and agricultural chemical problems. Off-flavor is most common in low salinity pond culture and is caused by blue-green algae (cyanobacteria) and microbes found in freshwater sediments. Off-flavor is common with intensive freshwater catfish culture, and in other low salinity, shrimp culture settings including those in Ecuador with *P. vannamei.* Off-flavor does not affect shrimp yield, but it does lower price.

Rice and other agricultural crops surround most inland shrimp farms. These crops are often sprayed with pesticides and insecticides, which can either drift into the shrimp ponds, or enter the pond with make-up water. Shrimp are especially sensitive to these agricultural chemicals since they are physiologically similar to the targeted pest organisms, which are usually insects. Other problems in inland shrimp farms include off-colored flesh, lower survival, soft exoskeleton, and smaller harvest size. Smaller harvest size and most other problems result in lower price.

Many rice and other traditional agriculturists became concerned by the rapid expansion of inland shrimp farms into their culture areas. They were most concerned about soil salinization caused by pond seepage, salinity increases in irrigation waters due to shrimp pond discharges, and sludge discharges from ponds into irrigation canals. Their concerns gained widespread public attention during April and May 1998 through a series of articles in Thai newspapers and on national TV. As a result, during the summer 1998 the Thai government banned all shrimp farming from freshwater inland areas. This resulted in protests by inland shrimp farmers and controversies between farmers, government departments, and among

academicians. Attempts are now ongoing to find a fair and equitable middle ground.

The inland shrimp farming controversy involves both economic and social considerations (Table 9). Terrestrial agriculture in Thailand, especially rice farming is a revered tradition that supports and engages a large portion of the rural population. These rice farmers, "...often remain poor and usually in debt", (Pongthanapanich 1999). Shrimp farmers; on the other hand are seen as opportunists that have become rich through application of modern technology and access to investment money. The majority of inland shrimp farmers lease land for their farms, and some are not native to their farming district. Complicating the issue is that many rice and orchard farmers have seen a business opportunity and have converted their farms to shrimp.

IV.2. <u>Other inland shrimp culture</u>. Traditional marine shrimp culture is in coastal areas where seawater or brackishwater is exchanged with pond water. All shrimp culture intensities relied on this water exchange (Table 1). As noted above, zero water exchange and low water exchange systems have evolved, but even then, they rely on water brought from the coast by pipeline or as brine in tanker trucks. In addition to these systems, there are some mostly experimental shrimp culture farms well inland that use slightly saline or even freshwater to culture marine shrimp (Jory 1999).

Arizona shrimp farm. The Wood Bros. Shrimp Farm near Gila, Arizona uses slightly saline well waters (1-2‰) at 25°C to culture SPF *P. vannamei* (Jory 1999). Effluent water from the farm is used to irrigate olive trees and Durham wheat. The farm includes an intensive greenhouse nursery to produce shrimp pond seed. Nursery tanks were stocked with about 20,000 PL8/m^2 in 17‰ salinity water, which was reduced to 2‰ over 26 days culture. Nursery survival was nearly 100%, with FCR of 0.7. The farm had 10 growout ponds (0.15 to 0.9 ha), which were stocked at both low (5 shrimp/m^2) and high (44-55 shrimp/m^2) densities. Aeration was with paddlewheels and air diffusers at 20-40 hp/ha. Water was exchanged at 0-1.9%/day, plus an additional 1.34-8.33%/day (3% average) to compensate for pond seepage. Shrimp survival and yields were reduced by high mortality a few days before harvest due to low, pond temperatures of 15.8° C. Average yields ranged from 484 kg/ha/crop for the low-density ponds to 3,070 kg/ha/crop for high-density ponds. The study demonstrated that *P. vannamei* can be successfully cultured in essentially freshwater, but that

greenhouse nurseries are critical for optimizing farm yield and profits in this location. Greenhouse operations allow the ponds to be stocked earlier with larger shrimp, thus reducing pond growout time. Two shrimp crops may be possible each year with fully integrated nursery and pond operations.

Harbor Branch Oceanographic Institution, Florida has conducted culture trials with *P. vannamei* in freshwater far remote from the ocean (Scarpa 1998). Motivation for this work was to develop a biosecure system, which would reduce disease transmittal. Inland shrimp culture in freshwater also addressed concerns about escapement of exotic shrimp species, and effluent discharges into coastal waters. Freshwater effluents have many applications for irrigation of agricultural crops without concern for salinization of soils or aquifers. *Penaeus vannamei* PL12-14 were first acclimated from seawater salinity (28-30‰) to freshwater (0.4-0.5‰) over a 5 to 6 day period. They were then placed in a recirculating freshwater (0.4-0.5‰) growout system and cultured to 15-16 g over a 4-5 month period. This work is now being extended to commercial operations, but it is still largely experimental.

IV.3. Summary: Inland shrimp farms located well away from coastal areas have certain advantages over traditional farms on the coast. Inland farms potentially have greater biosecurity, especially against disease transmittals and against escapement of exotic shrimp cultivars. Probably the three primary sources of disease infections on most farms are seed, source waters and vectors such as birds and other marine crustaceans. If an inland farm uses SPF seed and uses appropriate water management practices, it should enjoy greater biosecurity.

Inland farms in Thailand are the most convincing example that this approach is commercially viable on a large scale. Pongthanapanich (1999) found that inland shrimp farms were more profitable than coastal farms. Inland farms had disease problems, but apparently less than most coastal farms. Inland farms do, however, require more management attention, especially with saltwater conservation and water quality management. Inland farms are also more likely to experience social and environmental conflicts when they are sited in traditional agricultural areas.

Jory (1999) pointed out that integrating shrimp farming and agricultural crop irrigation is probably critical for the successful expansion of shrimp

farming into inland and coastal desert areas. Integrated shrimp farming with agriculture will undoubtedly mean using effluents and wastes from shrimp farms for crop irrigation and fertilization. This will be most easily accomplished if the effluents are nearly fresh with salinity of 2-3‰ or less (Glenn et al. 1998), although some potential forage crops can be grown at higher salinity of 31‰ (Brown and Glenn 1999).

The two shrimp species that have been cultured most widely and successfully in freshwaters are *P. vannamei* and *P. monodon*. Their ability to adapt to freshwater is well known. However, much additional work is needed for large scale, appropriate inland culture of these species without salt additions.

V. POND WATER RECYCLE AND RECIRCULATION SYSTEMS

Another alternative to high rates of water exchange and flushing is water recycling and recirculation. This allows water to be reused many times and reconditioned between each use. This has many advantages, which we will review.

V.1. Tank recirculation systems. Aquatic animal culture systems which use water recirculation and treatment have been used for 40 years or more (Allen and Kinney 1981; Timmons and Losordo 1994). Water recycling systems have been operated even longer for domestic and industrial wastewater treatment. Until recently, aquaculture recirculation systems were mostly confined to experimental culture applications involving relatively small water volumes, or to relatively small commercial ventures involving high value crops such as ornamental fish (Anonymous 1987; Adey and Loveland 1991; Moe 1992), fish and crustacean seed for conventional growout or stock enhancement (Menasveta et al. 1989, 1991; Colt and White 1991; Wang 1993; Tseng et al. 1998), or for research (Yang et al. 1989). These conventional aquaculture recirculation applications typically ranged from small aquarium (~200 l), to perhaps 100 m^3 culture tanks, silos or raceways (Table 10).

System components of conventional water recirculation systems generally include provisions for: containment of primary crop; solids removal; nitrification; and means of water recirculation and aeration (Table 10). Additional, optional components include: temperature control; denitrification; foam fractionation and biocidal treatments such as

ozonation and/or UV irradiation. Daily water exchange rates, or flow-through typically range from near zero with denitrification, to 10% or more without denitrification. Application of these conventional recirculation systems to marine shrimp culture have been largely confined to broodstock maturation (Menasveta et al. 1991), larviculture (Huguenin and Colt 1989) and nursery operations (Sturmer et al. 1992).

V.2. <u>Marine shrimp recirculating growout systems.</u> During the past 15 years, recirculation systems have been applied commercially to marine shrimp growout on much larger scales than previously envisioned. Reasons for these applications relate mostly to:

- control of diseases from source waters and organisms in source waters.
- scarcity of high quality source waters.
- control of water quality problems with source waters.
- improved growth performance due to greater control over water quality parameters.
- concerns about environmental degradation caused by shrimp pond effluents.

Serious shrimp diseases can come from source waters and/or organisms in source waters (Browdy and Bratvold 1998). Therefore, by limiting use of source waters and pre-treating these waters before use, and reconditioning effluent for recycle, chances of disease are greatly reduced. These precautions combined with use of specific pathogen free (SPF) or specific pathogen resistant (SPR) shrimp can greatly reduce disease incidence during growout. Recirculating pond systems can also reduce sedimentation within ponds, and improve discharge water quality to receiving waters.

A wide variety of shrimp pond recirculation schemes have been proposed and/or used (mostly proposed). These share many characteristics with conventional recirculation culture systems, but also differ in some respects (Table 10). In addition to size differences, conventional and pond recirculation systems perhaps differ most markedly with respect to photosynthesis. With conventional systems, photosynthesis is typically absent. Aeration and ammonia conversion with these systems are achieved by mechanical means and by bacteria. With pond recirculation systems, photosynthesis plays a dominant role in oxygentation and ammonia conversion with most systems. In addition, energy use and water exchange are typically much less with pond recirculation systems.

V.3. Ultra-intensive culture systems. Although no two pond recirculation system described in the literature to date were identical in all respects, they all had the same objectives of reducing water exchange, reducing settlable solids, and reducing metabolite toxicity (especially nitrogen compounds). Satisfactory achievement of these objectives should result in more successful shrimp culture with less environmental degradation.

Shigueno (1985) describes one of the earliest water recycle systems for ultra-intensive culture of karuma prawn (*P. japonicus*). This system developed during the 1970's used relatively small (≤0.2 ha), outdoor tanks. These circular concrete tanks had "false" bottoms with a sand layer through which water percolated and was collected for recirculation or discharge. The sand bottom functioned as a nitrification site (biofilter) and place for shrimp to burrow. Water flow-through was very high with 300% to 400% of pond volumes per day. Because of high capital and operating costs, these systems were often unprofitable despite very high yields (>35,000 kg/ha/yr) and high prices of U.S.$80/kg for live shrimp for the sushi market (Shigueno 1985, Fast 1992d).

Another ultra-intensive culture system using partial recirculation developed in the U.S. and Mexico about the same time as the Shigueno round culture tanks in Japan was the covered raceway system developed by University of Arizona and others, and described by Colvin (1985), Liao (1986) and Fast (1992d). This system used plastic lined raceways with aeration and partial water recirculation. An inflated plastic cover helped maintain optimal water temperatures. Yields of 70,000 kg/ha/crop with Pacific blue shrimp (*P. stylirostris*) were occasionally realized, but the system was plagued by disease problems of unknown origin, and ultimately proved unprofitable.

Photosynthetic activities were minimal or virtually non-existent with both of the above partial recirculation systems, and they were operated primarily as flow-through systems with only some recirculation.

V.4. Earthen and lined pond, intensive culture systems. More recently, Choroen Pakphand Group (CP) operated a recirculation pond system at their R&D center, Maeklong Area, Thailand (Anonymous 1996a). The system consisted of ten 0.5 ha shrimp culture ponds, two reservoir ponds, and four water treatment ponds (Fig. 8). Source water from a public canal

was pumped into Reservoir Pond I where solids settled. Water then flowed into Reservoir Pond II which supplied water to shrimp growout ponds, which in turn discharged into a drainage system. Water then either went to drain or was pumped into an aeration/sedimentation pond, then through two ponds with filter feeding bivalves such as green muscles (*Mytilus smaragdinus*) or oysters (*Crassostrea sp.*) for algal and suspended solids removal, and then through a sea weed pond (*Graciliaria sp., Polycavernosa sp.*) for nutrient stripping. Reclaimed water then flowed into Reservoir Pond II, and returned to the shrimp growout ponds. The water treatment process reduced suspend organic solids by 30%, ammonia by 90% and nitrites by 60%.

CP later modified this recirculation system as shown in Figure 9 (Anonymous 1996b). The revised configuration included green mussels on stakes in the drainage canal, two sedimentation ponds (A2,A3) following the aeration pond (A1). Sea bass (*Lates calcarifer*) stocked at 2.6 pcs/m^2 In pond A4 resulted in greater dissolved phosphorus concentrations in pond waters and more stable algal blooms. Stable algal blooms were important since healthy, rapidly growing algae absorb NH_3 and CO_2, and create more desirable water quality. Without adequate phosphorus and with senescent algae, algal populations often crash resulting in NH_3 spikes, poor water quality and shrimp mortalities. Using this recirculation system and *P. monodon* stocked at 30 PL/m^2, shrimp yields averaged 8,267 kg/ha/crop after 125 to 130 days culture. At the same time, sea bass yields were 6,288 kg/ha after 100 days culture.

Another, less intricate water circulation system used on a private Thai farm consisted of a supply canal pond, eight shrimp growout ponds ranging from 0.35 to 0.96 ha, and a drainage canal (Fig. 10; Anonymous 1996a). This farm was located in an area that received heavy pollution from industrial estates, factories and households. After the ponds and canals were initially filled, water was not exchanged until after three months, at which time water was circulated from the growout ponds through the drainage and supply canals, back to the growout ponds 3 to 4 times weekly. The canals served as sedimentation and aeration areas. Using this system, the farmer reported a single crop yield of 8,225 kg/ha with a 76% gross profit.

In Indonesia, shrimp pond recirculation systems were developed as a result of poor source water quality and resultant negative impacts on shrimp

yields. A typical Indonesian recirculation system consisted of 50% shrimp culture and 50% water reclamation, on an aerial basis (Fig. 11; Anonymous 1996c). Shrimp pond effluent first flowed through a sedimentation pond, followed by a fish/bivalve pond, and lastly through an aeration pond before return to the shrimp growout ponds. Milkfish (*Chanos chanos*), mullet (*Mugil spp.)* and/or green mussels or oysters were commonly used. The system was operated as closed, or partly-closed. Using this system, average shrimp yields of 8,600 kg/ha/crop were reported after about 145 days culture when stocked at 50 PL/m^2. Milkfish stocking densities were 1,000 pcs/ha.

Menasveta and Jarayabhand (1995) propose an in-pond treatment system (Fig. 12). This system consists of a floating oyster raft from which oysters are suspended on strings or trays. Oysters would remove excess algae and other suspended solids, and provide additional nitrification substrate. They also point out that oysters are preferred to green mussels because of their greater salinity tolerance and higher market value. Four paddlewheel aerators would move water in a circular motion, thus concentrating wastes in the center of the pond.

Another proposed system would consist of an intensive shrimp culture pond within an extensive culture pond (Fig. 13; A.W. Fast, unpublished). Water would enter the intensive culture pond using low-energy water movers (Rogers and Fast 1988), or commonly used push-pumps. The intensive pond would be deeper, and have mechanical aeration such as paddlewheels. Effluent from the intensive pond would return to the extensive pond where suspended solids would settle and nutrients would stimulate primary and secondary productivity for the secondary crop. Shrimp would be cultured at high densities in the intensive pond, while milkfish, mullet, tilapia, bivalves and/or shrimp would be cultured in the extensive pond at lower densities. A major advantage of this pond-in-pond system is that it could be retrofitted to existing extensive ponds without need to create more new pond areas from land now in other uses.

Chien and Liao (1995) describe a some what different conceptual farm concept. Their proposed system includes both water treatment components (bivalves, seaweed, marsh, mangroves and settling), as well as waste water recycling (Fig. 14). The reuse system includes first using reservoir or treated waters for intensive shrimp culture, followed by use in less intensive shrimp culture ponds before treatment and recycling. They also allow for a

wide range of operational options, which are probably beyond the scope of our present ability to operate optimally.

Sandifer and Hopkins (1996) proposed a conceptual model of a sustainable, marine shrimp culture system, which included: water recirculation and renovation; sludge removal, reclamation and use; and polyculture of shrimp, bivalves and fish (Fig. 15). This modular system, with no water discharges builds on research and other experience described in section III above. Their shrimp production goals include shrimp yields of 10,000 kg/ha/crop, a value already exceeded by >40% by McIntosh et al. (1999) in Belize. Each production module would include three 1.0-ha shrimp production ponds, one 1.0-ha water treatment pond for polyculture of bivalves (oysters) and fish (mullet or tilapia), a small phytoplankton inoculation tanks, a sludge and suspended solids settling basin, and a sludge drying bed. Water would be recycled between ponds, with water added to compensate for evaporation and seepage only. Water would be routinely cycled between shrimp production ponds and the polyculture pond. Sludge would be periodically pumped from production ponds through the settling pond where it would consolidate, and then to the drying bed where it would further de-water. Proposed stocking densities were 100 shrimp PL/m^2, oysters at 10^6 seed/ha, and mullet at 7,000 fish/ha. In South Carolina, they anticipated one shrimp crop per year (140-150 day crop cycle), and two year growout with oysters and mullet. Two year classes of oyster and mullet would be stocked, such that one crop each of oysters and mullet could be harvested each year. All sub-components of this system have been shown to work either on commercial farms, or in smaller, experimental trials. Farm management would be intensive, but computer sensing, monitoring and decision-making as proposed by Fast (1991) could assist. Computers could be used to monitor DO, turbidity, BOD, pH, temperature, algal densities, suspended particle sizes, salinity, water levels, and other water quality parameters. Water movement between ponds, and many other water management decisions could be pre-programmed and automated. Although human intervention cannot be replaced in the decision making at this time, computer assisted decision making is probably essential due to the complexities of optimizing such a system.

V.5. **<u>Raceway systems.</u>** Recently, Brune (1998) and Drapcho (1993) described their work at Clemson University with an algal based, Partitioned Aquaculture System (PAS) for freshwater channel catfish (*Ictalurus punctatus*). The PAS has been under development since 1988 and has

resulted in catfish yields of 15,000 to 22,000 kg/ha/yr, compared with about 5,500 kg/ha/yr with conventional, intensive culture of catfish (Tucker and Robinson 1990).

The PSA consists of a fish culture raceway component which is designed to have uniform, well mixed flow with high phytoplankton growth (Fig. 16). This provides high dissolved oxygen concentrations while at the same time reducing ammonia. Following the raceway is a settling area where wastes settle for removal from the system. Tilapia (*Oreochromis niloticus*) and/or other filter feeding fish and plants can also be cultured in this settling areas to further utilize algal production and wastes, and to provide additional cash crops. Highest yields were realized when catfish were co-cultured with tilapia. Oxygen transfer and other control functions are accomplished by computer control of the paddlewheel circulator.

Advantages of the PAS include: low water use and no water discharge; good disease control; good predator control; high crop yields; simplified grading of crop by size; easy crop harvest; and multi-cropping of more than one species. The PAS system has potential application to marine shrimp culture, but has not yet been used.

