ผลของแอลคาร์นิทีนต่อสาหร่าย *Tetraselmis suecica* โรติเฟอร์ *Brachionus plicatilis* และลูกปลากะพงขาว *Lates calcarifer* และการพัฒนาระบบการผลิตโรติเฟอร์

นางสาวชัชฎาภรณ์ สรรคอนุรักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECTS OF L-CARNITINE ON MICROALGA *Tetraselmis suecica*, ROTIFER *Brachionus plicatilis*, AND LARVAL SEABASS *Lates calcarifer* AND THE DEVELOPMENT OF ROTIFER PRODUCTION SYSTEM

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Marine Science
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Thesis Title	EFFECTS OF L-CARNITINE ON MICROALGA *Tetraselmis suecica*, ROTIFER *Brachionus plicatilis*, AND LARVAL SEABASS *Lates calcarifer* AND THE DEVELOPMENT OF ROTIFER PRODUCTION SYSTEM
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ชัชฎาภรณ์ สรรคอนุรักษ์ : ผลของแอลคาร์นิทีนต่อสาหร่าย *Tetraselmis suecica* โรติเฟอร์ *Brachionus plicatilis* และลูกปลากะพงขาว *Lates calcarifer* และการพัฒนาระบบการผลิตโรติเฟอร์. (EFFECTS OF L-CARNITINE ON MICROALGA *Tetraselmis suecica*, ROTIFER *Brachionus plicatilis*, AND LARVAL SEABASS *Lates calcarifer* AND THE DEVELOPMENT OF ROTIFER PRODUCTION SYSTEM). อ.ที่ปรึกษา : ศ. ดร. เปี่ยมศักดิ์ เมนะเศวต, อ. ที่ปรึกษาร่วม : รศ. ดร. ไทยถาวร เลิศวิทยาประสิทธิ, 137 หน้า.

การศึกษานี้แบ่งออกเป็น 3 การทดลอง ได้แก่ (1) การศึกษาผลของแอลคาร์นิทีนต่อสาหร่ายเตตราเซลมิส โรติเฟอร์ และลูกปลากะพงขาว (2) ศึกษาการใช้แอลคาร์นิทีนเพื่อเพิ่มการเติบโตของโรติเฟอร์ในระบบเลี้ยงแบบหมุนเวียนน้ำ และ (3) การพัฒนาระบบเพาะเลี้ยงสาหร่าย และโรติเฟอร์แบบต่อเนื่องด้วยระบบการหมุนเวียนน้ำแบบปิด

ผลของแอลคาร์นิทีนต่อสาหร่ายเตตราเซลมิส พบว่าชุดที่เสริมแอลคาร์นิทีนที่ระดับ 0.1, 1 และ 10 มก./ลิตรของอาหารเลี้ยง มีอัตราการเติบโตสูงกว่าชุดที่ไม่ได้เสริม โดยที่ระดับ 10 มก./ลิตร มีอัตราการเติบโตสูงที่สุด สำหรับปริมาณแอลคาร์นิทีนที่สะสมในเซลล์สาหร่ายมีค่าระหว่าง 258-1,813 ไมโครกรัมต่อกรัมน้ำหนักแห้ง โดยพบค่าสะสมสูงสุดที่ระดับการเสริมแอลคาร์นิทีน 10 มก./ลิตร ผลของแอลคาร์นิทีนต่อโรติเฟอร์ พบว่าเมื่อโรติเฟอร์กินสาหร่ายที่เสริมแอลคาร์นิทีน 1 มก./ลิตร จะมีการเติบโตดีที่สุด และพบปริมาณการสะสมแอลคาร์นิทีนในโรติเฟอร์มีค่าสูงสุดเมื่อโรติเฟอร์กินสาหร่ายที่เสริมแอลคาร์นิทีน10 มก./ลิตร สำหรับการทดลองโดยให้โรติเฟอร์กินอิมัลชั่นเสริมแอลคาร์นิทีน พบว่าที่ระดับ 10 มก./ลิตร ที่ระยะเวลา 4 ชม. มีผลการสะสมแอลคาร์นิทีนในโรติเฟอร์ดีที่สุด (เป็นโรติเฟอร์ที่ปรับให้กินสาหร่ายเสริมแอลคาร์นิทีน 10 มก./ลิตร มาโดยตลอด) ส่วนผลของแอลคาร์นิทีนต่อการเติบโต การรอด และปริมาณการสะสมแอลคาร์นิทีนในลูกปลากะพงขาว พบว่าลูกปลาชุดที่เลี้ยงด้วยโรติเฟอร์ที่ปรับให้กินสาหร่ายเสริมแอลคาร์นิทีน 10 มก./ลิตร โดยตลอด และกินอิมัลชั่นที่ไม่เสริมและเสริมแอลคาร์นิทีน 10 มก./ลิตร ที่ระยะเวลา 4 ชม. มีความยาวตัวมากกว่าชุดอื่นอย่างมีนัยสำคัญ ในขณะที่ลูกปลาเลี้ยงด้วยโรติเฟอร์ที่กินสาหร่ายไม่เสริมแอลคาร์นิทีนและไม่ได้กินอิมัลชั่น (ชุดควบคุม) มีความยาวตัวน้อยที่สุด ทั้งนี้น้ำหนักตัวทุกชุดทดลองไม่มีความแตกต่างอย่างมีนัยสำคัญ ส่วนอัตรารอดของทุกชุดทดลองสูงกว่าชุดควบคุม โดยชุดที่เลี้ยงด้วยโรติเฟอร์ที่ปรับให้กินสาหร่ายเสริมแอลคาร์นิทีน 10 มก./ลิตร โดยตลอด และกินอิมัลชั่นไม่เสริมแอลคาร์นิทีน มีอัตรารอดสูงสุด (39%) ผลของการวิเคราะห์ปริมาณการสะสมแอลคาร์นิทีน พบว่าลูกปลาในชุดควบคุม และลูกปลาชุดที่เลี้ยงด้วยโรติเฟอร์กินสาหร่ายไม่เสริมแอลคาร์นิทีนและกินอิมัลชั่นไม่เสริมแอลคาร์นิทีนตรวจไม่พบ จึงสรุปได้ว่าสามารถใช้วิธีการให้ลูกปลากินโรติเฟอร์ที่กินอิมัลชั่นเสริมแอลคาร์นิทีน 10 มก./ลิตร ที่ระยะเวลา 4 ชม. ได้เลยโดยไม่จำเป็นต้องใช้โรติเฟอร์ที่ปรับให้กินสาหร่ายเสริมแอลคาร์นิทีน 10 มก./ลิตร มาก่อน

การทดลองที่สองเป็นการใช้แอลคาร์นิทีน ในระบบเลี้ยงแบบหมุนเวียนน้ำ โดยเตรียมแอลคาร์นิทีน ความเข้มข้น 1 มก./ลิตร ในถังลี้ยงโรติเฟอร์ที่มีความหนาแน่น 500 ตัว/มล. ต่อถังเลี้ยง แช่ทิ้งไว้ 48 ชม. จากนั้นทำการล้างแอลคาร์นิทีนออกจากโรติเฟอร์แล้วนำโรติเฟอร์ไปเลี้ยงต่อในถังเลี้ยงเดิม พบว่าชุดทดลองที่โรติเฟอร์ผ่านการแช่ในแอลคาร์นิทีนมีความหนาแน่นโรติเฟอร์สูงกว่าชุดควบคุมอย่างมีนัยสำคัญ ตั้งแต่วันที่ 3 จนถึงวันที่ 9 ของการเลี้ยง

การทดลองสุดท้ายเป็นการพัฒนาและศึกษาการทำงานของระบบการเพาะเลี้ยงสาหร่ายและโรติเฟอร์แบบต่อเนื่องด้วยระบบการหมุนเวียนน้ำแบบปิด เพื่อใช้ในการเพาะเลี้ยงปลาวัยอ่อน ซึ่งเป็นการพัฒนาให้มีการทำงานแบบอัตโนมัติ โดยมีส่วนประกอบสำคัญ 3 ส่วน ได้แก่ ชุดเลี้ยงสาหร่าย ชุดเลี้ยงและเก็บเกี่ยวโรติเฟอร์ และชุดหมุนเวียนน้ำแบบปิด ซึ่งน้ำที่ผ่านการบำบัดแล้วมีคุณภาพเช่นเดียวกับน้ำใหม่ทั่วไป จากการศึกษาพบว่าระบบสามารถทำงานได้ต่อเนื่องเป็นเวลา 28 วัน โดยผลผลิตของสาหร่ายเฉลี่ย 1.63×10^{10} เซลล์/วัน ส่วนโรติเฟอร์มีผลผลิตเฉลี่ย 4.6×10^{8} ตัว/วัน ซึ่งระบบนี้มีข้อดีและได้เปรียบกว่าการเลี้ยงแบบเก็บเกี่ยวครั้งเดียวและแบบกึ่งต่อเนื่องที่ใช้กันทั่วไป

ภาควิชา	วิทยาศาสตร์ทางทะเล	ลายมือชื่อนิสิต.....ชัชฎาภรณ์ สรรคอนุรักษ์.....
สาขาวิชา	วิทยาศาสตร์ทางทะเล	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2550	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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CHATCHADAPORN SANANURAK: EFFECTS OF L-CARNITINE ON MICROALGA *Tetraselmis suecica*, ROTIFER *Brachionus plicatilis*, AND LARVAL SEABASS *Lates calcarifer* AND THE DEVELOPMENT OF ROTIFER PRODUCTION SYSTEM. THESIS ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D., THESIS COADVISOR: ASSOC. PROF. THAITHAWORN LIRDWITAYAPRASIT, Ph.D., 137 pp.