V.6. <u>Biosecure recirculation systems.</u>

"Biosecurity" is a term only recently used by the shrimp culture community to describe shrimp culture systems designed to prevent disease access into the system, and for maintaining pathogen free shrimp throughout their culture. With few if any exceptions, this means maintaining SPF shrimp in closed life-cycle culture. It also means developing growout systems that prevent disease access by any means, including those identified above in our Introduction. Biosecurity requires no water exchange, and a detailed set of procedures and protocols to prevent disease entry (Moss et al. 1998).

Samocha and Lawrence (1998) proposed two biosecure, growout conceptual designs. The first design consists of four, 20-m diameter, circular ponds of 300 m^2 each (Fig. 17). Each four pond module would be covered by an inflated, plastic sheet (60m x 60m) to prevent access by airborne disease carriers such as birds, insects or other animals. Pond bottoms and sidewalls would be lined with HDPE plastic membrane. In-pond aeration of 120 hp/ha would be provided by paddlewheel aerators and

air injection. There would be zero water exchange during operation, and drain waters at harvest would be discharged to a treatment reservoir for re-use or discharge to receiving waters. Projected yields from a 4-pond module are 2,821 kg/yr of 20-g shrimp. At $8.80/kg this would provide $25,000 gross income/yr. One worker could probably operate at least three or four modules.

A second biosecure, growout design proposed by Samocha and Lawrence (1998) consisted of a raceway with water flows around a central partition (Fig. 18). The bottom and sidewalls of the raceway would be epoxy coated concrete. The raceway would be under a greenhouse cover, and like the circular ponds could operate year around in areas like south Texas. Raceway water would be recirculated, filtered, and treated using sand filtration and a swirl particle separator to remove particulate matter; then ozonated as it returns to the raceway. This system would have no water exchange. Influent and drain waters would be treated the same as with circular ponds. One raceway is expected to produce 990 kg/yr of 20-g shrimp, with gross income of $8,712. One worker should be able to operate at least five of these raceway modules. Moss et al. (1998) and Ogle and Lotz (1998) proposed other biosecure raceway designs.

V.7. <u>Conclusions:</u> Shrimp pond water recirculation and recycling systems are being used, and will continue to be used for all the reasons identified above. However, it is also clear from review of existing literature that while their operating principals are well understood, there is little documentation of operating performance, and more importantly perhaps, design criteria needed to size a given system. This contrasts with conventional recirculation systems where these parameters are much better understood and where design criteria are described (Timmons and Losordo 1995). The reasons for this lack of detail for pond recirculation systems relates partly to uncertainties with performance predictability, and partly due to their relative newness. We anticipate much greater understanding of these pond systems over the next 10 years or so.

While shrimp pond water recirculation and recycling will increase, it is not practical for all shrimp farms. A 1994 farm survey by CP Shrimp Feed Marketing Department revealed that 70% of all intensive farms in Thailand were <1.6 ha (Anonymous 1996d). Most of these farms do not have adequate land area, nor perhaps skill levels to achieve meaningful water

recirculation. With these farms, some in-pond treatment methods seem more appropriate.

VI. PROBIOTICS FOR SHRIMP DISEASE PREVENTION

Probiotics are bacteria, microbes other than bacteria, or cellular products which when added to culture media such as pond or tank water, and/or when ingested by the cultivar species protects that species from pathogenic infections (Jory 1998). Probiotics are not harmful to the cultivar, and may benefit the target species in different ways. Probionts prevent infections by competitive exclusion, anti-microbial actions, or by some other means.

Probionts have been well recognized and used with terrestrial farm animals and humans (Morishita et al. 1971; Muralidhara et al. 1977; Sandine 1977; Gilliland 1979; Ellinger et al. 1980). These applications included ingestion of *Lactobacillus acdiophilus* to prevent pathogenic, intestinal infections. Most recently, the U.S. Food and Drug Administration approved use of a probiotic spray on chickens (Anonymous 1998). The spray is a mixture of 28 non-pathogenic bacteria which is sprayed on chicks soon after hatching. The chicks ingest the bacteria while pecking and cleaning their feathers. These bacteria then become established in chick guts and prevent establishment of pathogenic *Salmonella* and/or other bacteria during the bird's lifetime.

Probiotic applications to aquaculture mostly involve two approaches; pond water additives, and feed additives. Most of these applications have occurred during the past 10 years, with mixed results.

VI.1. Probiotics as pond water additives. There are a variety of microbial-based pond "supplements" marketed today which allege to do many wonderful things for shrimp, and for shrimp pond water quality. These pond water treatments are commonly referred to as "bioaugmentation" (Moriarty 1997). These products include bacterial inoculum, enzyme preparations, and plant extract products. Their manufacturers claim benefits which include such things as; improved water quality, off-flavor prevention, reduced blue-green algal growth, accelerated sediment decomposition, improved cultivar health, and increased crop yields. We know of no evidence substantiating these claims. Mostly, the manufacturers rely on testimonials from satisfied farmers, with little or no

statistical evidence to back-up alleged benefits. Virtually all careful studies show no benefits from these pond water additives (Tucker and Lloyd 1985; Boyd et al. 1994; Boyd 1995b, Boyd and Gross 1999). One possible exception was a recent trial with freshwater channel catfish (*Ictalurus punctatus*) in which Queroz and Boyd (1998) found increased survival in ponds with microbial supplements which consisted of three species of live *Bacillus* bacteria. They could not, however, establish a clear link between improved catfish survival and use of these materials. Even in this latter case, should benefits exist, high cost of the supplement preclude its use in commercial catfish production.

VI.2. Probiotics as feed or feed additives. Bacterial probiotics have been used successfully with oysters, fish and marine shrimp (Gatesoupe 1991; Maeda and Liao 1992; Douillet and Langdon 1994). In each case, the bacteria was ingested by the cultivar species and its survival and/or growth was improved. The following examples demonstrate two successful applications with marine shrimp.

Garriques and Arevalo (1995) isolated non-pathogenic *Vibrio alginolyticus* from raw seawater in Ecuador. *Vibrio alginolyticus* was then batch cultured and added to 25 and 60 m^3 *Penaeus vannamei* larviculture tanks. Control larviculture tanks containing oxytetracycline additions, or with no prophylactic additions were used for comparison. Average survival from the probiotic treatment was 90%, compared with 83% and 74% for antibiotic and no treatment tanks respectively. Water samples from larviculture tanks indicated no pathogenic *V. parahaemolyticus* in tanks receiving the *V. alginolyticus* probiotic, while the other two larviculture tanks both contained *V. parahaemolyticus* in 10% of the water samples. These findings clearly indicate that a probiotic bacteria could provide protection against infections by pathogenic bacteria during larviculture. They did not document, however, potential benefits during pond growout.

Rengpipat et al. (1998) demonstrated similar beneficial effects with black tiger prawns (*Penaeus monodon*). They isolated a *Bacillus* bacterium, designated S11 from marine sediments, ocean waters and from *P. monodon*. This bacterium showed anti-microbial properties in innoculums. It was fed to PL and juvenile shrimp in prepared diet in three forms; fresh, fresh in saline solution, and freeze-dried. Compared with controls, all three forms results in significantly greater ($P<0.05\%$) survival and growth of *P. monodon* during 100 days culture starting from PL-15. Furthermore,

shrimp feed *Bacillus* S11 fortified feeds had significantly greater ($P<0.05$) survival compared with controls when challenged with pathogenic, luminescent bacterium *Vibrio harveyi.* During these 10-day challenge tests using 115 day old *P. monodon*, survival was 100% with shrimp fed Bacillus S11 in all three forms, while survival was only 26% for shrimp not fed thee probiotic (Fig. 19). Similar benefits occurred when *Bacillus* S11 was fed to *P. monodon* during larviculture, although survivals during challenge tests with *V. harveyi* were much reduced for both control and probiotic treated larvae (Rengpipat et al. 1999). In Indonesia, Haryanti and Tsumura (1999) also isolated a bacterium, coded BY-9 and fed this to *P. monodon* during larviculture. Larvae fed BY-9 had 46% survival to PL stage compared with 11% survival for un-inoculated controls. Potential benefits of *Bacillus* S11 or other probionts with *P. monodon* during pond growout to market size was not evaluated.

VI.3. Summary and conclusions: We see no proof that probiotic pond supplements benefit shrimp growout. Probiotic feed supplements, however, have clearly defined benefits during larviculture and early rearing. What is needed now are growout trials in pond settings to document potential benefits of these feed supplements. In particular, we need to know: (1) will probiotic use during larviculture confer protection through growout, or should the probiotic be added to feeds during part or all of growout?, (2) will probiotic bacteria protect against viral pathogens?, and (3) are there other probionts that would be more effective against bacterial and/or viral pathogens? We fully expect commercial applications of probiotic feed additives within one to three years, and significant improvements in their efficacy thereafter.

VII. GENETIC IMPROVEMENTS FOR POND CULTURE

Sustained, world production increases of farmed shrimp requires use of genetically selected and improved shrimp. Genetic traits of interest include such things as; improved survival through disease resistance, improved growth, improved feed conversions, and increased meat yield. These improvements, especially disease resistance are necessary for further growth of the industry. Improved survival alone would result in increased shrimp production without any further increases in farming areas.

Selective breeding and genetic improvements of shrimp require long-term commitments to closed life-cycle culture, an appropriate selection

program (Cedeno et al. 1999), substantial capital investments, and many years wait before profitable returns on investment are realized. Lack of protection for investments is a major impediment for financial commitments by commercial interests. Since seed and/or broodstock sales to other companies is the primary means of recovering investments in genetic improvements, these investments can only be protected if improved seed cannot reproduce. Sale of fertile seed means that people purchasing seed can rear them to broodstock, and produce seed for sale in competition with the original investor. Those purchasing improved, fertile seed derive all the benefits of genetic improvements at a fraction of the cost experienced by the original investors. As a consequence, there is little or no incentive to be the first to make these investments. Sale of sterile seed would protect investments. Triploidy induction is one means of producing sterile seed, but as we will discuss below, mass production of triploid shrimp seed is not yet possible.

Unless means are perfected of inducing sterility in genetically improved marine shrimp seed, we are unlikely to see major improvements in shrimp stocks through genetic manipulations, unless these improvements are supported by governments and made available to industry. Some governments have made commitments in this regard, but as yet we do not see many widespread applications. Most shrimp seed still come from wild-caught broodstock or PL.

VII.1. <u>Closed life-cycle culture</u>. Quantum leaps in shrimp stock improvements are not possible without widespread use of closed-cycle, captive reproduction of shrimp. Continued reliance on wild-caught broodstock and seed could doom the shrimp culture industry to only marginal production increases, while at the same time risking continued disease problems. Crop losses create economic instabilities, and in the long run, higher shrimp prices.

During the past 10 years, perhaps >70% all cultured marine shrimp production consisted of four species: *P. monodon* and *P. orientalis* (fleshy prawn) in Asia; and *P. vannamei* and *P. stylirostrus* in the Americas. Closed life-cycle culture of *P. monodon* has been accomplished (Menasveta et al. 1991, 1993, 1994; Sangpradub et al. 1994; Withyachumnarnkul et al. 1999), but it is not widely used. Commercial culture of *P. monodon* relies almost entirely on wild-caught broodstock and seed. Closed life-cycle culture of *P. vannamei* and *P. stylirostrus* is routinely done in many places

such as the mainland U.S., Hawaii and Tahiti; but again, the vast majority of commercially production with these two species still relies on wild-caught broodstock and seed. Never the less, we are likely to see more genetic improvements in these latter two species because of their more widespread and reliable closed life-cycle culture.

VII.2. Genetic selection. When wild stocks of any species are cultivated by man in closed life-cycle conditions, natural selection still operates even without conscience selection for specific traits (Pullin et al. 1998). When captive-bred populations are also subjected to artificial selection in addition to natural selection, domestication is even more rapid (Doyle 1983). Domestication leads to reduced genetic diversity, but at the same time produces cultivars that perform better under specific culture conditions. Domestication, if it proceeds long enough invariably leads to animals which are unsuitable or unable to survive in the wild.

As we have already indicated, the vast majority of the world's farmed shrimp production comes from wild caught stocks that have no selection for any culture traits beyond the species level. Historically, this approach was necessary since penaeid life-cycles were not closed until about 1940 (Hudinaga 1942). It took another 30 years before this basic knowledge resulted in methods for mass producing marine shrimp seed. Today, most shrimp seed still come from the wild or from wild caught broodstock because these are lower priced. However, there is growing momentum towards greater reliance on hatchery produced seed from closed life-cycle stocks. The primary motivation for this change now is the need for disease free and disease resistant seed. Although that is the primary driving force, closed culture also allows selection, either conscience or un-conscience for other desirable culture traits.

Meaningful selective breeding programs for marine shrimp will of course require long-term commitments to shrimp culture for species of interest, especially *P. vannamei* and *P. monodon*. Breeding goals may differ somewhat depending on region, but two traits have universal importance; growth rate and disease resistance (Gjedrem and Fimland 1995). Although expected growth rate improvements in shrimp through selective breeding programs are unclear at this time, Pullin et al. (1998) indicated, "...rapid and significant positive response to selection for improved growth," for *P. japonicus* in Australia. Wyban (1992) and Benzie et al. (1997) reported indications of genetic variability in growth with *P.*

vannamei and *P. monodon* respectively. These observed variabilities are favorable indications that growth can be increased through selective breeding since selection for any improvement requires variability in the selected trait.

More is know about the effects of selective breeding on fish growth, including growth of salmon, trout and tilapia where increased growth rates of 10% to 23% per generation were found (Gjedrem and Erling 1995). Substantial yield increases have also occurred with other agricultural crops such as hens, pigs and dairy cattle through conventional genetic selection (Eknath et al. 1991). Economic returns on these investments in genetic selection can be substantial. With Atlantic salmon in Norway, a 10% increase in growth coupled with an 8% decrease in early maturation would result in $20 million/yr economic gain when the industry produced 100,000 MT/yr (Gjerde and Olsen 1990). Estimated cost for this selection program was $2.5 million/yr. One-year growth increases of 14.6% have been obtained in Norway through selection (Gjerde 1986).

Selection for disease resistance in marine shrimp has already occurred in the Western Hemisphere and Polynesia. The earliest selection program began in Tahiti during 1980 (Bedier et al. 1998). During 1980, IFREMER imported *P. vannamei* and *P. stylirostris* from Mexico. Culture performance of *P. vannamei* declined during the 1980s due to IHHNV infection, which resulted in runt deformity syndrome (RDS). At the same time, *P. stylirostris* cultured along with these *P. vannamei* gave good culture performance. Tests on *P. stylirostris* confirmed that they carried IHHNV but were not affected by it, since they produced yields of 20 MT/ha/yr under intensive culture. These *P. stylirostris* have now been bred for more than 15 generations in closed life-cycle culture without further imports. During 1993, Tahitian P. stylirostris were reintroduced into Mexico and proved superior to local *P. stylirostris* strains during pond growout comparisons. Tahitian *P. stylirostris* also demonstrated reduced susceptibility to TSV. Another captive *P. stylirostris* population in Venezuela has also proven IHHNV resistant. These strains and others like them could provide excellent SPR stocks for wider culture throughout the Americas.

During 1989, six U.S. institutions of the Gulf Coast Research Laboratory Consortium (known as the Shrimp Consortium) began developing SPF and genetically improved *P. vannamei* (Pruder et al. 1995).

They also developed a second SPF-TSV resistant strain of *P. vannamei*. Culture performance of the original SPF strain proved superior to non-SPF shrimp in Hawaii, Texas and South Carolina with greater growth, survival and yields, with reduced FCR (Beidier et al. 1998). However, large-scale trials in Ecuador proved less impressive, with these SPF shrimp providing poorer performance in may cases compared with non-SPF shrimp. The conclusion was that SPF shrimp perform well in SPF environments, but that most farming areas have little or no means of providing the necessary biosecurity needed during growout. In these areas, SPF shrimp may be more vulnerable, and SPR shrimp would be more appropriate. We also saw from the farm in Belize discussed earlier that the original SPF *P. vannamei* performed very well under SPF conditions, and it outperformed the SPF-SPR *P. vannamei* strain with respect to growth, survival and yields (Table 7).

Although SPR shrimp are not widely used yet, Rosenberry (1999b) reported that, "...at least two domesticated, genetically selected SPR strains of this species (*P. stylirostris*), which are resistant to IHHNV, are currently being developed and marketed in the Americas. In some regions, these SPR stocks of TSV and IHHNV resistant *L. stylirostris* are replacing *L. vannamei* stocks in culture." There is also reason to believe that some wild stocks have developed at least partial resistance to certain viruses in areas where these viruses caused earlier, widespread disease problems with cultured stocks (Rosenberry 1999b, Jory 1999b).

In Asia, domestication and selective breeding of *P. mondon* has just begun (Withyachumnarnkul et al. 1999). The ultimate goal is to provide SPF and SPR *P. mondon* seed at competitive prices. Realization of this goal will take perhaps five years or more. In the meantime, the strategy for dealing with WSSV and YHV are based on prevention by excluding the viruses and their carriers from culture ponds. The exclusion process begins with assessment of disease status of wild caught broodstock using sophisticated analytical techniques, including polymerase chain reaction (PCR) and others. Presently, only PCR provides reliable WSSV detection in broodstock, larvae and PL. Shrimp that test positive are eliminated. Farmers typically require a clean bill of health from the PL provider before they purchase PL and stock their ponds. Exclusion procedures are also practiced at farms. Farm management practices include: (a) quick lime (CaO) soil applications between crops to disinfect pond bottoms and to kill disease carriers; (b) disinfecting of pond source waters with chlorine and/or

short duration pesticides to kill disease carriers; (c) stocking disease-free PL at lower densities to reduce stress; (d) feeding only prepared (sterile) feeds; e. not exchanging water during the first two months after stocking PL; and (f) should infections occur, they take steps to prevent its spread to other ponds on the same farm, and/or to other farms in the area. Farmers who practice the shrimp farmer's code of ethics are required to notify farmers in their area who share common source waters, of disease outbreaks on their farm. These neighboring farmers are advised to stop pumping water into their ponds for at least one week while the infected farm decontaminates and disinfects its effluent waters. These disease exclusion practices, while not fail-safe, have proven effective. Shrimp production in Thailand increased during 1998/99, at least in part due to these disease management practices.

VII.3. <u>Ploidy induction</u>. Triploid animals are those with an added set of chromosomes (3 sets) rather than the normal two sets (diploid). This chromosome set addition is usually achieved through temperature, pressure or chemical shock treatments soon after fertilization to force 2nd polar body re-absorption into the fertilized egg (Purdon 1983). The 2nd polar body is produced during egg meiosis and contains a full chromosome set. However, shock induced triploidy is seldom 100% effective. An alternative method of triploidy induction is crossbreeding diploid (2N) animals with tetraploid (4N) animals to produce 100% triploids (3N). Tetraploid animals are produced by shock treatment just before first cell division, following egg fertilization and after the 2nd polar body has been lost (Myers 1986). At this time, both sets of (2N) chromosomes have replicated (4N) in preparation for first cell division. Shock at this time disrupts cell division and results in an organism with four chromosome sets (4N) in all its cells.

The single most important, likely benefit of triploidy with penaeid shrimp culture is seed sterility. Production and sale of sterile seed assures seed producers that their investment is protected from pirating. Without such assurance, a potential investor would be hesitant to make the substantial investment necessary to produce closed life-cycle, genetically improved shrimp stocks and seed. We are unsure at this time whether triploid penaeid shrimp will perform any better or worse than normal diploids in growout culture. Likewise, culture performance of tetraploid shrimp is unknown. With fish, triploids often have superior culture performance compared with diploids, including increased growth, lower FCR, and greater percentage yield of edible flesh (Fast et al. 1995, Qin et al.

1999); while tetraploid fish normally do not grow well. Perhaps the greatest value of tetraploid shrimp is their use in crossbreeding with diploids to produce 100% triploids.

Sterile shrimp seed have other benefits besides protection of investment in stock improvements. These added benefits include reduced risks from escapement of exotic shrimp species, and/or escapement of genetically altered or selected shrimp within their known range (Arthington and Blü hdorn 1998). In the first case, exotic shrimp that are fertile could potentially become established to the detriment of indigenous shrimp species. In the second case, inter-breeding of wild stocks with "improved" culture stocks could potentially result in undesirable, genetic impacts on wild shrimp.

Ploidy induction in penaeid shrimp is more difficult to achieve than with fishes. This difficulty is caused by the fragile nature of shrimp eggs soon after spawning. Newly spawned eggs are encased in a gelatinous layer that takes about 15 minutes to form into a semi-rigid membrane around the egg (Hudinaga 1942). Triploidy induction requires shock treatments during the first few minutes, at which time eggs are very sensitive to any handling. Furthermore, the rigid membrane that forms later is also sensitive to handling before first cell division. Never the less, several researchers claim to have produced triploids, mostly using chemical shocks for 2nd polar body retention. Triploidy was reportedly induced in Chinese fleshy prawn (*P. orientalis*) using cytocalacin B (CB) and 6-DMAP chemical shock treatments (Bao et al. 1993; Xiang et al. 1999), and with karuma prawn (*P. japonicus*) in Australia using temperature and chemical shocks (Nigel Preston, personal communications 1997). Preston indicated that they also produced tetraploid *P. japonicus* by these same means. The senior author of our present paper successfully induced tetraploidy in *P. vannamei* during 1999 using temperature shock (A.W. Fast, unpublished data). We know of no reported cases where triploid or tetraploid penaeid shrimp were cultured to adult size, and where their culture performance was evaluated. However, chances are good that the efficacy of large-scale ploidy induction in penaeids will be fully explored during the next few years. If these efforts prove successful, ploidy induction tactics will stimulate greater investments in genetic improvements of shrimp stocks, and they will reduce environmental risks associated with use of exotic or genetically selected or altered shrimp.

VII.4. Transgenic shrimp. Plants and animals which have genes (DNA) permanently incorporated into their genetic material are called transgenic. Genes are usually inserted at the one cell stage of eggs or sperm just before fertilization, or into the one or two cell stage just after fertilization. Genes are usually inserted using microinjection, retrovirus infection, particle gun bombardment, or electrical techniques (Chen et al. 1995). Growth hormone gene insertions are the most common transgenic transformations with fishes. Often, inserted genes do not become incorporated into chromosomes soon after they are inserted, but remain as extra-chromosomal units. If they incorporate into chromosomes during later development at the multi-cellular stage, this usually results in transgenic mosaics where only some cells have genes integrated into chromosomes. Delayed transgene integration and resultant mosaics are detected by mating these animals with animals which have not been injected with transgenes. If gene insertions were successfully incorporation into at least one chromosome of all cells, then at least 50% of the resultant crossbred offspring will carry at least one transgene. "Stable integration of the transgenes is an absolute requirement for continuous vertical transmission to subsequent generations and establishment of a transgenic fish line" (Chen et al. 1995).

Since 1992, dozens of transgenic plant cultivars have been approved for agricultural uses in the U.S., and perhaps hundreds more are under development (Yoon 1999). Transgenic plants use genes taken from viruses, bacteria, insects and other animals. During 1999, 20% to 45% of U.S. production of corn, soybeans and cotton were from transgenic plants. While progress with transgenic plants has been phenomenal during the past 10 years, there has as yet not been a single transgenic animal approved for human consumption by the U.S. Food and Drug Administration. Although many transgenic aquatic species have been produced, none have passed all the necessary certifications.

Transgenic work with crustaceans is in its infancy (Bachere et al. 1997). To date, almost all transgenic work with animals has involved nematodes, fruit flies, sea urchins, frogs, laboratory mice and farm animals (Chen et al. 1995). Transgenic research with marine shrimp is particularly difficult since successful outcomes rely on a number of necessary conditions, many of which are not met, including; closed life-cycle culture of shrimp over many generations, basic knowledge about a shrimps genome, basic knowledge about genetic make-up of shrimp disease bacteria and viruses, and reliable methods of inserting transgenes and their promoters into

shrimp genomes. This work is made even more difficult by the fact that as yet, there are no proven techniques for culturing crustacean cell-lines. If these difficulties can eventually be overcome, there are some advantages to producing transgenic shrimp from shrimp strains bred using conventional genetic selection methods. One of the main advantages is that transgenic transformations can be achieved without otherwise altering the shrimp's genetic make-up (Bachere et al. 1997). Conventional genetic selection strategies often result in counter-selection problems and loss of some desirable traits.

In addition to technical problems producing transgenic shrimp, there are also potential environmental, social and ethical concerns (Kapuscinski and Hallerman 1990a, Kohler et al. 1992, Yoon 1999). Environmental concerns include potential impacts connected with escapement of transgenic shrimp from commercial culture and/or research sites, as discussed above. The American Fisheries Society (Kapuscinski and Hallerman 1990b) is on record as urging caution with use of all transgenic fishes; and secondly recommends using sterile transgenic fish in commercial aquaculture. These same cautions apply to other aquatic cultivars such as shrimp. In addition to environmental concerns, there is considerable consumer concern about use of transgenic animals for food production. In Europe, consumer concern has resulted in public policies against importation and sale of transgenic food items. This policy has already impacted U.S. grain sales to Europe, and should be carefully assessed before large-scale production of transgenic shrimp is undertaken.

VII.5. Summary. Increased, stable world production of farmed shrimp ultimately requires large-scale use of domesticated and genetically improved shrimp for culture. Required genetic improvements include; disease resistance, increased survival and growth rates, better FCR, and perhaps increased meat yields. Use of disease resistant shrimp with increase survival and growth would greatly increase world shrimp production from existing farms without any further increases in farming areas. A fundamental requirement for these genetic improvements is use of closed life-cycle culture of shrimp species of interest, and selective breeding programs that achieve desired results. Closed life-cycle culture of SPF and SPR (for IHHNV and TSV) is most advanced with *P. vannamei* and *P. stylirostris*. We anticipate greatest near-term advances with these two species, and even more widespread use of hatchery reared, SPF and SPR seed. Genetic selection programs are currently underway for other

improvements and should provide measurable benefits within the next few years. Work with transgenic shrimp is still in its infancy, and many years away from possible commercial applications. With all genetically selected or altered shrimp, there are serious environmental, social and ethical concerns that must be addressed.

VIII. STATUS OF POND INNOVATIONS

We have reviewed some of the more interesting and relevant shrimp pond innovations that became prominent during the past 10 years or so. Some of these innovations are still evolving and developing, while others are either now adopted or are well on their way to widespread application. We have not reviewed every innovation, of course, but have tried to narrow our review to those that we felt had special relevance. We focused on those innovations that should help sustain healthy and profitable shrimp farming into the next millenium.

Clearly, environmental and sustainability issues became more important during the past 10 years with shrimp pond culture (Table 11). Shrimp culturists, researchers, government officials, politicians, and the general public all now have heightened awareness of potential environmental issues concerning aquaculture in general and shrimp farming in particular. Although this resulted in initial discomfort for the industry, it was a healthy development since it focused attention on ways to improve culture techniques that not only improve culture efficiencies, but also preserve and enhance the environment. This trend will continue.

Zero water exchange with intensive shrimp culture is a new concept that was perhaps unthinkable 10 to 15 years ago. It is now widely practiced with many inland shrimp farms. We are still learning how to optimize zero water exchange and water recycling with low feed protein levels, sludge management, and other aspects of pond management at yields >15,000 kg/ha/crop. However, proof now exists that these yields can be sustained while minimizing disease risk and environmental impacts.

Probiotics feed additives can prevent shrimp diseases and will become widely used in both hatchery and ponds. To date, most probiotic applications were in hatcheries, but we expect more widespread use in ponds as feed additives during the next three years. We do not expect

probiotic, pond water supplements or "bioaugmentation" of pond waters to produce many benefits in the near future. They have not as yet.

Disease free (SPF) and high health shrimp are and will be used on an even larger scale as culture systems are built to make optimal use of these shrimp strains. Disease resistant (SPR) shrimp are and will be used on existing farms more widely as they become available from hatcheries. Both SPF and SPR seed require long-term commitments to closed life-cycle culture, which will also promote other genetic selections and improvements in shrimp culture stocks. Triploidy induction is a promising means of protecting investments made for these genetic improvements, while at the same time reducing environmental risks. If mass production techniques for triploid seed are perfected, we expect to see widespread applications within 3 to 10 years. Work is also progressing with transgenic shrimp, but we do not anticipate any major applications within the next 10 years.

IX. ACKNOWLEDGEMENTS

The authors thank the Thailand Research Fund for the support of this study.

IX. LITERATURE CITED

Accion Ecologica, et al. 1997. Choluteca Declaration. World Aquaculture 28:38-39

Adey WH, Loveland K (1991) Dynamic aquaria. Academic Press, San Diego, CA, 643 p.

Allen LJ, Kinney EC (1981) Proceedings of the bio-engineering symposium for fish culture. American Fish. Soc., Bethesda, MD, 307 p.

Allen, G.L. and G.B. Maguire. 1993. The effects of water exchange on production of *Metapenaeus macaleayi* and water quality in experimental pools. Jour. World Aquaculture Soc. 24:321-328

Anonymous (1987) Practical koi keeping. Koi USA, Midway City, CA, 191 p.

Anonymous. 1997. The FAO Fisheries Department Code of Conduct for Responsible Fisheries. World Aquaculture 28:40-41

Anonymous (1996a) The recirculation system, new technology for shrimp culture. In: Lin, CK, Nash, GL (compilers), Asian Shrimp News Collected Volume 1989-1995. Asian Shrimp Culture Council, C.P. Tower, 313 Silom Rd., Bangkok 10500, p 44-46

Anonymous (1996b) The recycle system for shrimp culture. In: Ibid. p 52-54

Anonymous (1996c) Successful Indonesian experience in shrimp-fish integration in recycle system. In: Ibid. p 56-58

Anonymous (1996d) Update on shrimp farm size, distribution and feed performance in Thailand. In: Ibid. p 91-92

Anonymous. 1997. The FAO Fisheries Department code of conduct for responsible fisheries. World Aquaculture 28:40-42

Anonymous. 1998. Salmonella has met its match. Honolulu Star-Bulletin, Honolulu, Hawaii. (March 19):A-7

AQUACOP. 1984. Review of ten years of experimental penaeid shrimp culture in Tahiti and New Caledonia (South Pacific). Jour. World Mariculture Soc. 15:73-91

Arthington, A.H. and D.R. Blühdorn. 1998. The effects of species interactions resulting from aquaculture operations. Asian Fisheries Soc. 11:71-95

Bachère, E., V. Cedeno, C. Rousseau, D. Destoumieux, V. Boulo, J.P. Cadoret and E. Mialhe. 1997. Transgenic crustaceans. World Aquaculture (December):51-55

Bailey, C. 1997. Aquaculture and basic human needs. World Aquaculture 28:28-31

Bao, Z.M., Q.Q. Zhang, H. Wang, J. Gao and J.X. Dai. 1993. Studies on the triploid induction of Penaeus orientalis II. Cytocalasin B treatment. Acta Oceanologica Sinica 15(3):101-106

Bardach, J.E. 1997. Fish as food and thee case for aquaculture. In: Bardach, J.E. (Ed.), Sustainable Aquaculture, John Wiley & Sons, Inc., New York, 1-14 p.

Bédier, E., J.C. Cochard, G.L. Moullac, J. Patrois and AQUACOP. 1998. Selective breeding and pathology in penaeid shrimp culture: the genetic approach to pathogen resistance. World Aquaculture 29(2):46-52

Benzie, J.A.H., M. Kenway and A.T. Bechkenbach. 1997. Estimates of the heritability of size in juvenile Penaeus mondon prawn half-sib matings. Aquaculture 152:49-53

Borgstrom, G. 1962. Fish as food. Academic Press, New York. 777 p.

Boyd, C.W., W.D. Hollerman, J.A. Plumb and M. Saeed. 1984. Effect of treatment with a commercial bacterial suspension on water quality in channel catfish ponds. The Progressive Fish-Culturist 46:36-40

Boyd, C.E. 1995a. Bottom soils, sediment, and pond aquaculture. Chapman and Hall, New York.

Boyd, C.E. 1995b. Chemistry and efficacy of amendments used to treat water and soil quality imbalances in shrimp ponds. In: Browdy C.L.and J.S. Hopkins (eds.), Swimming through troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, pp. 183-199

Boyd, C.E. 1999a. Code of practice for responsible shrimp farming. Global Aquaculture Alliance, St. Louis, MO USA. 48 pgs.

Boyd, C.E. 1999b. Aquaculture sustainability and environmental issues. World Aquaculture 30:10-13, 71-72

Boyd, C.E. and A. Gross. 1999. Use of probiotics for improving soil and water quality in aquaculture ponds. In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. pp. 101-106

Brock, J.A., R.B. Remedios, D.V. Lightner and K. Hasson. 1997. Recent developments and an overview of Taura Syndrome of farmed shrimp in the Americas. In: T.W. Flegel and I.H. MacRae (eds), Diseases in Asian Aquaculture III. Fish Health Section, Asian Fisheries Society, Manila, Philippines. pp. 275-283.

Browdy, C.L., D. Bratvold, J.S. Hopkins and P.A. Sandifer. 1997. Aspects of microbial ecology and shrimp pond management. In: D.E. Alston, B.W. Green and H.C. Clifford (eds), IV Symposium on aquaculture in Central America: focusing on shrimp and tilapia, 22-24 April 1997, Tegucigalpa, Hondouras. Asociacion Nacional de Acuicultores de Hondouras and the Latin American Chapter of the World Aquaculture Society. pp. 138-139

Browdy, C.L. and D. Bratvold. 1998. Preliminary development of a biosecure shrimp production system. In: S.M. Moss (ed), Proceedings of the US Marine Srhimp Farming Program, Biosecurity Workshop, Feb. 14, 1998, Oceanic Institute, Waimanalo, HI. pp. 19-38

Brown, J.J. and E.P. Glenn. 1999. Reuse of highly saline aquaculture effluent to irrigate a potential forage halophyte, *Suaeda esteroa.* Aquacultural Engineering 20:91-111

Brune DE (1998) Recirculation in photosynthetic aquaculture systems. Aquaculture Magazine 24(3):63-71

Brune, D.E. and C.M. Drapcho. 1991. Fed pond aquaculture. In: Aquaculture systems engineering: proceedings of the World Aquaculture Society and the American Society of Agricultural Engineers Jointly Sponsored Session. American Society of Agricultural Engineers, St. Joseph, MI. p. 15-33

Cedeño, V., E. Bachère, J. Rodriguez, E. Bédier, E. Goyard, V. Boulo, J.P. Cadoret and E. Mialhe. 1998. Quantitative genetics and genetic transformation for the selection of pathogen-resistant shrimp. In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum,

Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. pp. 61-66

Chamberlain, G. 1998. The feeds industry assessment of Biosecure systems. In: S.M. Moss (ed), Proceedings of the US Marine Srhimp Farming Program, Biosecurity Workshop, Feb. 14, 1998, Oceanic Institute, Waimanalo, HI. pp. 81-84

Chen, T.T., J.K. Lu, M.J. Shamblott, C.M. Cheng, C.M. Lin, J.C. Burns, R. Reimschuessel, N. Chatakondi and R.A. Durham. 1995. Transgenic fish: ideal models for basic research and biotechnological applications. Zoological Studies 34(4):215-234

Chiang, P. and I.-C. Liao. 1985. The practice of grass prawn (Penaeus monodon) culture in Taiwan from 1968 to 1984. Jour. World Mariculture Soc. 16:297-315

Chien YH, Liao IC (1995) Integrated approach to prawn growout system design. In: Browdy CL, Hopkins JS (eds.), Swimming through troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, p 167-182

Chong, V.C. and A. Sasekumar. 1981. Food and feeding habits of the white prawn *Penaeus merguiensis*. Mar. Ecol. Prog. Ser. 5:185-191

Clifford, H.C. 1985. Semi-intensive shrimp farming. In: Chamberlain GW, Halby MG, Midget RJ (eds.), Texas Shrimp Farming Manual. Texas Agricult. Ext. Service, Texas A&M Univ., College Station, TX, IV-1-IV-14 p

Clifford, H.C. 1992. Marine shrimp pond management: a review. In: J. Wyban (ed), 1992 Proceedings of the special session on Shrimp Farming, World Aquaculture Soc., Baton Rouge, LA. Pp. 110-137

Colt J, White RJ (1991) Fisheries bioengineering symposium. American Fish. Soc., Bethesda, MA

Colvin LB (1985) Intensive growout systems for shrimp. In: Chamberlain GW, Halby MG, Midget RJ (eds.), Texas Shrimp Farming Manual.

Texas Agricult. Ext. Service, Texas A&M Univ., College Station, TX, IV-1-IV-14 p

Costanza, R. 1991. Ecological economics: the science and management of sustainability. Columbia University Press, New York.

Csavas, I. 1988. Shrimp farming in Asia. Presented at Seventh Session on the IPFC Working Party of Experts on Aquaculture. FAO Regional Office for Asia and thee Pacific, Bangkok, Thailand. IPFC WPA/11

Cummins, K.W., C.E. Cushing and G.W. Minshall. 1995. Introduction: an overview of stream ecosystems. In: C.E. Cushing, K.W. Cummins and G.W. Minshall (eds), River and stream ecosystems, Ecosystems of the World 22, Elsevier, Amsterdam. pp. 1-8

Dall, W. 1968. Food and feeding of some Australian penaeid shrimp. F.A.O. Fish. Rep. 57:251-258

Doyle, R.W. 1983. An approach to the quantitative analysis of domestication in aquaculture. Aquaculture 33:167-185

Drapcho CM (1993) Modeling of algal productivity and diel oxygen profiles in the partitioned aquaculture system. Ph.D. thesis, Dept. of Agri. & Biol. Engr. Dept., Clemson Univ., Clemson, SC.