This study consisted of three experiments, i.e. (1) Effect of L-carnitine on microalga *Tetraselmis suecia*, rotifer *Brachionus plicatilis* and larval seabass *Lates calcarifer* (2) The use of L-carnitine to enhance growth of rotifer *B. plicatilis* in recirculation culture system and (3) The development of closed-recirculating, continuous culture system for microalga *T. suecica* and rotifer *B. plicatilis*.

The results revealed that *T. suecica* enriched with 0.1, 1 and 10 mg l^{-1} L-carnitine exhibited significantly higher specific growth rate than the control. The best specific growth rate was found at 10 mg l^{-1} L-carnitine. L-carnitine content in *T. suecica* ranged from 258-1,813 $\mu g\ g^{-1}$ dw and the highest was at 10 mg l^{-1} L-carnitine. The results of the study on the effect of L-carnitine on rotifer showed that growth of rotifer fed on *T. suecica* ,that enriched with 1 mg l^{-1} L-carnitine, was higher than other treatments, while the highest L-carnitine content has been found in rotifer that fed on *T. suecica* enriched with 10 mg l^{-1} L-carnitine. The experiment on rotifers fed on emulsion diet showed that the highest L-carnitine content has been found in rotifers enriched with 10 mg l^{-1} L-carnitine emulsion for 4 h. The study on the effect of L-carnitine on growth, survival and L-carnitine content in seabass larvae showed that the larvae fed on rotifers (that consumed *T. suecica* enriched with 10 mg l^{-1} L-carnitine) and rotifers fed on both with and without emulsion diet with 10 mg l^{-1} L-carnitine for 4 h were significantly larger in total length than other treatments. While the larvae fed on rotifers that consumed *T. suecica* without L-carnitine enrichment and no emulsion (control) were smallest. No significant difference has been found in the weight of rotifers of all treatments. The survival rate of fish larvae in all treatments were higher than the control treatment. The treatment of larvae fed on rotifers that consumed *T. suecica* enriched with 10 mg l^{-1} L-carnitine and rotifers fed on emulsion without L-carnitine showed the highest survival rate (39%). L-carnitine content in fish larvae were not detected in the control treatment and the treatment of larvae fed on rotifers that consumed *T. suecica* without L-carnitine enrichment and rotifers fed on emulsion without L-carnitine. The results suggested that the treatment of rotifers fed on emulsion enriched with 10 mg l^{-1} L-carnitine for 4 hrs could be used for feeding of seabass larvae.

In the second experiment, the use of L-carnitine in rotifer recirculation culture system at the initial cell density of 500 ind.ml^{-1} and exposed to 1 mg l^{-1} L-carnitine for 48 h was conducted. The result showed that population density of rotifers exposed with L-carnitine was higher than control treatment from day 3 to day 9 of the culture period. There was no difference in water quality between two treatments, except ammonium concentration.

The final experiment was to develop and operate a closed-recirculating, continuous culture system to produce microalgae and rotifers for larval fish culture. This new, automated system had three sub-components, including an alga culture component, a rotifer culture and storage with harvest component and a water treatment and re-use component. Our trials with the closed-recirculating, continuous culture system demonstrated that this culture system is capable of sustained and acceptable levels of microalgae and rotifer production for at least 28 days. During a continuous culture trial, microalga *T. suecica* production averaged 1.63×10^{10} cells day^{-1} while rotifer *B. plicatilis* production averaged 4.6×10^{6} ind.day^{-1}. This 28-day culture trial demonstrated the benefits and advantages of this culture system compared with more commonly used batch or semi-continuous culture system for microalgae and rotifers.

Department	Marine Science	Student's signature.....
Field of study	Marine Science	Advisor's signature.....
Academic year	2007	Co-advisor's signature.....

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CHAPTER I

INTRODUCTION

L-carnitine, an amino acid like compound, is used as a carrier to transport long-chain fatty acids into the mitochondria of a cell for beta-oxidation to produce energy. Based on its role in vertebrates, the use of L-carnitine supplementation in fish diets in aquaculture has been advocated for multi functional purposes. As a growth promoter, it specifically aids in utilization of high fat levels in the diet, thus providing a protein sparing effect. In addition, it provides protection against toxic levels of ammonia and xenobiotics. It can also alleviate stress related to water temperature extremes and facilitate better acclimation to water temperature changes; moreover it can help prevent adverse changes in muscle structure/texture related to higher levels of swimming activity and enhance reproduction (Bremer, 1983; Harpaz, 2005; Rebouche, 1998).

Marine rotifers (*Brachionus plicatilis*) are used at first feed during the initial development stages of several marine fish, mainly due to their ideal size and availability of large quantities by easy mass cultivation. Many attempts have been made to improve the nutritional quality of the rotifers. Several methods to enhance rotifer population growth by environmental manipulations, chemical treatments and also diets have been investigated (Yoshimura et al. 1997; Dhert et al. 2001; Hagiwara et al. 2001). Nutritional quality of live foods is a crucial factor for survival and normal growth of many aquatic larvae because these prey microorganisms must supply adequate energy and essential nutrients. In order to more specifically evaluate nutritional value of live food organisms, not only fatty acids, vitamins and amino acids, but other important component, like L-carnitine, should also be considered because of its growth promoting potential for fish of small size (Harpaz, 2005). Studies have shown that L-carnitine diet supplementation in small fish tend to exhibit better results. It is expected that fish larvae have higher requirement for L-carnitine. Seabass (*Lates calcarifer*) is an economically important fish in Thailand, which is why we selected seabass in this study.

Despite being an excellent first food for fish and crustacean larvae, rotifers still have some problems related to their culture and use. Among these problems are unpredictability in rotifer mass production difficulties in management and harvest of large rotifer populations, and difficulties in producing clean rotifers. In order to solve these problems, much research has been conducted. Recently, new culture methods of rotifer production have been developed enabling high density populations in continuous culture systems (Abu-rezq et al. 1997; Fu et al. 1997; Yoshimura et al. 1997) and continuous culture with recirculation systems (Suantika et al. 2003).

Alga production is the critical first step in production of rotifers and other lives, foods for fish larval. Considerable effort has gone into development of substitutes for live microalgae, but as yet these substitutes have not proven satisfactory (Fu et al. 1997; Navarro, 1999; Suantika et al. 2001; Yoshimura et al. 1997). Microalga substitutes with adequate nutritional values, are difficult to maintain, or have other functional problems, especially in tropical areas like Thailand. Consequently, live microalgae culture of *Chlorella* spp., *Nannochloropsis* spp. and *Tetraselmis* spp. still provide the basis for rotifer culture (Kongkeo, 1991). To date, however, most of these closed systems are either too costly, and/or are too technically complex and difficult to operate in most commercial hatchery settings. No continuous, closed-recirculation system for algae and rotifers culture has been developed for Thai hatcheries.

The objectives of this study were to evaluate the effects of L-carnitine on microalga (*Tetraselmis suecica*), rotifer (*Brachionus plicatilis*) and larval seabass (*Lates calcarifer*) and to develop an efficient but simple production system for rotifer. This study consisted of three sections.

The first set of investigation (Chapter III) involved the study on the effects of L-carnitine on microalga (*T. suecica*) on rotifer *B. plicatilis*, and on larval seabass (*L. calcarifer*).

The second research effort (Chapter IV) evaluated the effects of L-carnitine on population growth of rotifer (*B. plicatilis*) in a rotifer recirculation culture system.

The third section (Chapter V) designed the development of closed-recirculating, continuous culture system for microalga (*T. suecica*) and rotifer (*B. plicatilis*).

CHAPTER II

LITERATURE REVIEWS

2.1 Microalga

2.1.1 Systematic and Classification of *Tetraselmis*

Tetraselmis is a marine green flagellate, belongs to the Division Chlorophyta, class Prasinophyceae, order Chlorodendrales, family Chlorodendraceae, genus *Tetraselmis* and species *Tetraselmis suecica* (Tomas, 1997)

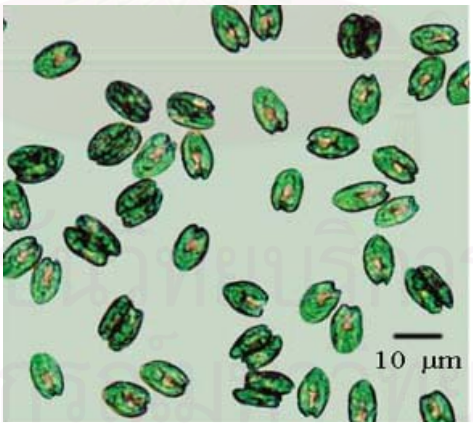

Figure 2.1 Photograph of *Tetraselmis suecica*

2.1.2 Culture Conditions

A. Physics condition

Temperature

The temperature at which cultures are maintained should ideally be as close as possible to the temperature at which the organisms were collected tropical (>20 ^{o} C). Most commonly cultured species of microalgae tolerate temperature between 16 and 27 ^{o} C, although this may vary with the composition of the culture medium, the species and strain cultured (Barsanti and Gualtieri, 2006). The temperature range of 15-32 ºC was the optimal growth condition for marine *T. suecica* (Weiss et al. 1985).