Douillet, P.A. and C.J. Langdon. 1994. Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). Aquaculture 119:25-40

Eknath, A.E., H.B. Bentsen, B. Gjerde, M.M. Tayamen, T.A. Abella, T. Gjedrem and R.S.V. Pullen. 1991. Approaches to national fish breeding programs: pointers from a tilapia pilot study. NAGA, the ICLARM Quarterly 723:10-12

Ellinger, D.K., L.D. Muller and P.J. Glantz. 1980. Influence of feeding fermented *Colostrum* and *Lactobacillus acidophilus* on fecal flora of dairy calves. Jour. Dairy Science 63:478-482.

FAO. 1991. Environmental and sustainability in fisheries. COFI/91/3. Document presented at the 19th Session of the Committee on Fisheries.

April 1-12. Food and Agricultural Organization of the United Nations, Rome. 23 p.

FAO. 1995a. Codes of conduct for responsible fisheries. FAO, Rome, Italy. 41 p.

FAO. 1995b. The state of world fisheries and aquaculture. FAO, Rome, Italy. 57 p.

FAO. 1995c. Fisheries statistics, catches and landings. FAO Fisheries Series 76, Rome, Italy. 41 p.

Fast, A.W., T. Pewnim, R. Keawtabtim, R. Saijit and R. Vejaratpimol. 1995. Comparative growth of diploid and triploid Asian catfish (*Clarias macrocephalus*) in Thailand. J. World Aquaculture Soc. 26:390-395

Fast, A.W., Y.C. Shang, I.C. Liao and G.L. Roger. 1989. Description of Taiwan intensive shrimp culture farms, and simulated transfer to Hawaii. Univ. Hawaii Sea Grant College Program Rep. Series, UNIHI-SEAGRANT-ME 89-02, 94 pgs.

Fast AW (1991a) Marine shrimp pond growout conditions and strategies: a review and prognosis. Reviews in Aquatic Sciences 3:357-399

Fast, A.W. 1991b. Development of appropriate and economically viable shrimp pond growout technology in the United States. pp. 197-240. *In:* P.F. DeLoach, W.J. Dougherty and M.A. Davidson (eds). Elsevier Science Publishers, Amsterdam, The Netherlands.

Fast AW (1992a) Penaeid growout systems: an overview. In: Fast AW, Lester LJ (eds). Marine Shrimp Culture: Principles and Practices. Elsevier Science Publishers, Amsterdam. 347-356 p

Fast AW (1992b) Penaeid semi-intensive growout systems. In: Ibid. 371-382 p

Fast AW (1992c) Penaeid intensive growout systems. In: Ibid. 383-392 p

Fast AW (1992d) Penaeid ultra-intensive growout systems. In: Ibid. 393-402 p

Fast, A.W. and L.J. Lester. 1992. Future of world shrimp culture. In: Ibid. 839-851 p

Fast, A.W., Y.C. Shang, K. Wanitprapha and P. Hanvivatanakit. 1995. Marine shrimp culture and trawling in Thailand during 1985/86: characterization and financial comparisons. J. Aquaculture in the Tropics 10:319-336

Fast, A.W. and P. Menasveta. 1998. Some recent innovations in marine shrimp pond recycling systems. In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. pp. 87-92

Flaherty, M. and P. Vandergeest. 1998. "Low salt" shrimp aquaculture in Thailand: goodby coastline hellow Khon Kaen! Environmental Management 22:817-830

Flegel, T.W., S. Sriurairatana, C. Wongteerasupaya, V. Boonsaeng, S. Panyim and B. Withyachumnarnkul. 1995. Progress in characterization and control of yellow-head virus of Penaeus monodon. In: Browdy C.L.and J.S. Hopkins (eds.), Swimming through troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, pp. 76-83

Folke C, Kautsky N (1992) Ecological economic principles for aquaculture development. In: Cowey CB, Cho CY (eds), Feeding fish in our water: nutritional strategies in management of aquaculture waste. Univ. of Guelph, Ontario, Canada

Garriques, D. and G. Arevalo. 1995. An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. In: Browdy C.L.and J.S. Hopkins (eds.), Swimming through troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, pp. 53-59

Garza, J.R., K.W. Hasson, B.T. Poulos, R.M. Redman, B.L. White and D.V. Lightner. 1997. Demonstration of infectious Taura Syndrome Virus in the feces of seagulls collected during an epizootic in Texas. Jour. Of Aquatic Animal Health 9:156-159

Gatesoupe, F.G. 1991. *Bacillus* sp. Spores: a new tool against early bacterial infection in turbot larvae, *Scophthalmus maximus*. In: P. Lavens, P. Sorgeloos, E. Jaspers and F. Ollevier (eds), Larvi '91-Fish and crustacean larviculture symposium. European Aquaculture Society, special publication No. 15, Ghent, Belgium. pp. 409-411.

Gjerde, B. 1986. Growth and reproduction in fish and shellfish. Aquaculture 57:37-55

Gjerde, B. and B. Olsen. 1990. Economic value of breeding program (Ø konomisk verdi av avlsarbeidet). Aktuelt fra Statens Fagtjeneste for Landbruket, Husdyrforsøtet 13-14 februar 1990:61-65

Gilliland, S.E. 1979. Beneficial interrelationships between certain microorganisms for use as dietary adjunts. Jour. Food Protection 42:164

Glantz, M.H. and J.D. Thompson. (eds). 1981. Resource management and environmental uncertainty; lessons from coastal upwelling fisheries. John Wiley & Sons, New York

Gleason, D.F. and G.M. Wellington. 1988. Food resources of postlarval brown shrimp (Penaeus aeus aztecus) in a Texas salt marsh. Mar. Biol. 97:329-337

Glenn, E.P., J.J. Brown and J.W. O'Leary. 1998. Irrigating crops with seawater. Scientific American. (August)

Gjedrem, T. and E. Fimland. 1995. Potential benefits from high health and genetically improved shrimp stocks. In: Browdy C.L.and J.S. Hopkins (eds.), Swimming through troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, pp. 60-65

Hambrey, J. 1996. The mangrove question. Asian Shrimp News, Asian Shrimp Culture Council, Bangkok, Thailand, Issue No. 26, 2nd Quarter.

Hargreaves, J.A. 1997. The quest for sustainable shrimp aquaculture: a moderator's perspective. World Aquaculture 28:43-47

Haryanti, K.S. and S. Tsumura. 1999. Use of BY-9 as a probiotic agent in the larval rearing of *Penaeus monodon*. In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. p. 183

Hill, B.J. 1990. Food and feeding. In: J.H.S. Blaxter and A.J. Southward (eds), The biology of the Penaeidae. Advances in Marine Biology, Vol. 27. Academic Press, New York. pp. 315-332

Hindley, J.P.R. and C.G. Alexander. 1978. Structure and function of the chelate pereiopods of the banana prawn *Penaeus merguiensis*. Mar. Biol. 48:153-160

Hirasawa, Y. 1985. Economics of shrimp culture in Asia. In: Y. Taki, J.H. Primavira and J.A. Llobrera (eds), Proceedings 1st International Conference on the Culture of Penaeid Prawns/Shrimp. Southeast Asian Fisheries Development Center, Iloilo, Philippines. p. 131-150

Hopkins, J.S., R.D. Hamilton II, P.A. Sandifer, C.L. Browdy and A.D. Stokes. 1993. Effects of water exchange rate on production, water quality, effluent characteristics and nitrogen budgets of intensive shrimp ponds. Jour. World Aquaculture Soc. 24:304-320

Hopkins, J.S., P.A. Sandifer and C.L. Browdy. 1994. Sludge management in intensive pond culture of shrimp: effect of management regime on water quality, sludge characteristics, nitrogen extinction, and shrimp production. Aquacultural Engineering 13:11-30

Hopkins, J.S., P.A. Sandifer and C.L. Browdy. 1995a. A review of water management regimes which abate the environmental impacts of shrimp farming. In: Browdy CL, Hopkins JS (eds.), Swimming through

troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, p 157-166

Hopkins, J.S., P.A. Sandifer and C.L. Browdy. 1995b. Effects of two feed protein levels and feed rate combinations on water quality and production of intensive shrimp ponds operated without water exchange. J. World Aquaculture Soc. 26(1):93-97

Hopkins, J.S., J.D. Holloway, P.A. Sandifer and C.L. Browdy. 1997. Results of recent controlled comparisons of intensive shrimp ponds operated with and without water exchange. In: D.E. Alston, B.W. Green and H.C. Clifford (eds), IV Symposium on aquaculture in Central America: focusing on shrimp and tilapia, 22-24 April 1997, Tegucigalpa, Hondouras. Asociacion Nacional de Acuicultores de Hondouras and the Latin American Chapter of the World Aquaculture Society. pp. 157-158

Hudinaga, M. 1942. The study of Penaeus-I. The development of Penaeus japonicus Bate. Japanese J. Zoology 10:305-393

Huguenin J, Colt J (1989) Design and operation guide for aquaculture seawater systems. Elsevier, Amsterdam.

Jory, D. 1998. World shrimp farming in 1997. Aquaculture Magazine Buyer's Guide. 27:32-41

Jory, D. 1999a. A review of world shrimp farming in 1998. Aquaculture Magazine Buyer's Guide. 25:40-59

Jory, D. 1999b. Shrimp whitespot virus in the Western Hemisphere. Aquaculture Magazine 25(3):83-91

Kapuscinski, A.R. and E.M. Hallerman. 1990a. Transgenic fish and public policy: anticipating environmental impacts of transgenic fish. Fisheries (Bethesda) 15(1):2-11

Kapuscinski, A.R. and E.M. Hallerman. 1990b. AFS position statement on transgenic fishes. Fisheries (Bethesda) 15(4):2-4

Kohler, C.C., W.L. Muhlach, P.C. Phillips and G.A. Paleudis. 1992. Environmental and ethical concerns associated with transgenic fishes. Jour. World Aquaculture Soc. 23:97

Leber, K.M. and G.D. Pruder. 1988. Using experimental microcosms in shrimp research: the growth-enhancing effect of shrimp pond water. Jour. World Aquaculture Soc. 19:197-203

Liao IC (1986) General introduction to the prawn system in Taiwan. Aquacultural Engineering 5:219-233.

Lightner, D.V. 1983. Diseases of cultured penaeid shrimp. In: J.P. Mcvey (ed.), CRC Handbook of mariculture: volume I, crustacean aquaculture. CRC Press, Boca Raton, Florida. pp. 289-320

Lightner, D.V., C.T. Fontaine and K. Hanks. 1975. Some forms of gill disease in penaeid shrimp. Proceedings World Mariculture Soc. 6:347-365

Lightner, D.V., R.M. Redman, B.T. Poulos, L.M. Nunan, J.L. Mari and K.W. Hasson. 1997. Status of the major virus diseases of concern to the shrimp farming industries of the Americas: known distributions, hosts and available detection methods. In: D.Ee. Alston, B.W. Green and H.C. Clifford (eds), IV Symposium of Aquaculture in Central America: focusing on shrimp and tilapia, April 22-24, Tegucigalpa, Honduras. Asociacion Nacional de Acuicultores de Honduras, Tegucigalpa, Honduras. pp. 36-48

Lightner, D. 1998. History of Marine Culture Enterprises. In: S.M. Moss (ed), Proceedings of the US Marine Srhimp Farming Program, Biosecurity Workshop, Feb. 14, 1998, Oceanic Institute, Waimanalo, HI. pp. 71-80

Lin, C.K. 1995. Progression of intensive marine shrimp culture in Thailand. In: Browdy C.L.and J.S. Hopkins (eds.), Swimming through troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, p 13-23

Ling, S.H. 1977. Aquaculture in Southeast Asia: a historical overview. University of Washington Press, Seattle

Lockwood, G.S. 1997. World shrimp production with environmental and social accountability: a perspective and proposal. World Aquaculture 28:52-55

Losordo TM (1998a) Recirculating aquaculture production systems: the status and future. Aquaculture Magazine (Jan./Feb.):38-45.

Losordo TM (1998b) Recirculating production systems: the status and future, part II. Aquaculture Magazine (March/April):45-53.

Macintosh, D.J. and M.J. Phillips. 1992. Environmental issues in shrimp farming. In: H. de Saram and T. Singh (eds), Shrimp '92, Proceedings of the 3rd Gobal Conference on the Shrimp Industry. Infofish, Kuala Lumpur, Malaysia.

Maeda, M. and I.C. Liao. 1992. Effect of bacterial population on the growth of prawn larvae, *Penaeus monodon.* Bulletin of the National Research Institute of Aquaculture 21:25-29

Marsh, J.S. 1998. Forced entry to the Garden of Eden. Biologist 45(3):144

McIntosh, R.P. D.P. Drennan and B.M. Bowen. 1999. Belize aquaculture: development of an intensive sustainable, environmentally friendly shrimp farm in Belize. In: B.W. Green, H.C. Clifford, M. McNamara and C.M. Montrain (eds), V Central American Symposium on Aquaculture, 18-20 August 1999, San Pedro Sula, Honduras, Asociacion Nacional de Acuicultores de Hondouras, the Latin American Chapter of the World Aquaculture Society, and Pond Dynamics/Aquaculture Collaborative Research Support Program, Choluteca, Honduras. pp. 85-99

Menasveta, P., S. Sangpradub, S. Piyatiratitivorkul and A.W. Fast. 1991. Gonadal maturation and spawning of giant tiger shrimp (*Penaeus monodon* Fabricius) in Thailand related to broodstock size and source. pp. 245-246. *In:* P. Lavens, P. Sorgeloos, E. Jaspers and F. Ollevier (eds). Larvi '91, Proceedings of Symposium on Fish and Crustacean Larviculture, Gent, Belgium. Aug. 27-30, 1991.

Menasveta, P., S. Piyatiratitivorakul, S. Rungsupa, N. Moree and A.W. Fast. 1993. Gonadal maturation and reproductive performance of giant tiger prawn (*Penaeus monodon* Fabricius) from Adaman Sea and pond-reared sources in Thailand. Aquaculture 116:191-198.

Menasveta, P., S. Sangpradub, S. Piyatiratitivorakul and A.W. Fast. 1994. Effects of broodstock size and source on ovarian maturation and spawning of *Penaeus monodon* Fabricius from the Gulf of Thailand. Jour. World Aquaculture Society 25:41-49.

Menasveta, P. 1997. Intensive and efficient shrimp culture systems-the Thai way- and save mangroves. Aquaculture Asia 2(1):38-44

Menasveta, P., P. Aranyakanonda, S. Rungsupa and N. Moree. 1989. Maturation and larviculture of penaeid prawns in closed recirculating seawater systems. Aquacultural Engineering 8:357-368

Menasveta, P. and A.W. Fast. 1998. The evolution of shrimp culture and its impacts on mangroves. Infofish International 1/98:24-29

Menasveta P, Aranyakanonda P, Rungsupa S, Moree N (1991) Maturation and larviculture of penaeid shrimp in closed recirculating seawater system. Aquacultural Engineering 8:357-368

Menasveta P, Fast AW, Piyatiratitivorakul S, Rungsupa S (1991) An improved, closed seawater recirculation maturation system for giant tiger prawn (*Penaeus monodon* Fabricius). Aquacultural Engineering 10:173-181.

Menasveta P, Jarayabhand P (1995) Environmental planning in the coastal area for sustainable shrimp culture operation. Thai Jour. Aquatic Sci. 2(1):48-58.

Miller, P.J., M. Flaherty and B. Szuster. 1999. Inland shrimp farming in Thailand. Asia Aquaculture IV:27-32

Moav, R., G. Wohlfarth, G. Schroeder, G. Hulata and H. Barash. 1977. Intensive polyculture of fish in freshwater ponds. 1. Substitution of expensive feeds by liquid cow manure. Aquaculture 10:25-43

Moe MA, Jr. (1992) The marine aquarium reference, systems and invertebrates. Green Turtle Publications, Plantation, FL, 512 p

Moriarty, D.J.W. 1997. The role of microorganisms in aquaculture ponds. Aquaculture 151:333-349

Morishita, Y., T. Mitsouoka, C.C. Kaneuchi, S. Yamamoto and M. Ogata. 1971. Specific establishment of lactobacilli in the digestive tract of germ free chichens. Japanese Jour. Microbiology 15:531

Moss, S.M., G.D. Pruder, K.M. Leber and J.A. Wyban. 1992. The relative enhancement of *Penaeus vannamei* growth by selected fractions of shrimp pond water. Aquaculture 101:229-239

Moss, S.M. 1995. Production of growth-enhancing particles in a plastic-lined shrimp pond. Aquaculture 132:253-260

Moss, S.M., W.J. Reynolds and L. Mahler. 1998. Design and economic analysis of a prototype biosecure shrimp growout facility. In: S.M. Moss (ed), Proceedings of the US Marine Srhimp Farming Program, Biosecurity Workshop, Feb. 14, 1998, Oceanic Institute, Waimanalo, HI. pp. 5-18

Muralidhara, K.S., G.G. Sheggeby, P.R. Elliker, D.C. England and W.E. Sandine. 1977. Effect of feeding lactobacilli on the coliform and lactobacillus flora of the intestinal tissue and feces from piglets. Jour. Food Protection 40:288

Musig, Y. and S. Boonnom. 1999. Low salinity culture of Penaeus monodon Fabricius and its effect on the environment. In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. p. 123

Myers, J.M. 1986. Tetraploid induction in *Oreochromis spp.* Aquaculture 57:281-287.

National Research Council. 1992. Marine aquaculture: opportunities for growth. National Academy Press, Washington, DC

New, M.B. 1999. Global aquaculture: current trends and challenges for the 21st century. World Aquaculture 30(1):8-13, 63-79

New, M.B. and H.R. Rabanal. 1985. A review of the satatus of penaeid aquaculture in South East Asia. In: Roghlisberg, P.C., B.J. Hill and D.J. Staples (eds), 2nd Australian National Prawn Seminar, NPS2, Cleveland, Queensland, Australia, 22-26 Oct. 1984. 307-326

Nunan, L.M., B.T. Poulos and D.V. Lightner. (unpublished). The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported shrimp. (cited by C.L. Browdy and D. Bratvold 1998)

Ogle, J.T. and J.M. Lotz. 1998. Preliminary design of a closed, biosecure shrimp growout system. In: S.M. Moss (ed), Proceedings of the US Marine Srhimp Farming Program, Biosecurity Workshop, Feb. 14, 1998, Oceanic Institute, Waimanalo, HI. pp. 39-47

Perez Farfante, I. and B. Kensley 1997. Penaeoid and Sergestoid shrimps and prawns of the world. Keys and diagnoses for the families and genera (Les crevettes peneides et sergestides du monde, Cles d'identification et diagnose des familles et genres). Memoires du Museum National d'Histoire Naturelle (France), Vol. 175. 235 pp.

Pike, I.H. 1997/8. Future supplies of fish meal and fish oil: quality requirements for aquaculture, with special reference to shrimp. International Aquafeed Directory and Buyer's Guide, Turret RAI, Uxbridge, UK. p. 39-49

Pongthanapanich, T. 1999. Inland shrimp farms: how profitable, what really are their impacts of the environment, is inland shrimp farming worth a second look? Thailand. Asia Aquaculture IV:33-36

Pullin, R.S.V., M.J. Williams and N. Preston. 1998. Domestication of crustaceans. Asian Fisheries Society 11:59-69

Purdon, C.E. 1983. Genetic engineering by the manipulation of chromosomes. Aquaculture 33:287-300.

Queiroz, J.F. and C.E. Boyd. 1998. Effects of a bacterial inoculum in channel catfish ponds. Jour. World Aquaculture Soc. 29:67-73

Qin, J., A.W. Fast and H. Ako. 1998. Growout performance of diploid and triploid Chinese catfish *Clarias fuscus*. Aquaculture 166:247-258.

Reid, B. and C.R. Arnold. 1992. The intensive culture of penaeid shrimp *Penaeus vannamei* Boone in a recirculating raceway system. Jour. World Aquaculture Soc. 23:146-153

Rengpipat, S., W. Phianphak, S. Piyatiratitivorakul and P. Menasveta. 1998. Effects of probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167:310-313

Rengpipat, S., S. Rukpratanporn, S. Piyatiratitivorakul and P. Menasveta. 1999. Probiotics in aquaculture: a case study of probiotics for larvae of the black tiger shrimp (Penaeus monodon). In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. pp. 177-182

Robertson, A.I. and M.J. Phillips. 1995. Mangrove as filters of shrimp pond effluents: predictions and biogeochemical research needs. Hydrobiolgia 295:311-321

Rogers GL, Fast AW (1988) Potential benefits of low energy water circulation in Hawaiian prawn ponds. J. Aquacultural Engineering 7:155-165.

Rosenberry, R. 1990. World shrimp farming 1989. Aquaculture Digest, San Diego, CA. 28 pp.

Rosenberry, R. 1998. World shrimp farming 1998. Annual Report, Shrimp News International, San Diego, CA

Rosenberry, R. 1999. Strategies for controlling shrimp viruses. Aquaculture Magazine 25(4):22-23

Samocha, T.M. and A.L. Lawrence. 1998. Preliminary design and operating specifications for a biosecure shrimp growout facility in Texas. In: S.M. Moss (ed), Proceedings of the US Marine Srhimp Farming Program, Biosecurity Workshop, Feb. 14, 1998, Oceanic Institute, Waimanalo, HI. pp. 49-58

Sandifer, P.A., J.S. Hopkins and A.D. Stokes. 1988. Intensification of shrimp culture in earthen ponds in South Carolina: progress and prospects. Jour. World Aquaculture Soc. 19:218-226

Sandifer, P.A. and J.S. Hopkins. 1996. Conceptual design of a sustainable pond-based shrimp culture system. Aquacultural Engineering 15:41-52

Sandine, W.E. 1979. Roles of lactobacillus in the intestinal tract. Jour. Food Protection 42:259

Sangpradub, S., A.W. Fast, S. Piyatiratitivorakul and P. Menasveta. 1994. Effects of different feeding regimes on ovarian maturation and spawning of pond-reared giant tiger prawn in Thailand. Aquaculture International 2:49-58.

Scarpa, J. 1998. Freshwater recirculating systems in Florida. In: S.M. Moss (ed), Proceedings of the US Marine Srhimp Farming Program, Biosecurity Workshop, Feb. 14, 1998, Oceanic Institute, Waimanalo, HI. pp. 67-70

Schroeder, G.L. 1978. Autotrophic and heterotrophic production of microorganisms in intensively-manured fish ponds, and related fish yields. Aquaculture 14:303-325

Schroeder, G.L. 1987. Carbon pathways in detrital systems. In: D.J.W. Moriarty and R.S.V. Pullin (eds), Detritus and microbial ecology in aquaculture, ICLARM Conference Proceedings 13, International Center for Living Aquatic Resources Management, Manila, Philippines. pp. 317-236

Shigueno K (1985) Intensive culture and feed development in *Penaeus japonicus*. In: Taki Y, Primavera JH, Llobrera JA (eds), Proceed. First Internat. Confer. on the Culture of Penaeid Prawns/Shrimp. Southeast Asian Fish. Devel. Center, Iloilo City, Philippines, p 115-122

Sturmer LN, Samocha TM, Lawrence AL (1992) Intensification of penaeid nursery systems. In: Fast AW, Lester LJ (eds). Marine Shrimp Culture: Principles and Practices. Elsevier Science Publishers, Amsterdam, p 321-344

Tacon, A.G.J. 1994. Dependence of intensive aquaculture systems on fishmeal and other fishery resources—trends and prospects. FAO Aquaculture Newletter 6:10-16.

Tacon, A.G.J. 1997/8. Global trends in aquaculture and aquafeed production 1984-1995. International Aquafeed Directory and Buyer's Guide, Turret RAI, Uxbridge, UK. p. 5-37

Tang, Y.A. 1970. Evaluation of balance between fishes and available fish foods in multi-species fish culture ponds in Taiwan. Trans. American Fish. Soc. 99:708-718

Teichert-Coddington, D.R., D.B. Rouse, A. Potts and C.E. Boyd. 1999. Treatment of harvest discharge from intensive shrimp ponds by settling. Aquacultural Engineering 19:147-161

Timmons MB, Losordo TM (1994) Aquaculture water reuse systems: engineering design and management. Elsevier Science Publishers, Amsterdam. 346 p

Tseng KF, Su HM, Su MS (1998) Culture of Penaeus monodon in a recirculating system. Aquacultural Engineering 17:138-147.

Tucker, C.S. and S.W. Lloyd. 1985. Evaluation of a commercial bacterial amendment for improving water quality in channel catfish ponds. Mississippi Agriculture and Forestry Experiment Station, Mississippi State University, Res. Rpt. 10:1-4

Tucker, C.S. and E.H. Robinson. 1990. Channel catfish farming handbook. Van Nostrand Reinhold, New York. 454 p.

Vondruska, J. 1987. Shrimp situation and outlook. U.S. National Marine Fisheries Service, St. Petersburg, FL. mimeo.

Wang JK (1993) Techniques for modern aquaculture. American Soc. Agricultural Engineers. Niles, MI, 604 p

Westerman, P.W., J.M. Hinshaw and J.C. Barker. 1993. Trout manure characterization and nitrogen mineralization rate. In: J.K. Wang (ed), Techniques for modern aquaculture, Proceedings of an Aquacultural Engineering Conference 21-23 June 1993, Sokane, WA. p. 35-43.

Withyachumnarnkul, B., V. Boonsaeng, T.W. Flegel, S. Panyim and C. Wongteerasupaya. 1999. Domestication and selective breeding of *Penaeus monodon* in Thailand. . In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. pp. 73-77

Wyban, J.A. 1992. Selective breeding of specific pathogen free (SPF) shrimp for high health and increased growth. In: W. Fulks and K.L. Main (eds), Diseases of cultured penaeid shrimp in Asia and the United States. The Oceanic Institute, Waimanalo, Hawaii. pp. 257-268

Wyban, J.A., J.N. Sweeney and R.A. Kanna. 1988. Shrimp yields and economic potential of intensive round pond systems. Jour. World Aquaculture Soc. 19:210-217

Wyban, J.A. and J.N. Sweeney. 1989. Intensive shrimp growout trials in a round pond. Aquaculture 76:215-225

Yang WT, Halon RT, Lee PG, Turk PE (1989) Design and function of closed seawater systems for culturing loliginid squid. Aquacultural Engineering 8:47-65.

Yoon, C.K. 1999. Gene-altered crops a concern. The San Diego Union-Tribune. (Nov. 3, 1999)

Xiang, J., L. Zhou, F. Li and C. Wu. 1999. Triploidy induction in Chinese shrimp (Penaeus chinensis) with special reference to a new chemical inducer 6-DMAP. In: T.W. Flegel (ed), Advances in shrimp biotechnolgy, Proceedings of the Special Session on Shrimp Biotechnology, 5th Asian Fisheries Forum, Chiengmai, Thailand, 11-14 November 1998. BIOTEC, Bangkok, Thailand. p. 71

Table 1. Four levels of shrimp culture intensity and typical operational characteristics of each level (modified from Fast 1991).

Parameter	Extensive	Semi-Intensive	Intensive	Ultra-intensive
Yields (kg/ha/yr)	<100-300	500-2,500	5,000-15,000	30,000-150,000
Stocking Rates (Pl/m^2/crop)	0.1-1.0	3-20	20-60	>100
Feed	natural	natural + Suppliments	formulated	formulated
Water Exchange	tidal or Pump	pump	pump	pump
Aeration	natural	water exchange &/or aerators	aerators	aerators
Energy Use (HP/ha)	0-2	2-5	15-20	>25

Table 2. Estimated world production of farm raised, marine shrimp in 1999.

Area	Percent of World Total	Production Heads-on (Metric Tons)	Grow out Area (ha)	Average Production (kg/ha)	Estimated No. Hatcheries	Estimated No. Farms
Eastern Hemisphere						
Thailand	24.6%	200,000	80,000	2,500	1,000	20,000
China	13.5%	110,000	180,000	611	2,000	10,000
Indonesia	12.3%	100,000	350,000	286	300	225,000
India	8.6%	70,000	130,000	538	225	100,000
Philippines	4.9%	40,000	60,000	667	120	4,000
Vietnam	4.9%	40,000	200,000	200	1,000	6,000
Taiwan	2.5%	20,000	5,000	4,000	220	3,000
Malaysia	0.7%	6,000	4,000	1,500	100	800
Iran	0.3%	2,500	4,000	625	10	150
Australia	0.3%	2,400	600	4,000	8	45
New	0.2%	1,850	450	4,111	5	11
Other E.H.	6.1%	50,000	100,000	500	500	5,000
E.H Total		642,750	1,114,050		5,488	374,006
Average				577		
Global percent	78.9%	78.9%	89.0%		95.0%	99.5%
Western Hemisphere						
Ecuador	10.4%	85,000	100,000	850	200	1,200
Brazil	1.8%	15,000	6,000	2,500	20	110
Nicaragua	0.5%	4,000	6,000	667	5	130
Venezuela	0.5%	4,000	2,000	2,000	3	12
Panama	0.2%	2,000	3,000	667	11	35
United States	0.2%	1,500	400	3,750	10	20
Other W.H.	7.4%	60,000	20,000	3,000	40	400
W.H. Total		171,500	137,400		289	1,907
Average				1,248		
Global percentage	21%	21%	11.0%		5.0%	0.5%
World Total	100%	814,250	1,251,450	651	5,777	375,913

Table 3. Land use during 1993 of previously existing mangrove areas before 1961 (Menasveta 1997).

Land Use	Area (ha)	Percentage of land
Shrimp Farms	64, 992	17.5
Community Use	4,961	1.4
Other[1]	133,813	35.9
Mangrove remaining	168,683	45.3

[1]Other uses include agricultural, salt farms, mining, and infrastructure development.

Table 4. Shrimp yield data during 1991 for four ponds with water exchange (0%, 2.5% and 25% per day), and two stocking densities (22 and 44 PL/m^2). Table from Hopkins et al. (1993).

Water Exchange and shrimp stocking rate	Shrimp Harvest Size (g)	Survival (%)	Yield (kg/ha)	FCR (dry:wet)
25% exchange and 44 PL/m^2	15.9	81.9	5,718	2.6
2.5% exchange and 44 PL/m^2	18.2	79.5	6,375	2.3
0% exchange and 44 PL/m^2	18.6	0.2	18	n/a
0% exchange and 22 PL/m^2	17.7	81.6	3,219	2.3

Table 5. Shrimp yield data during 1991 for three ponds with three different sediment management practices during shrimp growout: **REMAIN**—sediment was not removed from pond; **REMOVE**—sediment was removed by pump; **RESUSPEND**—sediment was resuspended by moving a paddlewheel aerator daily. Table from Hopkins et al. (1994).

Shrimp data	REMAIN	REMOVE	RESUSPEND
Harvest size (g)	23.3	16.7	14.6
Survival (%)	0.2	32.8	54.1
Yield (kg/ha)	18	2,406	3,474

Table 6. Shrimp yield data from six 0.1-ha ponds during 1995 and 1996. Three ponds (S-06, S-07, S-08) had water exchange of 15%/day, while the other three (S-10, S-11, S-12) had no water exchange. Penaeus vannamei were stocked at 38 Pl/m^2 during 1995, and 78 Pl/m^2 during 1996. Data from Hopkins et al. (1997).

	1995						1996					
	Water exchange			No exchange			Water exchange			No exchange		
Pond number	S-06	S-07	S-08	S-10	S-11	S-12	S-06	S-07	S-08	S-10	S-11	S-12
Mean Wt. (g)	15.4	17.5	16.7	15.1	15.1	16.3	9.4	9.9	9.5	8.5	9.7	9.9
Survival (%)	93.8	91.3	95.1	87.0	90.6	96.1	94.9	99.9	97.8	87.6	93.0	88.1
Yield (mt/ha)	5.51	6.10	6.06	5.13	5.23	5.97	6.97	7.72	7.26	5.82	7.05	6.81
FCR	1 .58	1.43	1.44	1.70	1.66	1.46	2.73	2.47	2.62	3.27	2.70	2.80

Table 7. Production data from a zero water exchange farm in Belize for two strains of *Penaeus vannamei* and one strain of *P. stylirostris*. All shrimp were SPF, while one *P. vannamei* strain was also TSV resistant. Data for first two years of operation (August 1997/July 1999). Data provided by Robins McIntosh (personal communications, 1999).

	P. vannamei (Mexican)	*P. vannamei* (Mexican)	*P. vannamei* (TSV resist.)	*P. stylirostris* (Ecuador)
Seasons	Sept.-Nov.	Dec.-Aug.	Dec.-Aug.	Nov.-Dec.
Areas harveste (ha)	2.84	7.68	3	1.6
Growout days	142	139	136	145
Stocking (Pl/sq. m)	122	118	112	93
Survival (%)	82	77	56	60
Growth (g/wk)	0.72	0.9	0.84	0.72
Size at Harvest (g)	14.6	17.9	16.4	15.1
Yields (kg/ha/crop)	13,751	14,629	10,340	7,450
FCR	1.9	2	2.6	2.5
Mean temp. C	27.5	28.8	29.7	27.4

Table 8. Summary of water quality data from one pond during one crop with zero water exchange on a shrimp farm in Belize. Data from McIntosh et al. (1999).

Parameter	Initial Value	Final Value
pH	7.9	7.4
CO_2 (mg/l)	30	75
Alkalinity (mg/l)	100	35
DO AM (mg/l)	5.0	4.8
DO PM (mg/l)	8.0	5.5
Transparency (cm)	40	30
Ammonia-N (mg/l)	1.4	0.8
Nitrite-N (mg/l)	0.8	8.5
Nitrate-N (mg/l)	0.8	13.0
Total Nitrogen-N	4.0	30.0
Total Phosphorus-P	1.0	8.7

Table 9. Comparisons between inland shrimp farming and rice farming in Thailand. Modified from (Pongthanapanich 1999). Monetary exchange rate used here is U.S$1.00:35 baht.

INLAND SHRIMP FARMING	RICE FARMING
1. Thailand is the world's leading shrimp producer and exporter, valued at $1.3 billion during 1997.	1. Thailand is the world's largest rice exporter, valued at $1.9 billion during 1997.
2. Total shrimp farming area is 73,600 ha in 1997 or 0.2% of the total land area of Thailand.	2. Rice farming area is 9,120,000 ha in 1995 or 20% of total land area of Thailand.
3. Shrimp farmers usually earn $17,857/ha from one crop.	3. Rice farmers usually earn $1,785/ha from two crops, but most produce only one crop.
4. Shrimp farming is a high-risk, high-return enterprise. Shrimp farmers pay higher land rents than rice farmers ($536 verses $107/ha respectively). A farmer can go bankrupt with one complete crop failure, or with two consecutive bad crops.	4. Many rice farmers are still poor. A land loan is usually used to guarantee a loan and if the crop fails, the farmer loses the land.
5. Shrimp farming is capital and management intensive. Good farm design and management are essential to prevent negative impacts on the farm and its surrounding environment.	5. Rice farming requires low capital inputs. Its environmental impacts are unclear, but hazardous chemicals are used and the pondsemit large amounts of greenhouse gases.
6. Domestic shrimp consumption is low because shrimp prices are high. Most shrimp is exported.	6. Rice is a staple food in Thailand Thai households spend 17% of their total income on rice.
7. Shrimp farms use more labor per unit land area than rice farming, and labor is higher paid .	7. Rice farming labor is seasonal and low paid. Man-hrs per kg rice produced is less than for shrimp.

Table 10. Comparisons between typical characteristics of conventional recirculation aquaculture systems, and commercial scale, marine shrimp recirculation pond culture systems. Table from Fast and Menasveta (1998).

Characteristic	Conventional Recirculation Systems	Shrimp Pond Recirculation Systems
1. Size	small (aquarium, tanks, silos, raceways)	large (ponds >0.5 ha)
2. System Components		
a. culture containment	tanks	pond
b. solids removal	settling, pressurized mechanical filtration	settling
c. aeration	mechanical	photosynthesis and mechanical
d. nitrification (biofilter; $NH_3 \rightarrow NO_3$)	inert sub-component	photosynthesis and pond surfaces
e. denitrification ($NO_3 \rightarrow N_2$)	requires anaerobic sub-component	pond muds
f. ozonation/UV	possible	not likely
3. Energy I/P	intensive	less intensive
4. Solar I/P	none to small	large and important
5. Water Replacement (% daily flow-through)	0 to 10%	0 to <5%

Table 11. Approximate time frames for large-scale, commercial application of recent pond growout innovations.