Light

Light is the source of energy which drives photosynthetic reactions is algae and in this regard intensity, spectral quality and photoperiod need to be considered. Light intensity plays an important role but the requirements greatly vary with the culture depth and the density of the algal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture. Too high light intensity (e.g., direct sunlight, small container close to artificial light) may result in photoinhibition. (Barsanti and Gualtieri, 2006). Grima et al. (1994) showed that the light saturated for growth, highest protein and chlorophyll of *Tetraselmis* sp. was between 80 and 100 Wm^{-2}. Moreover, overheating due to both natural and artificial illumination should be avoided. May be natural or supplied by fluorescent tubes emitting either in the blue or the red light spectrum, as these are the most active portions of the light spectrum for photosynthesis. Aidar et al. (1994) reported that *T. gracilis* showed a different growth response to light color and grew faster in red light. Although, *T. gracilis* cells grown in white light could have increased the cellular pool of pigments and proteins. Although cultivated phytoplankton develops normally under constant illumination, and hence a light/dark (LD) cycle is used (maximum 16:8 LD, usually 14:10 or 12:12). Meseck et al. (2005) found that *T. chui* were exposed to only 8 h of light had the slowest growth and

utilization of nutrients. These findings suggest that day length is important in determining growth and nutrient uptake in *T. chui*.

pH

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2-8.7. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The latter is accomplished the culture. In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth. *T. chui* showed the division rate is higher when the pH is maintained by bubbling carbon dioxide into the culture system and division of *T. chui* was greater at a pH ranged of 7.0-8.0 (Meseck, 2007; Meseck et al. 2007).

Salinity

Marine algae are extremely tolerant to changes in salinity. Most species grow best at a salinity that in slightly lower than that of their native habitat, which is obtained by diluting sea water with tap water.

Aeration/Mixing

Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification and to improve gas exchange between the culture medium and the air. Mixing of microalgal cultures may be necessary under certain circumstances. Cells must be kept in suspension in order to grow in concentrated cultures to prevent nutrient limitation effects due to stacking of cells and to increase gas diffusion. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test-tubes, Erlenmeyer), aerating (bags, tanks), or using paddle wheels and jet pumps (ponds). Not all algal species can tolerate vigorous mixing. Most cultures do well without mixing, particularly when not too concentrated, but when possible, gentle manual swirling (once each day) is recommended (Barsanti and Gualtieri, 2006; Lavens and Sorgeloos, 1996).

B. Chemical condition

Nutrients, Trace Metals, and Chelators

Nitrate is the nitrogen source most often used in culture media, but ammonium can also be used and indeed is the preferential form for many algae because it does not have to be reduced prior to amino acid synthesis, the point of primary intracellular nitrogen assimilation into the organic linkage. Ammonium concentrations greater than 25 μM however, often reported to be toxic to phytoplankton, so concentrations should be kept low. Inorganic (ortho) phosphate, the phosphorus form preferentially used by microalgae, is most often added to culture media. The trace metals that are essential for microalgal growth are incorporated into essential organic molecules, particularly a variety of coenzyme factors that enter into photosynthetic reactions. Of these metals, the concentrations of Fe, Mn, Zn, Cu and Co in natural waters may be limiting to algal growth (Kaplan et al. 1986). As radioactive selenite-75 has been used to investigate the metabolic transformation of inorganic selenium by the marine phytoplankton *T. tetrathele* (Wrench, 1978).

Vitamins

Roughly all microalgal species tested have been shown to have a requirement for vitamin B_{12} which appears to be important in transferring methyl groups and methylating toxic elements such as arsenic, mercury, tin, thallium, platinum, gold, and tellurium, around 20% need thiamine and less than 5% need biotin. It is recommended that these vitamins are routinely added to seawater media. No other vitamins have ever been demonstrated to be required by any photosynthetic microalgae (Kaplan et al. 1986).

2.1.3 Culture Method

Mass cultivation of *Tetraselmis* has been carried out in different kinds of open ponds. The main problem in outdoor cultivation in open systems is contamination of the culture by other algal species. At present, culture methods used in hatcheries for *Tetraselmis* production rely mainly on polyethylene bags and transparent glass-fibre cylinders (up to 500 l) usually kept indoors with artificial light (Fulks and Main, 1991). These systems are inefficient, leading to low productivities and little reliable cultures. In the last decade, research efforts have been directed towards the development of more efficient, high surface to volume ratio photobioreactors for microalgae cultivation. Some of these systems have been used, in laboratory or at small scale level outdoors, to cultivate *Tetraselmis* spp. (Borowitzka, 1997). Different types of algal cultures are used worldwide, the most routinely adopted include batch, continuous, and semicontinuous ponds and photobioreactors (Lavens and Sorgeloos, 1996; Zittelli et al. 2006). In addition, there are several types of culture system. Factors to be considered include: the biology of the alga, the cost of land, labor, energy, water, nutrients, climate (if the culture is outdoors) and the type of final product (Borowitzka, 1992). The various large-scale culture systems also need to be compared on their basic properties such as their light utilisation efficiency, ability to control temperature, the hydrodynamic stress placed on the algae, the ability to maintain the culture unialgal and/or axenic and how easy they are to scale up from laboratory scale to large-scale (Borowitzka, 1999).

Continuous culture is basically a method of prolonging the exponential growth phase of an organism in batch culture. The technique involves feeding the organisms with fresh nutrients and at same time removing spent indium plus cells from the system in such a fashion that several factors remain constant with time, i.e. culture volume, cell concentration, product concentration, and culture environment (e.g. pH, temperature or dissolved oxygen). This condition is known as steady state. Providing the culture remains free from contamination and the organism is stable then the system can be operated for long periods of time. The examples of continuous culture system for microalga *Tetraselmis* spp. are detailed in Table 2.1.

Table 2.1 Examples of continuous culture system for microalga *Tetraselmis* spp.

Type/Name	Highest productivity/ Cell density	Location	Reference
-Closed Photobioreactor Annular column	0.49 g l^{-1} day^{-1}	Italy	Zittelli et al. 2006
-Cyclostat	0.2 g l^{-1} d^{-1}/ 2.5×10^{6} cell ml^{-1}	Spain	Fábregas et al. 1995
-Turbidostat	0.56×10^{11} cells day^{-1}	England	Laing and Jones, 1988
-Photobioreactor Helical tubular (Biocoil)	1.2 g l^{-1} day^{-1}	Australia	Borowitzka, 1997

The main advantage of such a system is that cell mass and other products can be produced under optimal environment conditions. Depending on the control parameter and the operation mode, continuous culture can be classified into four general types (Figure 2.2). A common feature of these cultures is that they consist of one or more culture vessels into which fresh medium or culture from a preceding vessel is continuously introduced at a rate, F, expressed in liters per hour and that the culture volume, V, expressed in liters, is kept constant by continuous removal of the culture. The general concept and theory of these four types of continuous culture are described in more detail as follows.

Chemostat (described by Zeng, 1999)

The chemostat (Figure 2.2A) is defined as a continuous culture system in which the feed rate is set externally and cell growth is limited by a selected nutrient. The second condition means that the specific growth rate, μ ($hour^{-1}$), of the organism is a function of a single growth-limiting nutrient. However, this definition may be relaxed to include continuous culture limited simultaneously by more than one nutrient component (Gottschal, 1992). Continuous cultures that are fed with an

inhibitory nutrient or that form toxic products can be limited by growth inhibition under the condition of excess of all nutrients.

A chemostat is usually started as a batch culture. Before a nutrient becomes limiting, the nutrient feed is started. Cells grow until the chosen nutrient becomes limiting. After this, cell growth is limited by the rate of addition of medium. The specific growth rate of a chemostat culture can be determined from a material balance for biomass.

Cell accumulation	=	Cells In	+	Cells growth	-	Cell out	-	Cell death

In mathematical form this is:

$$\frac{dX}{dt} = \frac{F}{V}X + \mu X - \frac{F}{V}X - \alpha X \qquad (1)$$

Where X_0 and X are the cell mass (g/l) in the feed and the fermentor respectively, F is the medium flow rate (l/hr), V is the fermentor volume (l), μ and α are the specific growth and death rates (hr^{-1}) respectively and t is time (hr).

Usually, with a chemostat, the feed stream is sterile and $X_0 = 0$. Also in most continuous cultures, the specific growth rate is much greater than the death rate ($\mu >> \alpha$), so that equation l may be simplified as shown in equation 2.

$$\frac{dX}{dt} = -\frac{F}{V}X + \mu X \qquad (2)$$

As a consequence, at steady state, when $\frac{dX}{dt} = 0$,

$$\mu = \frac{dX}{dt} \qquad (3)$$

Thus the specific growth is determined by the flow rate of the medium divided by the culture volume. This ratio is defined as the dilution rate D or

$$D = \frac{dX}{dt} = \mu \qquad (4)$$

and at steady state the specific growth rate is equal to the dilution rate.

Thus, by maintaining a constant volume and changing the nutrient feed rate, one can precisely control the specific growth rate of a culture over a range up to the maximum specific growth rate (μ_{max}) and let the system come to a steady state. This is one of the most important properties of a chemostat. Note that the following additional assumptions are made in the derivation of equations 1 to 4.

1. Cells are distributed randomly in the bioreactor, i.e., the cells do not adhere to each other or to the walls of the reactor and the suspension is well mixed.

2. The population is non segregated and consists of physiologically identical cells.

3. The population density, *X*, is a continuous variable, i.e., the number of cells is sufficiently high and the size of each cell is sufficiently small for the discreteness of biomass to be ignored.