	Present	1⇒3 years	3⇒10 years	>10 years
1. Pond Water Recirculation & Reuse	X			
2. Zero Water Exchange	X			
3. Brine Based, Inland Pond Culture	X			
4. Probiotics for Disease Control		X		
5. Use of Genetically Altered Shrimp				
a. Triploids			X	
b. Genetically Selected			X	
c. Transgenic				X
6. Polculture				
a. Shrimp/Shrimp	X			
b. Shrimp/Fish	X			
c. Shrimp/Bivalves		X		
d. Shrimp/Fish/Bivalves			X	
7. Computerized Pond Management			X	

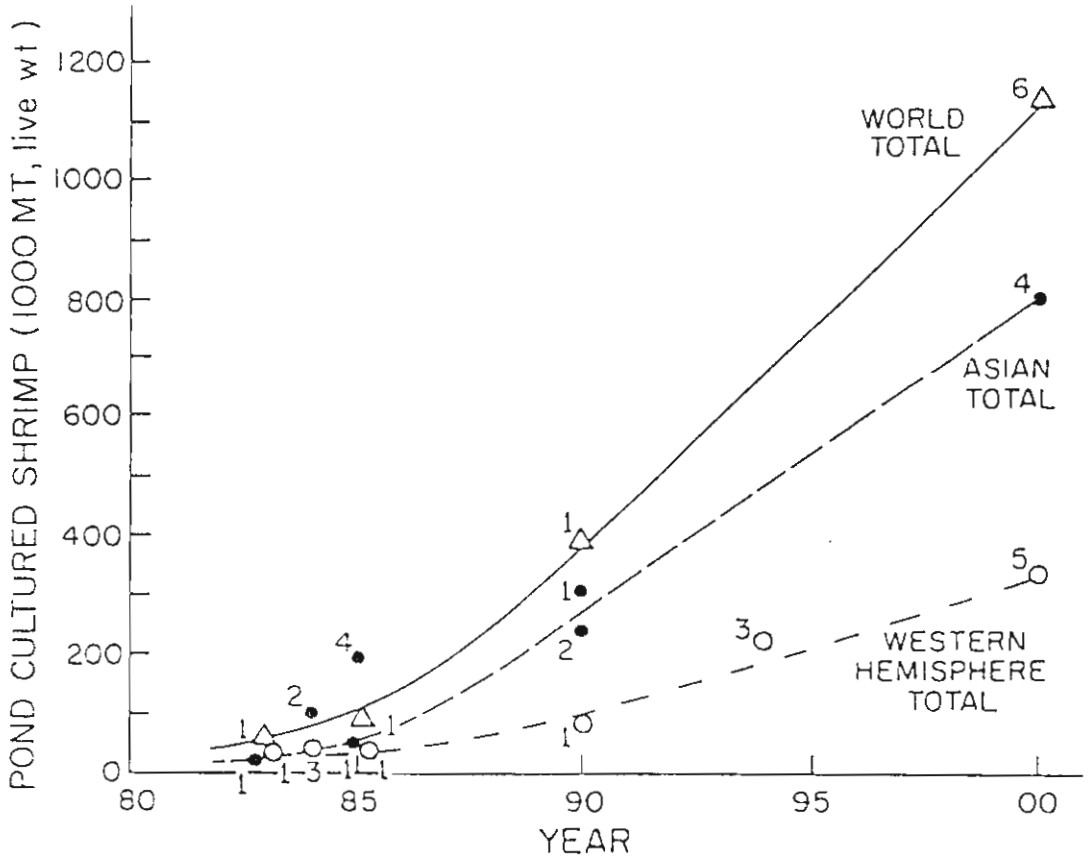


Figure 1. Observed and projected production of pond culture shrimp during 1990 (Fast and Lester 1992). Estimates and projections were based on composited values from: **1.** Vondruska (1984); **2.** New and Rabanal (1985); **3.** Lawrence (1984); **4.** Csavas (1988); and **6.** Sum of estimates from 4 & 5.

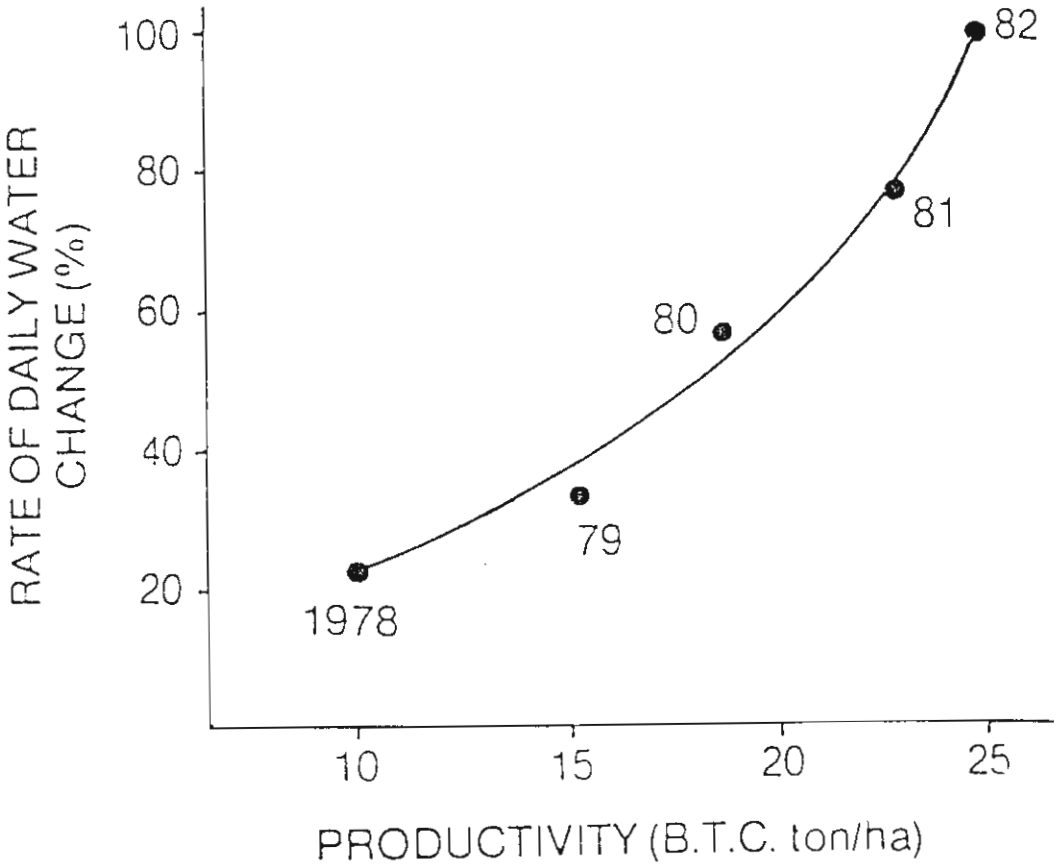


Figure 2. Relationship between rate of pond water exchange (% per day) and yearly production rate (BTC in MT/ha/yr). Figure from Hirasawa (1985).

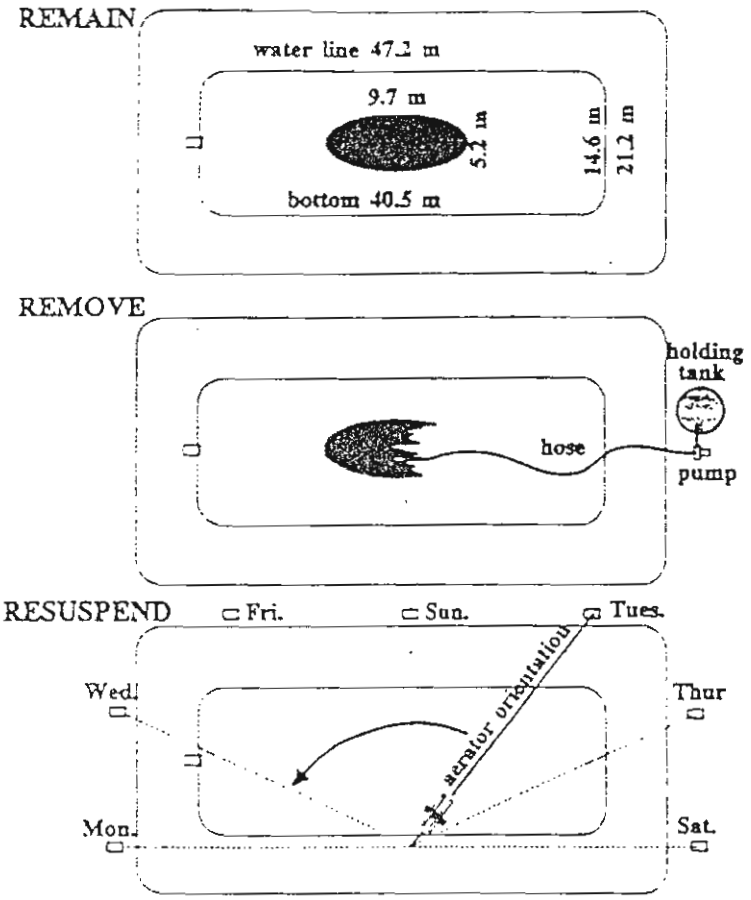


Figure 3. Three ponds with three different sediment management practices during shrimp growout: **REMAIN**—sediment was not removed from pond; **REMOVE**—sediment was removed by pump; **RESUSPEND**—sediment was resuspended by moving a paddlewheel aerator daily. Figure from Hopkins et al. (1994).

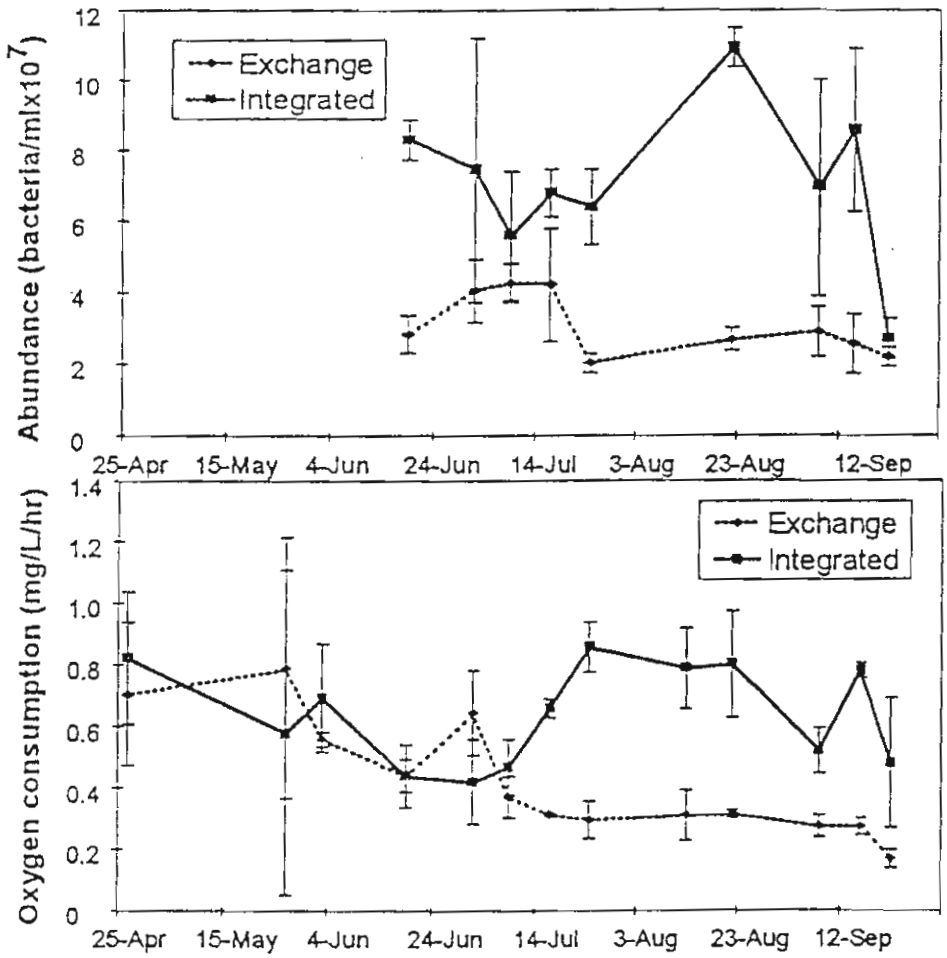


Figure 4. Microbial abundance and oxygen consumption rates in three ponds without water exchange, and three ponds with water exchanges of 15%/day during 1996. Figure from Browdy and Bratvold (1997).

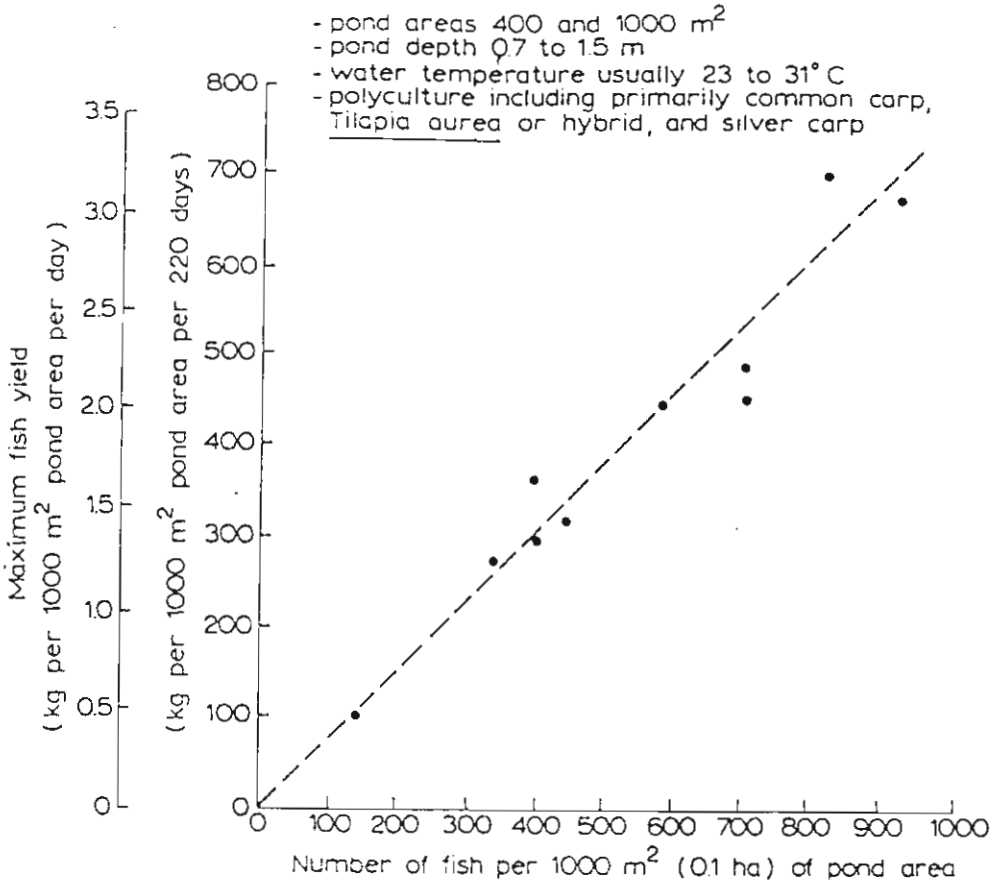


Figure 5. Relationship between number of fish stocked and manure applied, and fish yields in a 0.1-ha pond. This manure fed pond had no water exchange and relied on the heterotrophic food web for fish nutrition. Figure from Schroeder (1978).

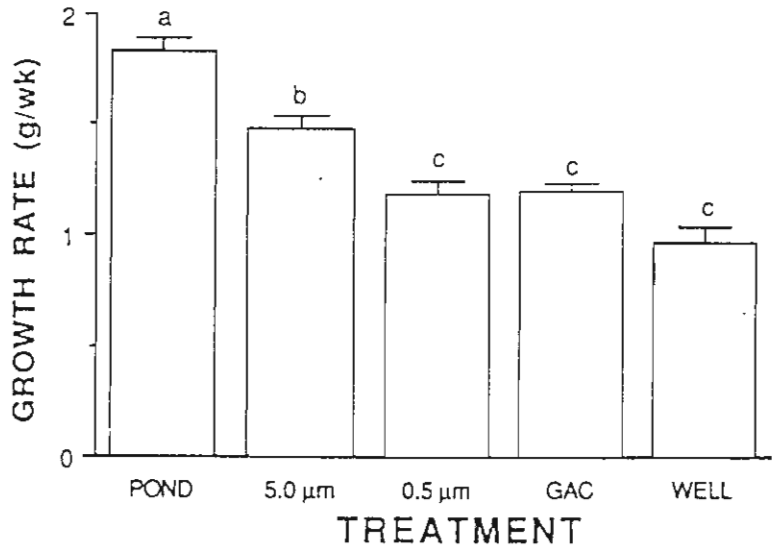


Figure 6. Average weekly growth rates of Penaeus vannamei held in microcosms and provided different combinations of pond water (filtered and unfiltered) and a complete diet. Shrimp cultured in well water and pond waters filtered using 0.5 µm mesh grew much slower than shrimp cultured in unfiltered pond water or pond water filtered through a 5.0 µm mesh. Figure from Moss et al. (1992).

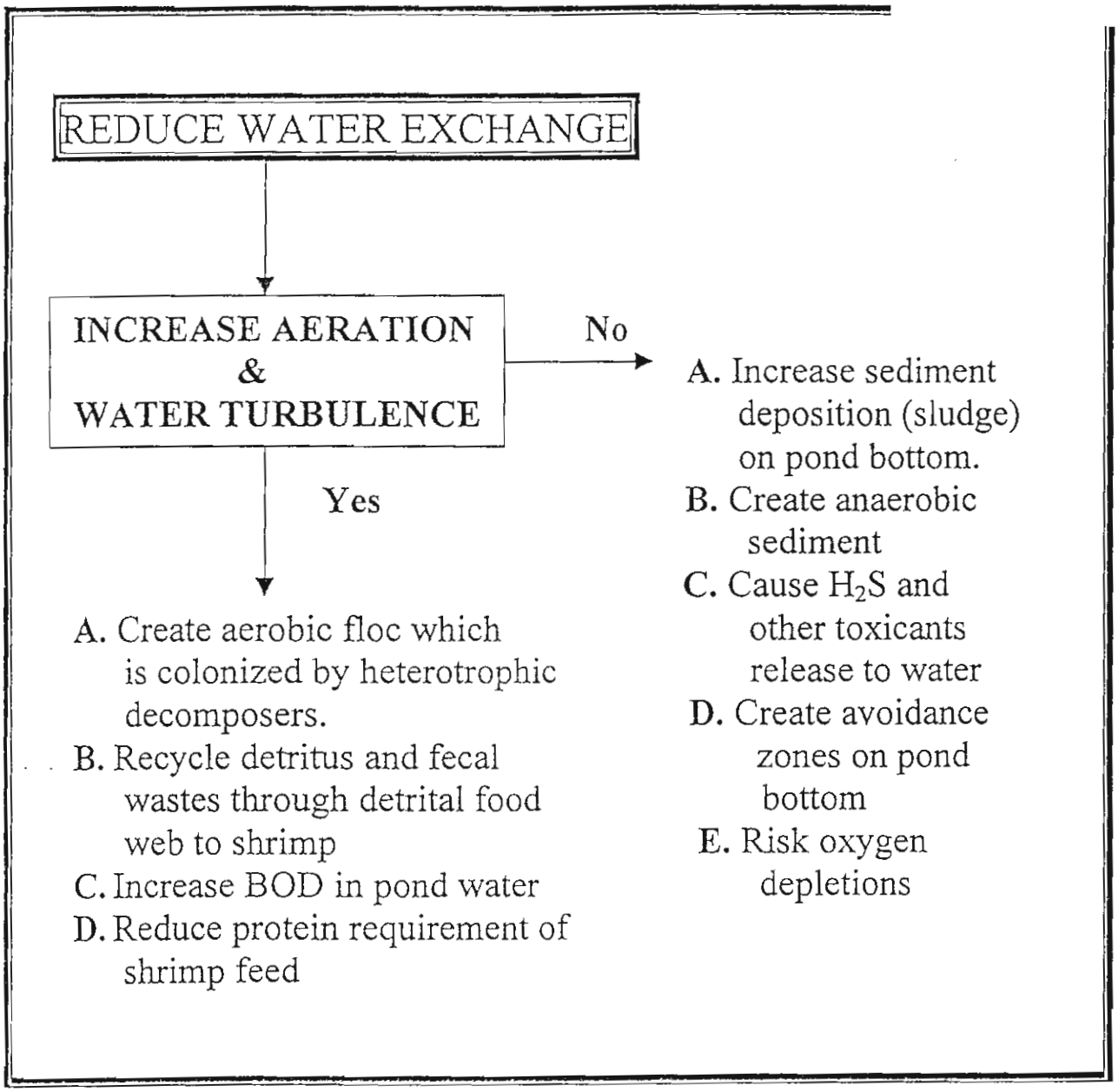


Figure 7. Summary of the effects of reduced water exchange and aeration/turbulence on pond water quality and shrimp yields.

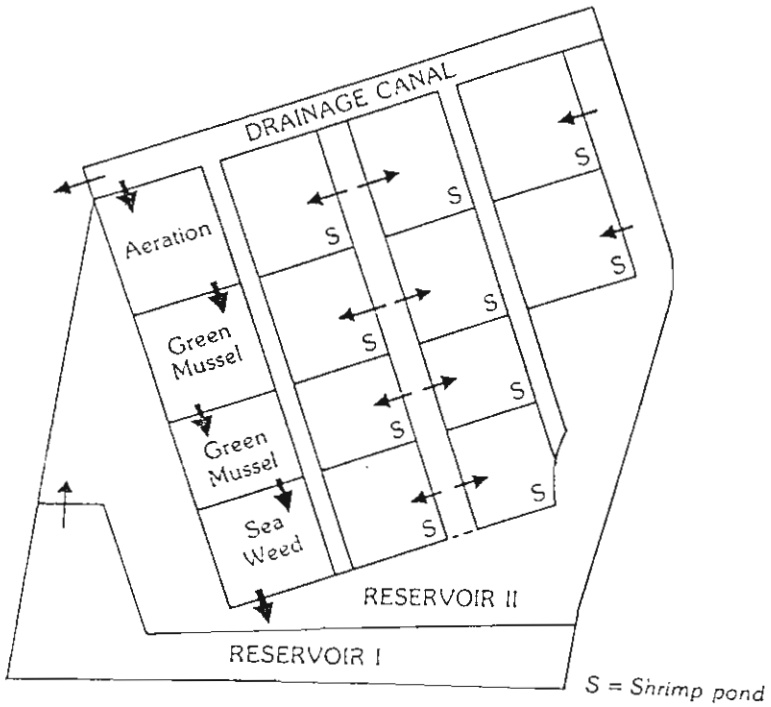


Figure 8. Recirculating shrimp pond system operated by Choroen Pakphan Group (CP) in Thailand (Anonymous 1996a). Source water entered Reservoir Pond I for sedimentation and conditioning before flowing into Reservoir Pond II, and then into shrimp growout ponds (S). Effluent from shrimp growout ponds went to the Drainage Canal where some or all of this water was then circulated through four water treatment ponds for nutrient stripping. Water then flowed into Reservoir Pond II again and returned to the shrimp growout ponds (S).

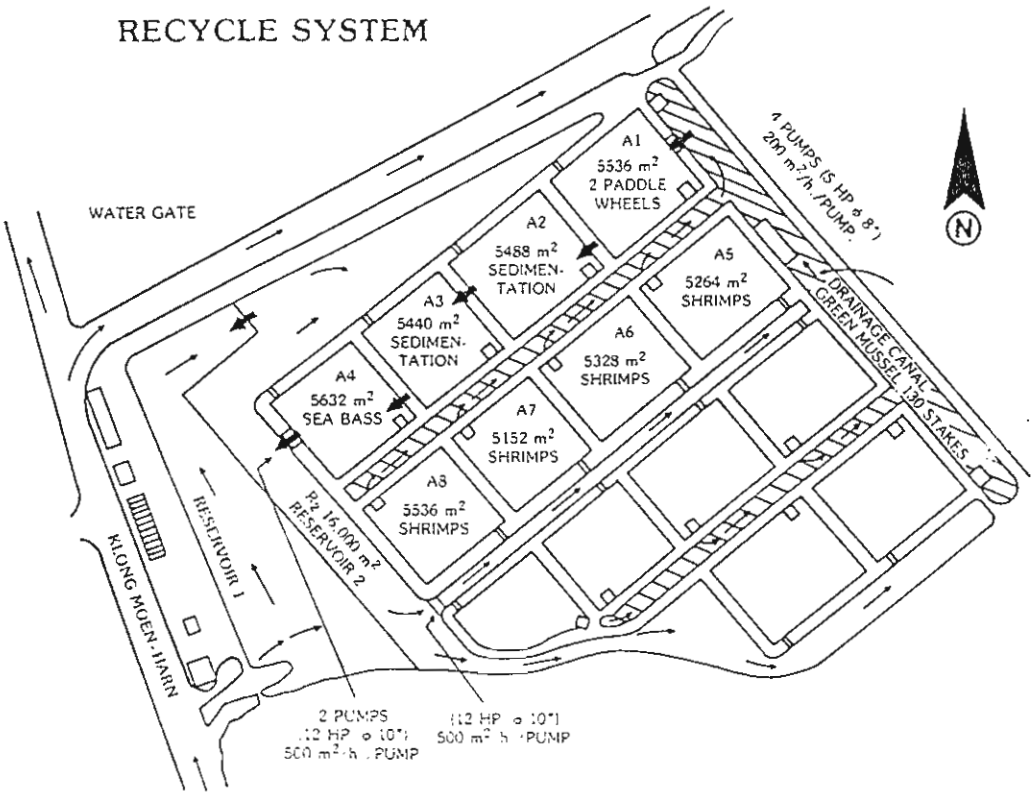


Figure 9. Modified shrimp recirculation system shown in Fig. 8 (Anonymous 1996b). Green mussels were placed in the drainage canal, and water treatment ponds A2, A3 were used for sedimentation. Water treatment pond A4 contained sea bass which created better water quality conditions for sustained phytoplankton growth. [note: pump water volumes should be in m^3 rather than m^2 as shown].

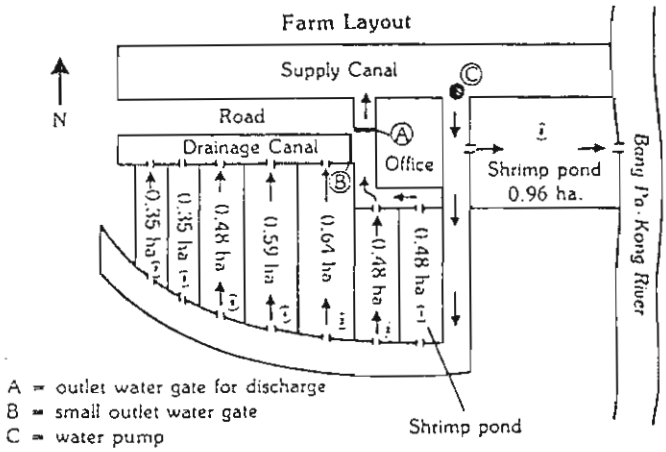


Figure 10. A private shrimp farm in Thailand which used water recirculation (Anonymous 1996a). Source waters in the vicinity of the farm were heavily polluted with industrial and household wastes. After initially filling the ponds, water was not circulated during the first three months of shrimp culture.

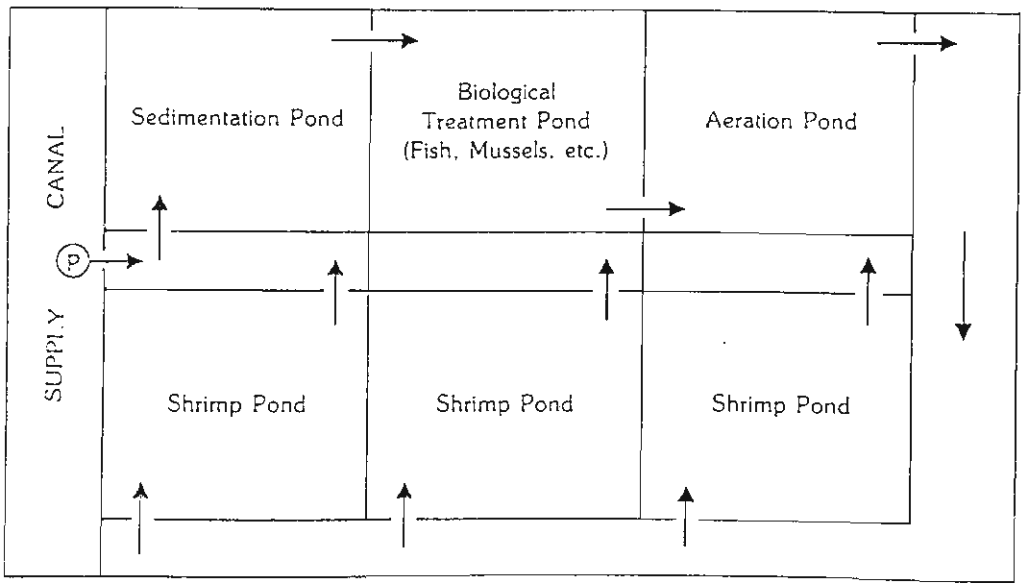


Figure 11. Typical recirculating shrimp farm system in Indonesia (Anonymous 1996c). Shrimp pond effluent flowed to a sedimentation pond, followed by a treatment pond with fish and/or bivalves. Milkfish, mullet, green mussels and/or oysters were commonly used. Waters were then aerated before returning to the shrimp growout ponds.

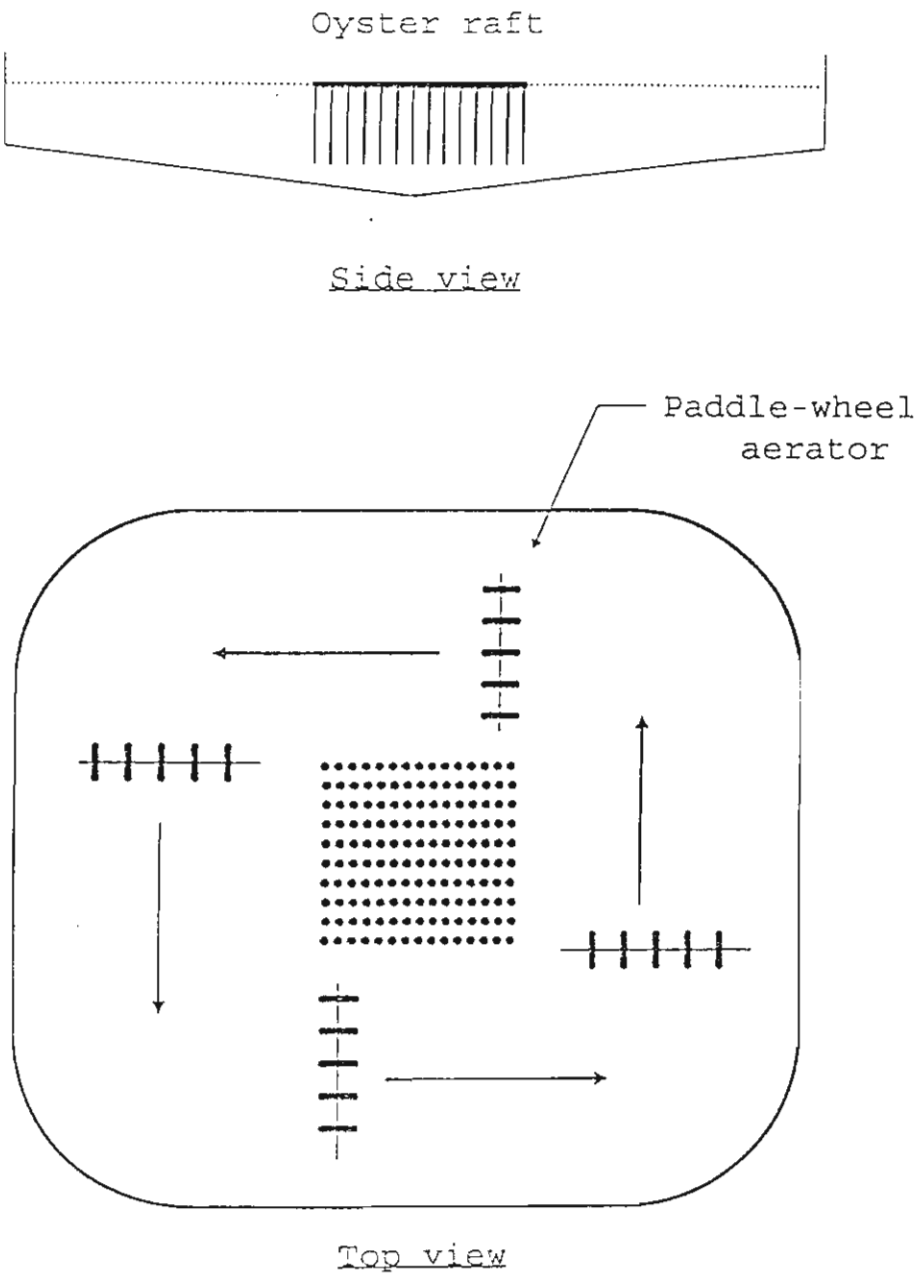


Figure 12. In-pond treatment system using an oyster raft proposed by Menasveta and Jarayabhand (1995). Oysters are preferred to green mussels because of their greater salinity tolerances and higher market value. Paddlewheel aerators would create circular water motions and concentrate wastes in the pond center.

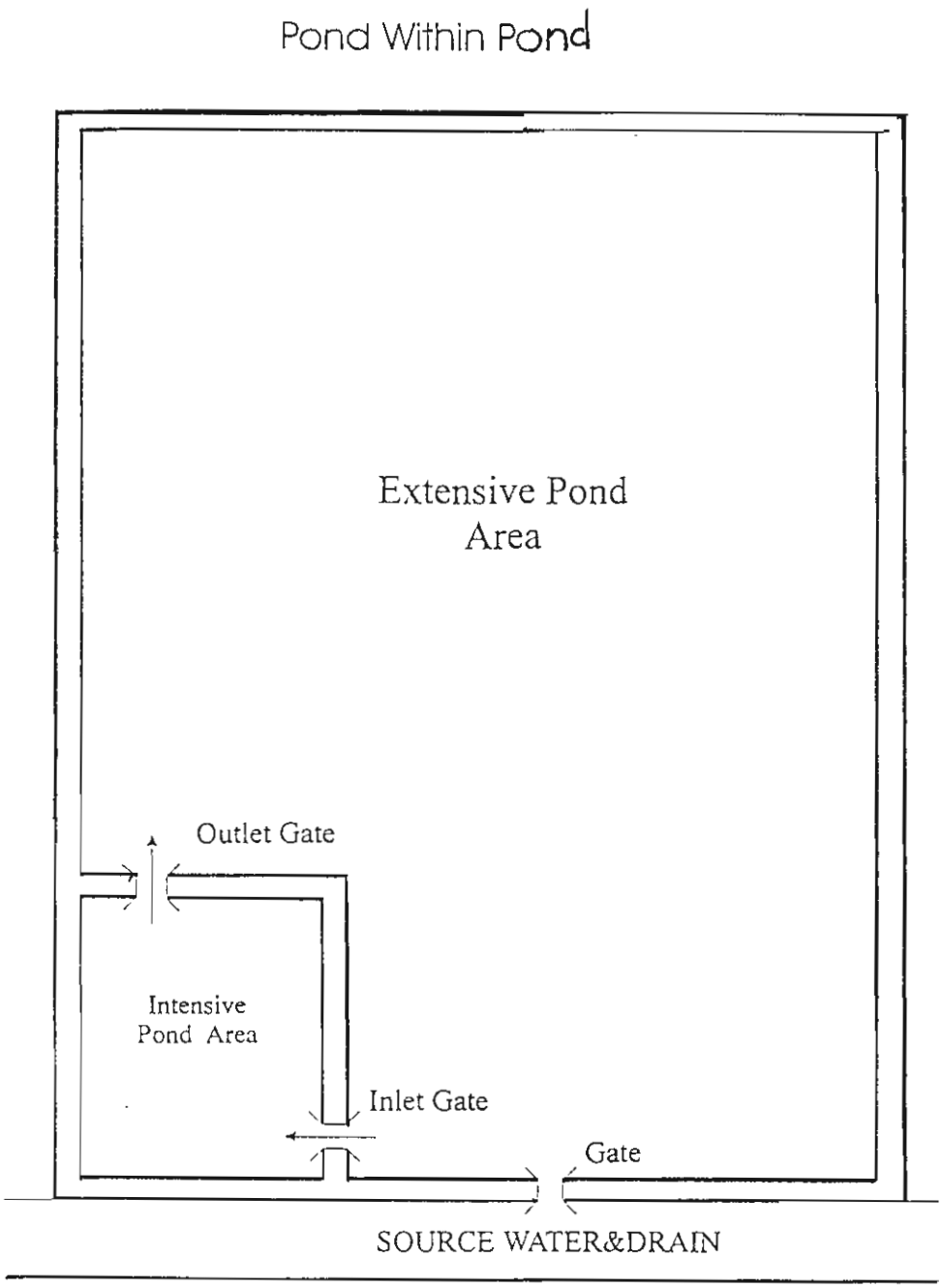


Figure 13. A proposed pond-in-pond culture system where shrimp are cultured intensively in a small pond, while source water and effluents recycle between a much larger extensive pond. The extensive pond serves as a water treatment component and provides a secondary cash crop. This system could easily be retrofitted to existing extensive ponds.