4. The volume occupied by the cells is negligible compared to the total volume of the culture.

Auxostat

An auxostat is a continuous culture in which a growth dependent parameter is kept constant by adjusting the feeding rate of medium (Figure 2.2B). The dilution rate and hence the specific growth rate of the culture adjust accordingly. The choice of the feedback parameter for an auxostat is quite broad. It includes cell density (turbidity), pH, dissolved oxygen concentration, CO_2 in effluent gas, and concentrations of nutrients and products (Gostomski et al. 1994). Sometimes, the term nutristat is also used to refer to auxostats using a nutrient concentration as the feedback growth parameter. Of the different kinds of auxostats, the turbidostat and the pH auxostat have so far found the most applications.

In a turbidostat, the biomass (cell density) is used as a control parameter. A sensor detecting the biomass density gives a signal to a pump to add more medium when the biomass density rises above a chosen level. By means of turbidostat control, therefore, the biomass density is set and the dilution rate adjusts itself to the steady-state value, in contrast to the chemostat in which the dilution rate is fixed and the biomass concentration adjusts itself to the steady-state level.

In a pH-autostat, the addition of fresh medium is coupled to pH control. As the pH drifts from a given set point, fresh medium is added to bring the pH back to the set point, fresh medium is added to bring the pH back to the set point. The rate of addition of medium is determined by the buffering capacity and the concentration of nutrients in the medium. Buffering capacity is defined as the equivalents of titrant required to change the medium pH to the culture pH. The governing equation is the mass balance on the H^+ ion concentration, expressed as milliequivalents (meq) per liter, in the bioreactor.

Continuous Culture with Cell Recycle (Perfusion)

Cell recycle is a useful means for increasing the concentrations of biomass and product in a continuous culture (Figure 2.2C). This system can be operated at a dilution rate higher than the maximum specific growth rate, leading to a much higher output of the reactor. Another property is that the dilution rate is almost independent of the growth rate. Recycle of biomass can also protect against shock loading with an inhibitory substrate, because the critical dilution rate is particularly advantageous in the following cases: (i) the growth-limiting substrate is un avoidably dilute, in the treatment of effluents; (ii) the substrate has a low solubility, such as when a gaseous is used; (iii) the concentration of growth-limiting substrate has to be limited because of the formation of inhibitory product (s); and (iv) product formation is not associated with growth.

Several methods can be used for the retention of biomass, such as filtration, centrifugation, and immobilization. Depending on the position of the separation device inside or outside of the reactor, the methods can be further divided into internal

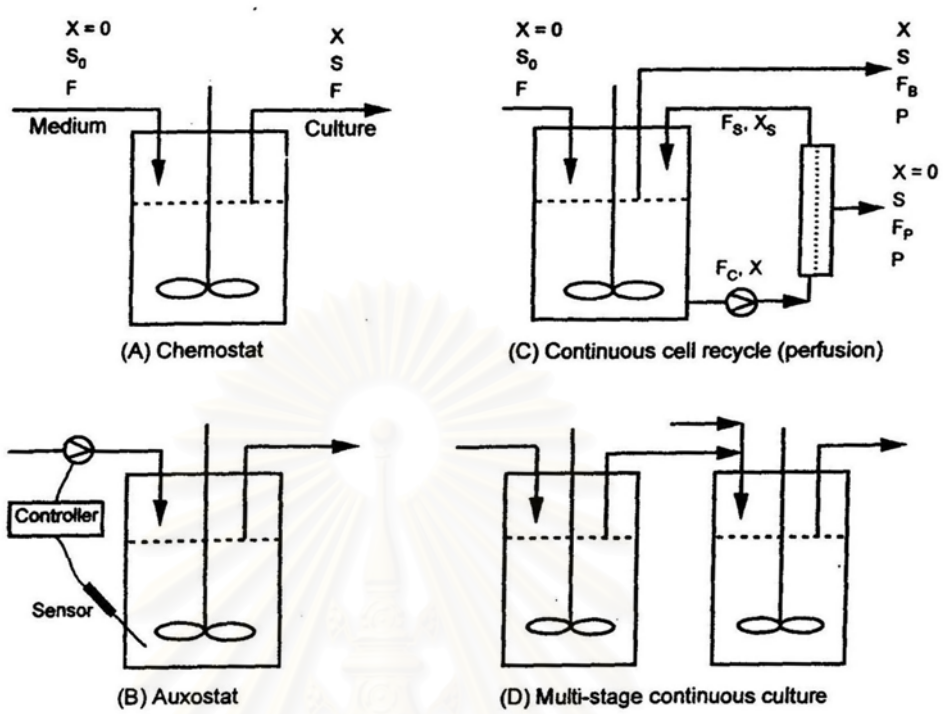

Figure 2.2 Schematic diagram of four types of continuous culture. (A) Chemostat, (B) Auxostat, (C) Continuous culture with cell recycle and (D) Multistage continuous culture

and external systems. The concentration of culture effluent outside of the reactor by means of membrane filtration has so far found the most frequent application, except for the biological treatment of wastewater, in which the recycling of sludge after sedimentation has been used for a long time.

Multistage Continuous Culture

The two-stage continuous culture system shown in Figure 2.2D can extend the range of application of continuous culture. For example, the second stage may be used to extend the growth rate downward to zero, and the first stage may be used to achieve stable conditions with maximum growth rate, both of which conditions may be desired in certain cases but are impossible in the simple chemostat. The latter property is particularly useful when the substrate is also a growth inhibitor. In the production of secondary metabolites and enzymes by continuous culture, the second

stage may be used to provide a nongrowing situation in which product formation occurs. For products of foreign gene expression, the second stage can be used for induction of expression. Another useful application of the two-stage continuous culture is for reactor scale-down studies. The two-stage culture system can be extended to include more stages and more feeding streams with or with or without biomass recycle (Zeng, 1999).

2.2 Rotifer

2.2.1 Systematic and Classification

The phylum rotifera is a relatively small group of microscopic aquatic or semi-aquatic invertebrates, encompassing about 2,000 species of unsegmented, bilaterally symmetric, pseudocoelomates. Rotifers belong to the phylum rotifera. The most commonly used system of taxonomy for rotifers is the system proposed by Koste and Shiel (1987) and modified by Wallace and Snell (1991). The phylum rotifera is divided as follows:

Class	Digononta		
		Order	Bdelloidea
		Order	Seisonacea
Class	Monogononta		
		Order	Collothecacea
		Order	Floscullariacea
		Order	Ploimida

Brachionus plicatilis, an important species belongs to the class of the Monogononta, order Ploimida and family Brachionidae.

2.2.2 Morphology

Rotifers are noted for being the smallest metazoan ranging from 40 to 2,000 μm (Pechenik, 1996) and are often smaller than ciliates. Males, in general, are much smaller in size and structurally much simpler or less developed than females. The rotifer's body is differentiated into three distinct parts consisting of the head, trunk and foot (Figure 2.3).

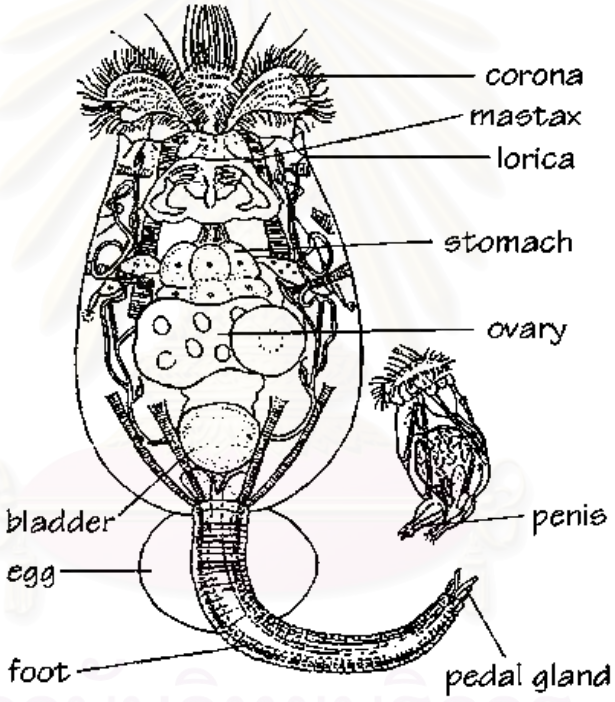

Figure 2.3 *Brachionus plicatilis*, female and male (modified from Koste and Shiel, 1987)

The males have no digestive tract and no bladder but have an over-proportionate single testis. Whereas the fertilized eggs have a larger size and thick shell (resting egg) which enables them to support unfavorable conditions such as drought, cold, food shortage, etc. The resting eggs will hatch into amictic females when incubated in optimum condition for hatching.

The broad or narrowed anterior end, generally called a head, is not well delimited and carries several organs such as the ciliated corona (rotatory organ that serves as locomotory and food-collecting organ from which the original name of rotifer), the mouth opening and several sensory organs and appendages. The presence of the corona distinguishes rotifers from all other metazoans. The rotatory organ or corona can easily be recognized its annular ciliation. Moreover, the characteristics of the corona distinguishes different group of rotifers. Next to the anterior part is the trunk (the body proper) which forms the major part of the body. The trunk contains the digestive, excretory organs and reproductive organs. A characteristic organ for the rotifers is a muscular pharynx, the mastax, possessing a complex set of hard jaws that is very effective in grinding ingested particles. The foot or the terminal portion of the body is a ring-type retractable structure without segmentation ending in one or four toes which is considerably narrower than the trunk region.

2.2.3 Biology and Life History

Their reproduction occurs either sexually or asexually known as parthenogenesis. Parthenogenesis is a characteristic of most groups, and in most species males are present in the population only at a certain time (Pechenik, 1996).

The life cycle of rotifers is simple under favorable conditions, consisting of production of amictic eggs by amictic female and hatching of the eggs to generate the diploid monogenic generation. This vegetative, amictic cycle can be influenced by some external factors such as temperature, food and population density (Pourriot and Snell, 1983), and also by genetic factors which play an important role in the sensitivity of strains to mictic stimuli (Gomez and Serra, 1995; Lubzens et al. 1985). Therefore, under particular conditions the female rotifers can undergo more complicated sexual reproduction resulting in mictic and amictic females. Mictic and amictic females are morphologically indistinguishable (Figure 2.4). The mictic females produce haploid (n chromosomes) eggs by meiosis and the hatched-out larvae of these unfertilized mictic eggs develop into haploid males (Figure 2.4). The life span of rotifers is temperature dependent. As the temperature of water rises, the life span period becomes shorter. A rotifer survives for 3.4 to 4.4 days under the culturing

conditions at 25 ºC, and produces about 10 eggs (maximum 25) which hatch within 0.5 to 1.0 day. The newly-hatched larvae become adult within 0.5 to 1.5 day (Hagiwara et al. 1995).

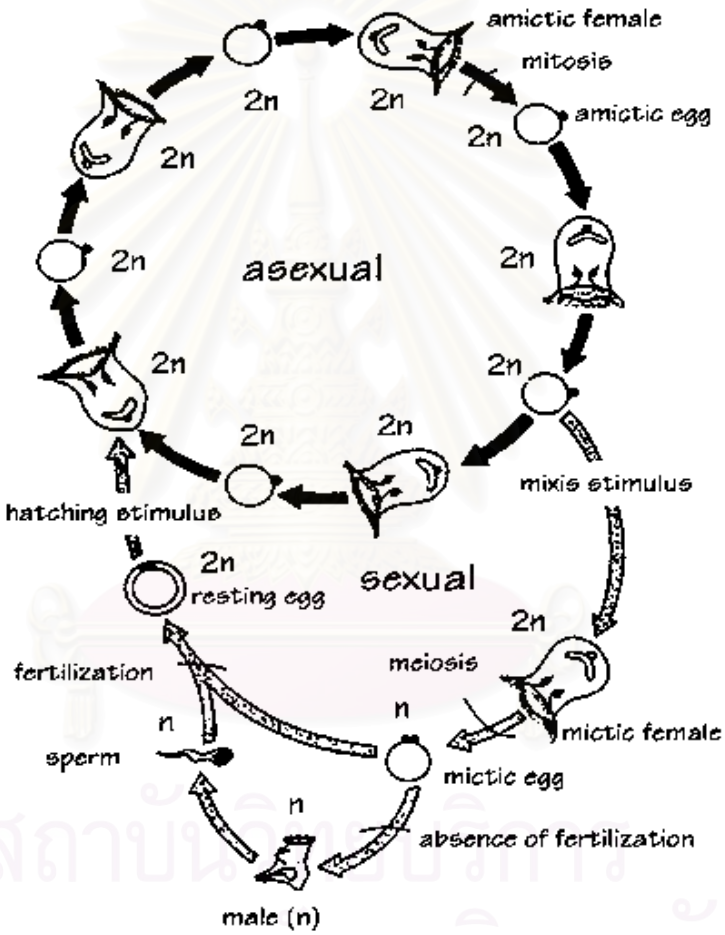

Figure 2.4 Parthenogenetical and sexual reproduction in *Brachionus plicatilis* (modified from Hoff and Snell, 1987)

2.2.4 Marine Rotifer *Brachionus plicatilis*

One of the most important species of marine rotifers is *Brachionus plicatilis*, a cosmopolitan in inland saline and coastal brackish water. *B. plicatilis* plays an important role in the natural food chain in marine or brackishwater habitats and modern aquaculture (Sorgeloos et al. 1994). *B. plicatilis* is a species complex, which includes different morphotypes; culturists describe them based on lorica size as L (large), S (small), and SS (super small) types, and biological traits were compared among morphotypes by Hagiwara et al. (1995). Based on information on morphology (Fu et al. 1991a), allozyme pattern (Fu et al. 1991b) and karyotypes (Rumengan et al. 1991). Segers (1995) classified L-type as *B. plicatilis* and others as *B. rotundiformis*. Recent studies indicate that the so-called S- and SS-type rotifers can be classified as different species; the former as *B. ibericus* and the latter as *B. rotundiformis* (Ciros-Perez et al. 2001; Kotani et al. 2005). It should be mentioned, however, that despite the strong species boundary observed between *B. plicatilis* and others, a weak species boundary was observed between *B. ibericus* and *B. rotundiformis* based on the male mating behavior when challenged with females of different species (Kotani et al. 1997). Recent studies in molecular phylogeny using ITS1 and COI indicate that *B. plicatilis* sp. complex may include at least 9 species (Gomez et al. 2002; Hagiwara et al. 2007). Their results indicate that so-called L-, S- and SS-type rotifers include 4, 4 and 1 species, respectively. But their morphological differences among species have not been clarified and species names have not been given. It is important to continue and further confirm the molecular phylogenic results as the current analyses are based on only two partial DNA sequences. As the current results suggest that the taxonomy of *B. plicatilis* sp. complex is not yet clear. The terms of L-, S- and SS-type, are commonly used among scientists and technologists in the area of aquaculture biology (Hagiwara et al. 2007).

2.2.5 Culture Conditions

Temperature

The optimal culture temperature for rearing rotifers is strain dependent. Each rotifer strain has a different range of temperature tolerance. However, physiological changes that occur at high temperatures are likely to be similar amongst strains. Increasing the temperature, until a certain limit, generally results in an increased reproduction activity. Rearing rotifers below their optimal temperature slows down the population growth considerably. Serra et al. (1998) reported that *B. rotundiformis* grow best at higher temperature (>25 ºC) while *B .plicatilis* shows a greater tolerance below 20 ºC. Optimal temperature for *B. plicatilis* is 25 ºC (Lubzens et al. 1985) and for *B. rotundiformis* reproduction stops under 15 ºC whereas *B. plicatilis* is still reproducing at this temperature.

Salinity

In general, salinity has an effect on reproduction, nutrition and growth of aquatic organisms. Growth may be optimal at a restricted salinity range depending on the species. The rotifer *B. plicatilis* is able to tolerate a wide range of salinities (euryhaline organism). Optimal reproduction, can only take place at salinities below 35 ppt (Lubzens, 1987). Although *B. plicatilis* has a very wide salinity tolerances range, transferring of the rotifers directly from low to high salinity may cause stress and immobilization of the rotifers and can result in high mortality rates (Øie and Olsen, 1993; Snell, 1986).

Dissolved oxygen

In rotifer cultures, dissolved oxygen is also one of the most important chemical characteristics. Most rotifers can survive in water containing as low as 2 mg 1^{-1} of dissolved oxygen (Dhert, 1996). The oxygen solubility in culture water depends on the temperature, salinity, rotifer density and type of food. Oxygen solubility correlates inversely with temperature and salinity. Increasing temperature results in decreasing dissolved oxygen concentration in culture water, whereas at high temperature the demand for dissolved oxygen increases due to the increased rotifers

metabolic rate. In a high density culture of rotifers (>10^3 ind. ml^{-1}) the supply of oxygen is crucial and it is difficult to maintain an optimum dissolved oxygen level (Yoshimura et al. 1997).

pH

Fukusho (1989) stated that rotifers can survive in an environment having a pH range from 5 to 9. In their natural environment rotifers live at pH levels above 6.6 and in culture conditions the best results are obtained at a pH above 7.5 (Dhert, 1996). In a high density culture of rotifers a pH of 7.0 is optimal for rotifer population growth (Yoshimura et at. 1995). The pH level is related to the toxicity of excretion products, for example, ammonia (NH_3).

Ammonia

The temperature and pH of the water influence the NH_3/ NH_4^+ ratio. High levels of unionized ammonia (NH_3) are toxic to rotifers but rearing conditions with ammonium (NH_4^+) concentration below 1 mg l^{-1} appear to be safe (Dhert, 1996). The toxicity of ammonia for rotifers is not clear.

2.2.6 Diets used in rotifer cultures

Microalgae

In their natural environment rotifers feed on microalgae, bacteria, yeast and protozoa (Fukusho, 1989). Microalgae are used to produce mass quantities of zooplankton (rotifers, copepods and brine shrimps) which serve in turn as food for larval and early juvenile stages of crustacean and fish (Dhert, 1996). For the cultivation of rotifers, food that can be produced in a large amount under artificial cultivation conditions and can be effectively utilized by rotifers is most desirable, since rotifers have very fast filtration capacity. Undoubtedly, marine microalgae are the best diet for rotifers and very high yields can be obtained if sufficient algae are available and an appropriate management is followed. The most common algae used in rotifer cultures are *Nannochloropsis oculata* (Lubzens, 1987; Fukusho, 1989) with

a size of 2-3 μm in diameter and a relatively high content in 20:5n-3 fatty acid (EPA), *Tetraselmis tetrahele* or *T. suecica* which have a cell diameter of 20-30 μm and high EPA content, *Isochrysis galbana* containing high level of 22:6n-3 fatty acid (DHA). Some other micro-algae including *Dunaliella tertiolecta*, *Pavlova lutheri*, *Chlorella* sp. and *Stichococcus* sp. have also been used as food for rotifer cultures. Microalgae are believed to play a role in stabilizing the water quality, influence the nutrition of the larvae and control the microbial composition.