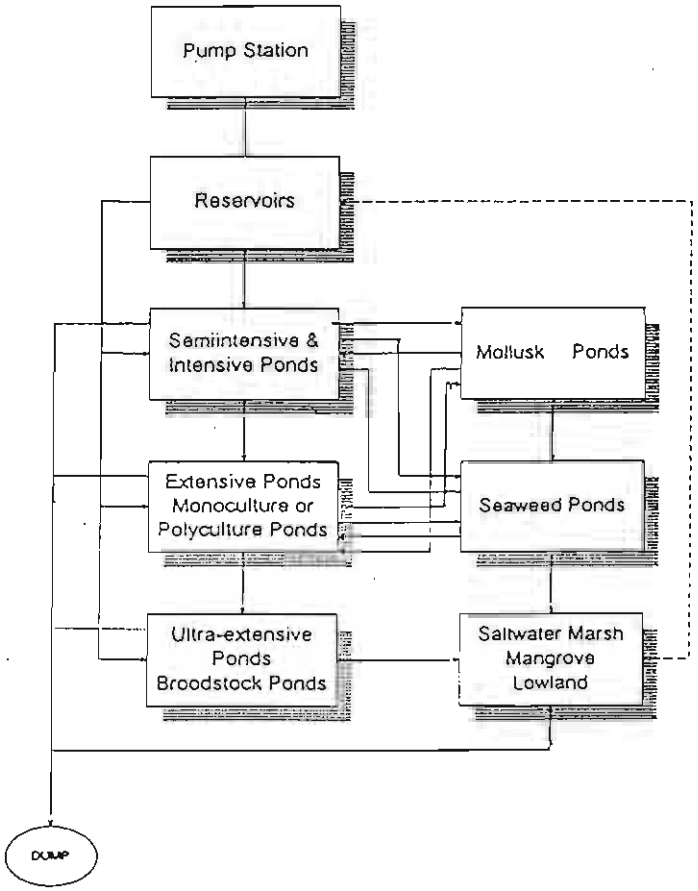


Figure 14. Conceptual design of a water recirculation system for shrimp culture (Chien and Liao 1995). This system differs from other described systems in that water can be circulated from intensive to less intensive ponds, as well as through treatment ponds before reuse.

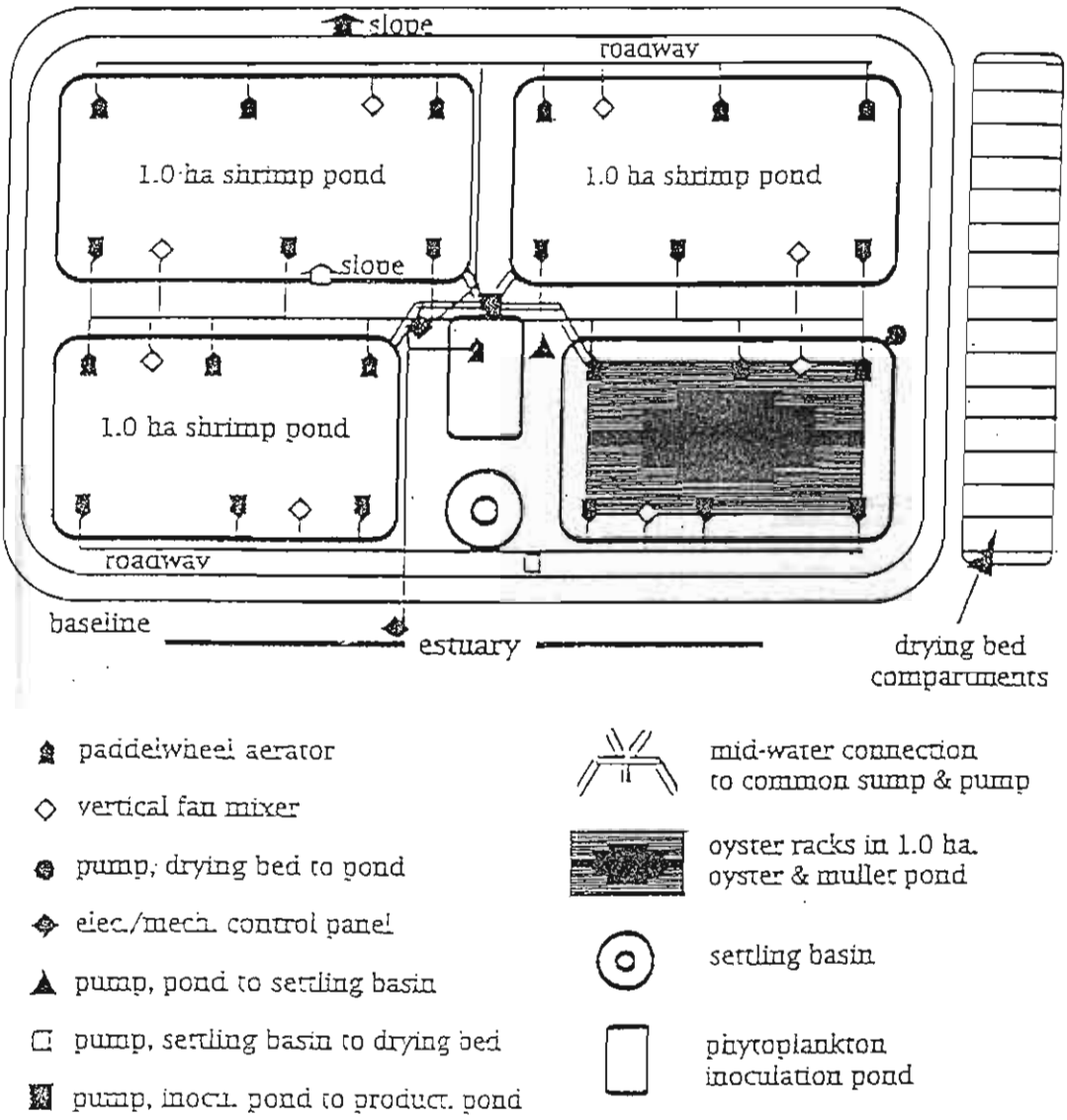


Figure 15. Schematic layout of a proposed 4-ha modular farm for intensive culture of marine shrimp (Sandifer and Hopkins 1996). This system would incorporate water recycling, polyculture, and sludge removal. There would be no water or sediment discharges from the farm.

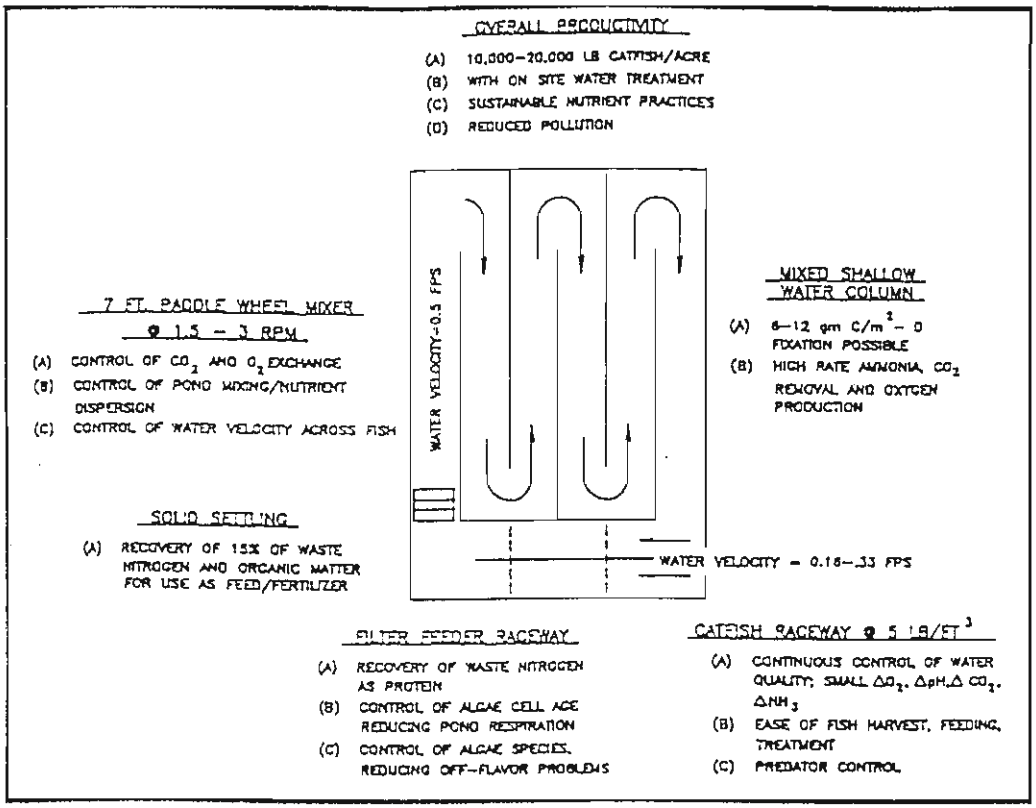


Figure 16. Schematic of Partitioned Aquaculture System (PAS) for pond culture under development at Clemson University. Figure courtesy of D.E. Brune (1998).

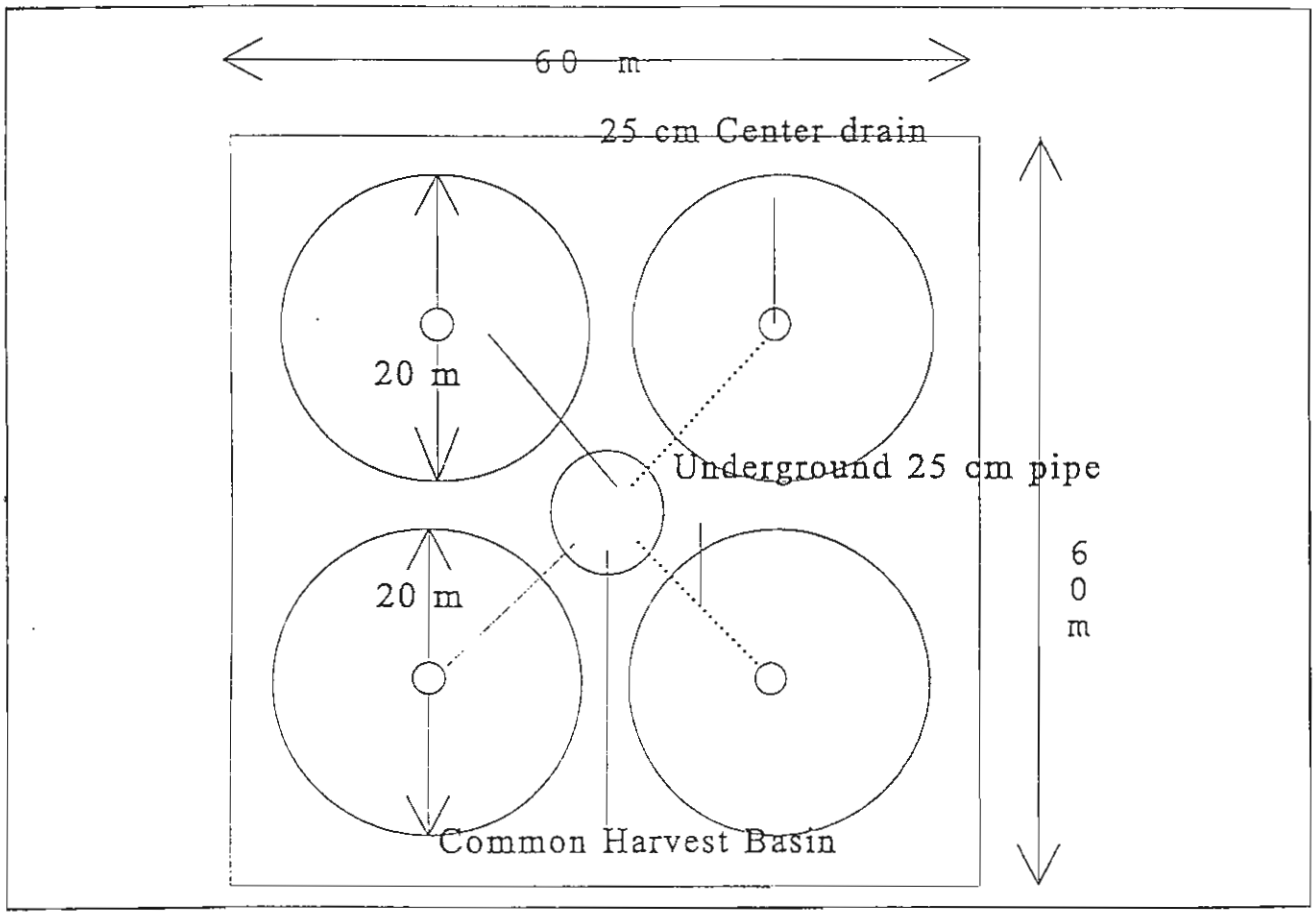


Figure 17. A conceptual design of a proposed 4-pond, biosecure shrimp growout system (Samocha and Lawrence 1998). The four ponds would be covered by an inflated plastic roof.

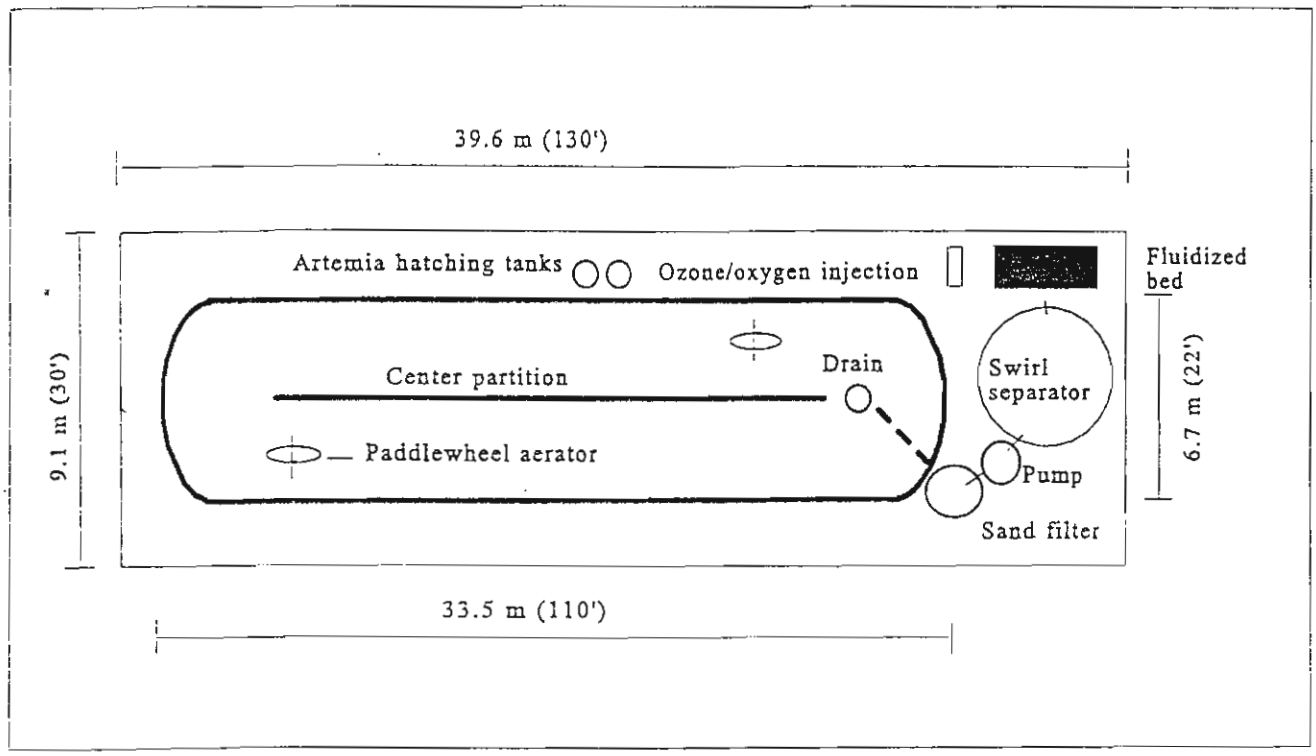


Figure 18. A conceptual design of a proposed, biosecure shrimp raceway growout system (Samocha and Lawrence 1998). The raceway would be under a plastic greenhouse cover.

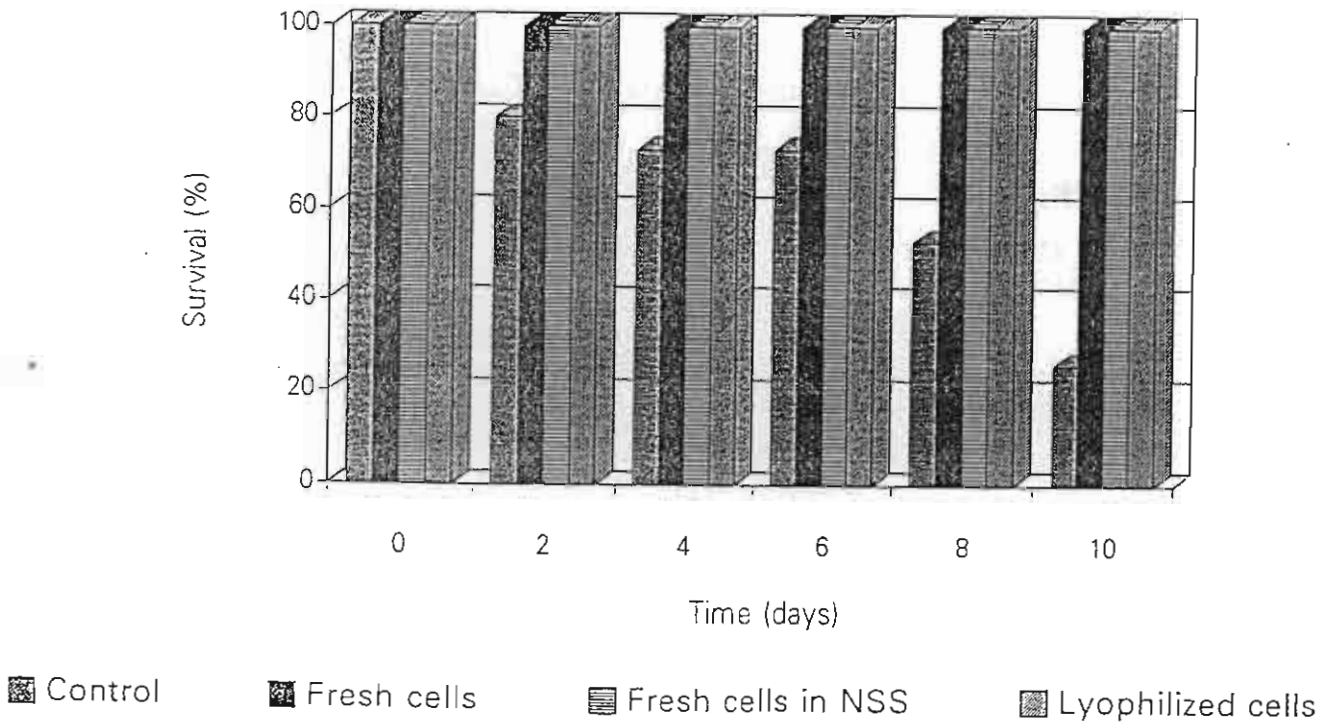


Figure 19. Survival of 115 day old *Penaeus monodon* during a 10-day challenge test with the pathogenic, luminescent bacterium *Vibrio harveyi*. Three groups of shrimp were fed the probiont Bacillus S11 in their feed as either fresh cells, fresh cells in normal saline solution (NSS), or as freeze dried cells (lyphilized cells). Control shrimp were not fed any probiotics. Figure from Rengpipat et al. (1999).