Yeast

Baker's yeast is commonly being used. The experiments on the use of baker's yeast as food for rotifers. They reported that the rotifers could grow on a mixed food (50% *Chlorella* and 50% baker's yeast) as well as with 100% *Chlorella* (Hirata, 1980). There are several yeasts that can be used as rotifer feed, e.g. baker's yeast (fresh and instant) (*Saccharomyces cerevisiae*), caked yeast (*Rhodotorula*) and marine yeast (*Zygosaccharomyces marina*, *Torulopsis candida* var. marina, *T. larvae*, and *Saccharomyces acidosaccharophill*). Baker's yeast has been used as a suitable algal substitute for *Brachionus* (Hirayama, 1987), because of its small particle size of 5-7 μm in diameter, high content of protein and also the presence of bacteria growing on the yeast surface. Although yeasts have been accepted as food for rotifer cultures, they contain low concentrations of HUFA of the n-3 series, mainly 20:5n-3 (Fukusho, 1983) as well as vitamin B_{12} (Hirayama and Funamoto, 1983). Yoshimura et al. (1996) stated that the supplementary feeding of baker's yeast makes the rotifer cultures less stable. The reason why baker's yeast has been used for rotifers is attributable to its supplemental nutritional effects to other microalgae and bacteria (Fukusho, 1989). In order to improve the nutritional value of rotifers the administration of baker's yeast for mass production of rotifers needs to be combined with algae (Lie et al. 1997).

Formulated diets

The bottlenecks in the optimal use of rotifers are mainly related to reliable and cost effective techniques for continuous mass production. A break-through in

production technology has been the development of an artificial diet which completely eliminates the need of an extra enrichment period for enhancement of the rotifers' dietary value (Lavens and Sorgeloos, 1996; Dhert et al. 2001). The most frequently used formulated diet in rotifer cultures is the yeast-based product Culture Selco® (CS) (INVE N.V., Belgium) available under a dry form. Candreva et. al. (1996) reported that Culture Selco® is widely used by hatcheries in Europe.

2.2.7 Culture method

Batch culture

Batch culture systems seem to be the most common type of rotifer production used in hatcheries. The size of the rearing tanks varies from 500 to 1,000 l plastic tanks up 10,000 l for concrete tanks. In these systems the rotifers are inoculated at a density of 50 to 200 ind. ml^{-1}. The density at harvest time is about 600 ind. ml^{-1} after 4 days culture (Dhert, 1996).

Semi-continuous culture

Semi-continuous culture systems are usually performed in larger tanks (50-200 m^3) than the ones used in the batch culture. The culture period is longer than that in the batch culture system. Morizane (1991) reported that they could continue culturing rotifers without changing tanks, harvesting a large of number rotifers for an entire year. The inoculated density of rotifers varies from 50 to 200 ind. ml^{-1} and can reach up to 300 to over 1,000 ind.ml^{-1} in 3 to 7 days at harvesting time, using microalgae and baker's yeast as food sources.

Continuous culture

James and Abu-Rezeq (1989) reported that the continuous culture systems have higher productivity than batch and semi-continuous culture systems. The initial density of rotifers varies, and during the culture period the rotifer density is maintained constant and the production is dependent on other factors such as feeding regime and water quality.

Ultra-high density culture

Japanese scientists have developed ultra-high density culture technology with fully automated systems. Yoshimura et al. (1995) reported that very high rotifer productions could be achieved in a l m^3 tank in a batch culture method in 2 day intervals with a initial density of 10,000 ind.ml^{-1}. The latest, ultra-high density (maximum density from 20,000 up to 40,000 ind.ml^{-1}) rotifer mass culture has been developed based on concentrated freshwater *Chlorella* as food (Yoshimura et al. 1994, 1997; Fu et al. 1997).

2.3 Seabass Larva

Lates calcarifer (Bloch), an important species, belongs to Phylum Chordata**,** Sub-phylum Vertebrata, the class of the Pisces, sub-class Teleostomi, order Percomorphi and family Centropomidae. *L. calcarifer*, commonly called the giant sea perch or seabass, is an economically important food fish in the tropical and subtropical regions of Asia and the Pacific. It is commercially cultivated in Thailand, Malaysia, Singapore, Indonesia, Hong Kong and Taiwan, in both brackish water and freshwater ponds, as well as in cages in coastal waters. Techniques for the propagation of seabass were originally developed in Thailand in the 1970s and this species is now cultured throughout most of its range. The considerable research and development efforts that have gone into culturing seabass over recent decades (Copland & Grey, 1987) have resulted in reliable and consistent techniques for the aquaculture of this species. Production of Asian seabass increased during the past ten years, and FAO statistics estimated that 26 000 tonnes were produced in 2004 (FAO, 2006).

2.3.1 Egg and larval development

Fertilized eggs undergo rapid development and hatching occurs 12-17 h after fertilization at 27-30°C (Tattanon and Tiensongrusmee, 1984; NICA, 1986; Ruangpanit, 1987; Parazo et al. 1990). Hatching rates for seabass induced using environmental and hormonal manipulation range between 40-85% and 0.1-85% respectively (Kungvankij et al. 1986). Newly hatched larvae have a large yolk that is absorbed rapidly over the first 24 h after hatching, and is largely exhausted by 50 h after hatching (Kohno et al. 1986). The oil globule is absorbed more slowly and persists for about 140 h after hatching. The mouth and gut develop the day after hatching (day 2) and larvae commence feeding from 45 to 50 h after hatching (Kohno et al. 1986; Parazo et al. 1990).

2.3.2 Larval Rearing (described by Rimmer and Russell, 1998)

The main requirements for the successful larval rearing of seabass are the same as those for other finfish species, providing a stable environment suitable for survival and growth of the larvae; prey organisms of suitable size and at suitable densities as a food source for the larvae. It is possible to meet these requirements using various culture procedures, but larval-rearing techniques can generally be divided into either intensive or extensive techniques. Intensive larval rearing involves the culture of larvae in a controlled environment, such as a hatchery, where the fish larvae are supplied with prey organisms that are also cultured under controlled conditions. In contrast, extensive larval rearing involves the culture of larvae in fertilized marine or brackish water ponds where the culturist has little direct control over factors such as water quality and prey organism density.

The physicochemical tolerances of seabass larvae are poorly known. The recommended levels of these parameters for larval rearing are listed in Table 2.2. These are based on experience in the larval rearing of seabass and on the few studies that have been carried out on the physicochemical tolerances of marine fish larvae. Newly hatched larvae have the lowest tolerance to various physicochemical

parameters and even moderate deviation from optimum conditions, while not directly lethal, may substantially reduce first feeding success and hence survival. In addition, two or more factors that deviate slightly from optimal conditions may act synergistically to reduce survival; for example increases in pH will increase the proportion of the toxic unionized form of ammonia (NH_3).

Table 2.2 Recommended water quality criteria for larval rearing of seabass. (Kungvankij et al. 1986).

	Optimum	**Minimum**	**Maximum**
Temperature (°C)	26-30	25	31
Salinity (ppt)	28-31	20	35
pH	8.0	7.5	8.5
Dissolved oxygen (mg 1^{-1})	Saturation	2	-
Ammonia (NH_3) (mg 1^{-1})	0	-	0.1
Nitrite (mg 1^{-1})	0	-	0.2
Nitrate (mg 1^{-1})	0	-	1.0

Note: These figures are only a guide, as the precise physicochemical, tolerances of seabass are poorly known.

2.4 Enrichment

Many attempts have been made to improve the nutritional quality of the rotifers in the last few year and two enrichment methods have been described: the “indirect method” which attempts to improve the biochemical composition of the algae or yeast by changing its culture medium and the “direct method” which supplies the rotifers with baker’s yeast plus some specific nutritional compounds, mainly emulsion of marine oils (Watanabe et al. 1983). The enrichment of rotifers, several approaches can be followed: (1) the adjustment of the lipid and vitamin content of the rotifers just before feeding them to other organisms is referred to as short-term

enrichment (generally less than 8 h exposure) and (2) the feeding of rotifers on a complete diet or long-term enrichment (rearing of the rotifers on the enrichment diet for more than 24 h). Many authors have elaborated on both techniques and each of them has its benefits and disadvantages. The short-term enrichment technique has the advantage of being fast and flexible but very often produces lower quality rotifers with too high lipid content (Dhert et al. 1990; Park et al. 2006; Robin, 1998) and low hygienic quality. The biggest problem in this enrichment technique that a lot of rotifers are lost when they are concentrated (sticking of the rotifers) at high density. Also, transfer of oil to larval rearing tanks with consequent loss of water quality. On top of that, the retention time of the nutrients, which are mainly accumulated in the digestive tract of the rotifers is very short and can create problems when the rotifers are not eaten immediately. The types of enrichment for rotifers are summarized in Table 2.3.

Table 2.3 Type of enrichment for rotifers.

Source	**Reference**
Microlgae	Ben-Amotz et al. 1987; Dhert et al. 1998; Koven et al. 1990; Lubzens et al. 1995; Mourente et al. 1993; Øie et al. 1994; Sukenik and Wahnon, 1991; Sukenik et al. 1993; Watanabe et al. 1983; Whyte and Nagata, 1990
Oil emulsions	Dhert et al. 1990; Kanazawa, 1993; Lie et al. 1997; Park et al. 2006; Reitan et al. 1994
Vitamins	Merchie et al. 1995; Merchie et al. 1997
Proteins	Øie and Olsen, 1997; Watanabe et al. 1983
Formulated diets	Fu et al. 1997; Suantika et al. 2001; Yoshimura et al. 1997

2.5 L-carnitine

2.5.1 Chemical Properties of L-carnitine

Carnitine (CAS: 1-propanaminium, 3-carboxy-2-hydroxy-N,N,N-trimethyl-hydroxide, inner salt) exists in the form of optically active enantiomers (R)-carnitine [L(−)-carnitine] and (S)-carnitine [D(+)-carnitine and as the racemic compound (RS)-carnitine (DL-carnitine). L-carnitine is widespread in the tissues of animals, plants and microorganisms. L-Carnitine (Figure 2.5) is an extremely hydrophilic compound.

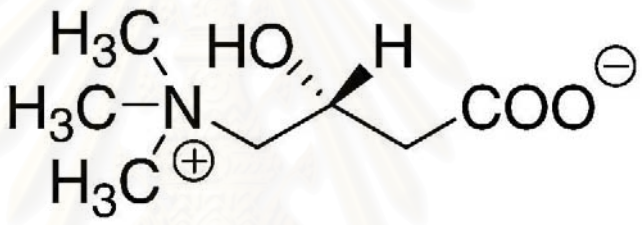

Figure 2.5 Structure of L-carnitne

L-carnitine (Latin: caro, carnis = meat) is found in high concentrations in the muscle of both vertebrates and invertebrate animals (Murray et al. 1980) and is a characteristic component of the skeletal muscles of animal tissues and the liver.

L-carnitine is an essential cofactor of fatty acid metabolism. The effect of L-carnitine is based on its ability to stimulate fatty acid oxidation, thereby increasing oxygen consumption. Within fatty acid metabolism, carnitine serves as a carrier for acyl groups through the mitochondrial membrane. The acyl groups are transferred by acyltransferase from acyl-coenzyme A onto the hydroxyl group of L-carnitine. Transporting L-carnitine and acyl-L-carnitin through the membrane is performed via the transport protein, translocase (Fritz et al. 1963). L-Carnitine is essential for certain insects, e.g. mealworm and therefore it was formerly called Vitamin BT (T for *Tenebrio molitor*) or mealworm factor (Budavari, 1989). Today, L-carnitine and acetyl-L-carnitine are applied in cosmetics (water reservoir, electrolyte, “stiffening”

effects) and in pharmacy, e.g. in myocardial disorders (Bahl and Bressler, 1987), geriatrics (Bartolomucci and Weltevreden, 2000) and certain types of diabetic disease (Bresica et al. 2002). L-carnitine is prepared in different ways by extraction, by chemical methods, e.g. from carbohydrates (Bellamy et al. 1990) or from glycerin (Marzi et al. 2000), and by microbial conversion from its precursors by microorganisms and enzymes and by enantioselective synthesis from achiral precursors (Jung et al. 1993).

2.5.2 Source and Biosynthesis Pathways of L-carnitine

L-Carnitine is synthesized from lysine and methionine in the human liver. Biosynthesis research based on L-carnitine started a long time ago: γ-butyrobetaine was administered to dogs, resulting in 3% carnitine in the urine (Linneweh, 1928). The origin of the body's own γ-butyrobetaine is ε-N-trimethyllysine, which is in turn closely related to lysine. Based on these studies, a biosynthesis of carnitin was developed, starting with lysin in Neurospora crassa (Broquist 1980). Three main approaches are known using biological systems for the enantioselective synthesis of L-carnitine from achiral precursors: they are described in detail in an excellent review (Jung et al. 1993) and therefore are only briefly mentioned now. First, enzymes are used which are involved in L-carnitine biosynthesis from lysine and methionine (Figure 2.6, reaction I). Second, microorganisms and enzymes are applied which are involved in the assimilation of achiral quarternary ammonium compounds, where L-carnitine is produced as an intermediate, thereby blocking the reaction after L-carnitine formation (reaction IV). Third, the return reactions are integrated in L-carnitine degradation under physiological conditions (reactions II, IIIa). The enantioselective hydroxylation of γ-butyrobetaine and crotonbetain via an Agrobacterium-like strain is also used for L-carnitine synthesis. Detailed studies shows that it proceeds via a conventional β-oxidation pathway involved in fatty acid metabolism (Jung and Kleber, 1991; Jung et al. 1993).

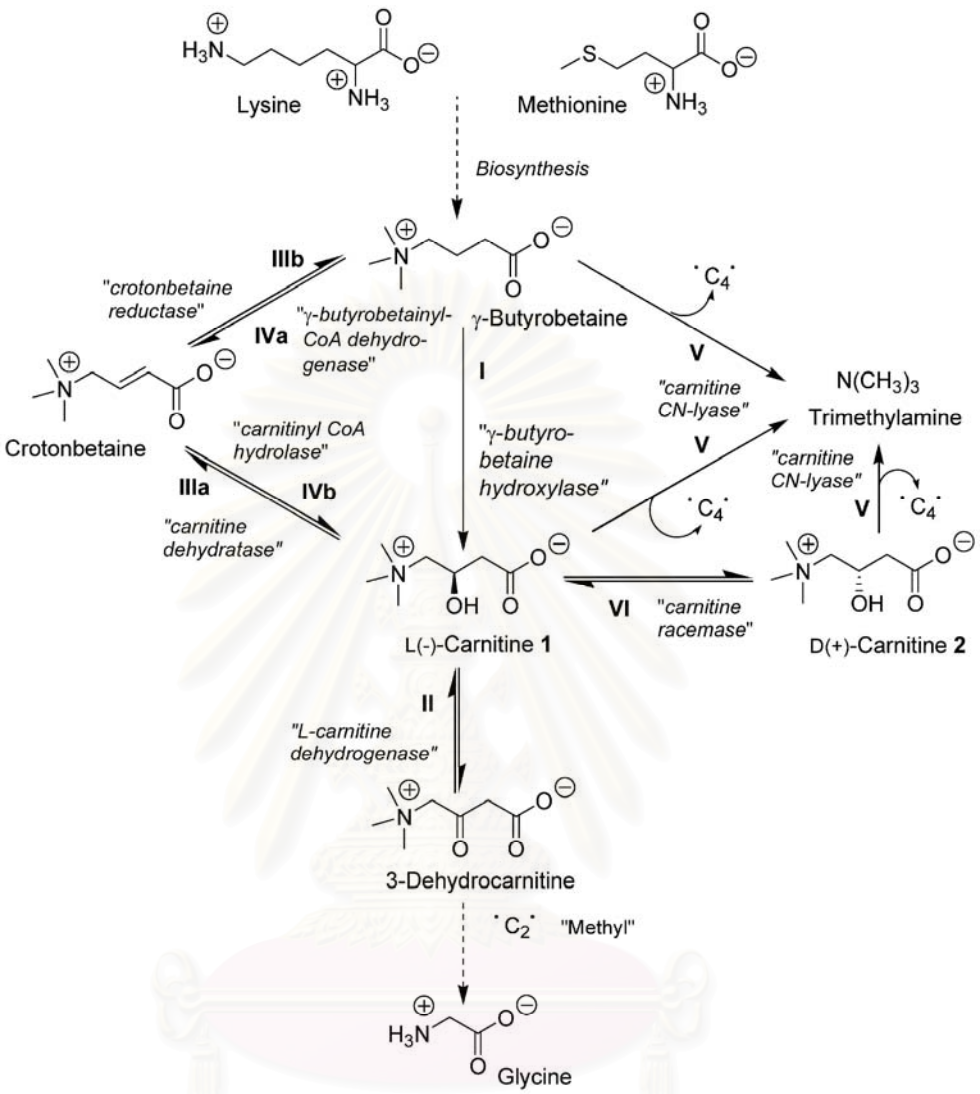

Figure 2.6 Microbial metabolism of L-carnitine

2.5.3 Role of L-carnitine in fish

Based on its role in vertebrates, the use of L-carnitine supplementation in fish diets in aquaculture has been advocated for multi functional purposes: As a growth promoter, specifically aiding in the utilization of high fat levels in the diet and thus providing a protein sparing effect, providing protection against toxic levels of ammonia and xenobiotics, alleviating stress related to water temperature extremes and facilitating better acclimation to water temperature changes, changes in muscle structure/texture related to higher levels of swimming activity and enhancing reproduction.

Effect on fish growth

Growth is of great importance to fish growers and therefore deserves special attention with respect to carnitine supplementation. The growth promoting effects of carnitine supplementation in fish feeds have been attributed to the increase in utilization of energy as a result of the increase in fatty acid oxidation by the mitochondria. This process has been demonstrated in isolated mitochondria of trout (Bilinski and Jonas, 1970). A growth promoting effect of L-carnitine supplementation was found by a number of researchers in Table 2.4.

Table 2.4 Fish and crustacean that growth promote (significant, not significant and no effect) by L-carnitine supplementation.

Species	**Growth promote** (+/-/0)	**Reference**
European seabass	+	Santulli and D'Amelio, 1986
Hybrid striped bass	+	Twibell and Brown, 2000
African catfish	+	Torreele et al. 1993
Red sea bream	+	Chatzifotis et al. 1995
Indian major carp	+	Keshavanath and Renuka, 1998
Mossambique tilapia	+	Jayaprakas et al. 1996
Hybrid tilapia	+	Becker et al. 1999
Common carp	-	Becker and Focken, 1995
Common carp	-	Focken et al. 1997
European sea bass	0	Dias et al. 2001
African catfish	0	Ozorio, 2001; Ozorio et al. 2001a,b
Rainbow trout	0	Rodehutscord, 1995
Rainbow trout	0	Chatzifotis et al. 1997
Channel catfish	0	Burtle and Liu, 1994
Hybrid striped bass	0	Gaylord and Gatlin, 2000a,b
Atlantic salmon	0	Ji et al. 1996
Ornamental cichlid	0	Harpaz et al. 1999
Guppy fish (*Poecilia reticulata*)	0	Dzikowski et al. 2001
Hybrid tilapia	0	Schlechtriem et al. 2004
Tiger prawn (*Penaeus monodon*)	-	Groth, 1997

+ significant, - not significant, 0 no effect

CHAPTER III

EFFECTS OF L-CARNITINE ON MICROALGA (*Tetraselmis suecica*), ROTIFER (*Brachionus plicatilis*), AND LARVAL SEABASS (*Lates calcarifer*)

3.1 Introduction

This study was undertaken to determine the effects of L-carnitine on microalga (*Tetraselmis suecica*) rotifer (*Brachionus plicatilis*), and on growth, survival and L-carnitine content of larval seabass (*Lates calcarifer*). The study consisted of three experiments.

3.2 Materials and Methods

Experiment 1:

Effect of L-carnitine on Microalga (*Tetraselmis suecica*)

Microalga Culture

Microalga (*Tetraselmis suecica*) was obtained from the Department of Marine Science, Chulalongkorn University, Thailand. *T. suecica* was maintained in Conway medium (Walne, 1966) at 25 ‰, 28 ± 2°C, 24:0 h (Light:Dark cycle) with white fluorescent light at the intensity of 40 µmol photon m^{-2} s^{-1} under continuous aeration.

Experimental Procedure

T. suecica was acclimated in culture medium enriched with L-carnitine for at least three generation. L-carnitine (crystalline USP Grade) was purchased from Lonza

Biotec s.r.o., Czech Republic. Enrichment concentrations were 0 (control), 0.1, 1 and 10 mg l^{-1} L-carnitine in culture medium of *T. suecica* at the initial cell density of 3-4×10^4 cells ml^{-1}. Batch culture was carried out in 2 l Erlenmeyer flask, working volume is 1 l. Growth of *T. suecica* was monitored daily by sampling the cultures triplicately from each culture flasks. Cell samples were kept in the small vials and preserve with Lugol's Iodine solution and then enumerated with a haemacytometer slide under light microscope. The specific growth rate (SGR: μ) of each treatments will be calculated by the equation 1 (Fogg and Thake, 1987). The specific growth rate was estimated as the slope of In N vs. time (equation 5) in the initial linear phase by linear regression.

$$\mu = \frac{\ln N_t - \ln N_0}{t} \qquad (5)$$

Where:

N_0 = the initial cell density of microalga

N_t = the final cell density of microalga

t = day between N_0 and N_t

All treatment of *T. suecica* were cultured until exponential growth phase and then harvested by centrifugation (3500×g for 15 min). Samples were rinsed with 0.5 M ammonium formate to remove residual salts from the seawater medium (Volkman et al. 1993), centrifuged again, then freeze-dried and kept at -20 °C before analyzed by HPLC.

L-carnitine Analysis

Method was modified from Prokorátová et al. (2005).

A. Chemicals

All chemicals were analytical grade. L-carnitine inner salt 98% ($C_7H_{15}NO_3$) was purchased from Fluka. Acetic acid, (CH_3COOH), nitric acid (HNO_3) and Sodium

hydrogen carbonate ($NaHCO_3$) were obtained from Sigma–Aldrich. FMOC (9-fluorenyl-methylchlorformate) and acetonitrile were obtained from Merck.

B. Equipment and Chromatography

The HPLC system was a Shimadzu HIC-6A Ion Chromatograph with conductivity detector. The analytical column was Shim-pack IC-PCI/IC-GCL/IC-CI and temperature maintained at 30 °C. The mobile phase was 5 mM nitric acid in 5% acetonitrile and flow rate was 1 ml/min. The run time took 20 min.

C. Standard Solutions Preparation

The standard solution stock was prepared by drying L-carnitine standard in the oven at 100 °C for 1 h and cooled in desiccators for 2 h. The 0.05 mg of cooled L-carnitine was dissolved in 100 ml deionized water. This concentration was approximately 50 ppm. This stock solution was stable at 4°C for a month.

D. Sample Preparation and Derivatization for HPLC Analysis

Samples approximately 100-500 mg dry weight were weighed and homogenized in 1 ml deionized water. Dilute with mobile phase and mix. Filter the solution through microfilter 0.45 µm.

Fresh stock 100 mM solution of FMOC was prepared in acetonitrile before derivatization, 50 mM carbonate buffer (pH 10.4) and 1 M acetic acid were prepared in water and stored at ambient temperature. The procedure was following: 1 ml of the sample was mixed with 1 ml of carbonate buffer, after the addition of 2 ml of FMOC solution the sample was derivatized for 1 h at 50 ºC. To complete the reaction, 2 ml of acetic acid buffer and 4 ml of water were added to a final volume of 10 ml. The solution was then filtered and injected.

Data Analysis

One-way ANOVA with the Duncan multiple comparison test was used to compare differences in growth rate of microalga among the four treatments of L-carnitine. Differences were considered significant at the $p < 0.05$ level.

Experiment 2:

Effect of L-carnitine on Rotifer (*Brachionus plicatilis*)

Rotifer Culture

Rotifers (*Brachionus plicatilis*) was obtained from Bangsaen Institute of Marine Science, Burapha University, Thailand. Batch cultures of *B. plicatilis* were maintained in 25‰ filtered seawater at 28±2°C with continuous aeration and fed *T. suecica* daily.

Experimental Procedure

Trial 1. Indirect Method (L-carnitine uptake from microalgae)

A. Growth, egg ratio and body size characteristics

The batch culture was carried out in 2 l Erlenmeyer flask, working volume was 1 l filtered seawater. Rotifer cultures were grown under the condition of 28±2°C, 25‰ and provided with continuous aeration. The initial cell concentration of rotifer for each treatment was 100 ind.ml^{-1}. Four L-carnitine levels accumulated in microalgae *T. suecica* from experiment 1 were used for this experiment in three replicates. *T. suecica* enriched with different levels of L-carnitine was added every day by centrifuged to remove culture medium and cell density was adjusted to 2-3×10^{5} cells ml^{-1} for each treatment.

Growth of rotifer densities were measured daily by sampling the culture from each treatments then kept in the small vials and preserved with Lugol's Iodine solution. One ml of the preserved sample was filled into the Sedgewick-Rafter slide and counted three replicate. 100 rotifers for each treatment was used for determination of body size (lorica length and width) (Fu et al. 1991). The egg ratio was calculated from the equation 6. Calculation for the specific growth rate of rotifers is given by equation 5.

$$\text{Egg ratio} = \frac{eggs}{rotifers} \quad (6)$$

Where Eggs is expressed in egg ml^{-1}

Rotifer is expressed in ind. ml^{-1}

B. L-carnitine content

For the study on L-carnitine content, rotifers were cultured in four 50 l vessels, using the same cultured condition as described earlier.

All rotifers in each L-carnitine levels were sampled on day 10 by using a 58 μm nylon sieve, washed with filtered seawater, 0.5 M ammonium formate then freeze-dried and kept at -20°C before analyses by HPLC as described in experiment 1.

Trial 2. Direct Method (L-carnitine enrichment from oil emulsion)

Preparation of oil emulsion was the method described by Watanabe et al. (1983). L-carnitine with oil emulsions were prepared by mixing fish oil, egg yolk, seawater and sufficient L-carnitine to obtain concentration of 0.1, 1 and 10 mg l^{-1} in rotifer medium. We chose the treatment that has highest L-carnitine content from experiment 1. Rotifers were divided into two groups i.e. rotifer fed *T. suecica* without L-carnitine (B0) and rotifers fed *T. suecica* acclimated in culture medium enriched with 10 mg l^{-1} L-carnitine (B10). B0 and B10 were enriched with four levels of emulsions i.e. without L-carnitine, added L-carnitine 0.1, 1 and 10 mg l^{-1} and at 1, 2, 3 and 4 h. Rotifer enrichments were carried out in the small 10 l vessels at a density of 500-600 ind. ml^{-1}. After enrichment, rotifer were harvested after 1, 2, 3 and 4 h, using the same procedure as described for trial 1 (B). Most of rotifers were dead after exposure to with emulsion for 5 h (immobilized).

These experimental designs by testing a two-way analysis of variance indicated below.

Rotifer	0	0.1	1	10 (mg l^{-1})
B0	1, 2, 3 and 4 h	1, 2, 3 and 4 h	1, 2, 3 and 4 h	1, 2, 3 and 4 h
B10	1, 2, 3 and 4 h	1, 2, 3 and 4 h	1, 2, 3 and 4 h	1, 2, 3 and 4 h

Emulsion (L-carnitine level)

Data Analysis

One-way ANOVAs with the Duncan multiple comparison test were used to compare differences in density, egg ratio, body size and L-carnitine content of rotifer among the levels of L-carnitine. Differences were considered significant at the $p < 0.05$ level.

Two-way ANOVAs with the Duncan multiple comparison test were used to determine the statistical significance of L-carnitine levels of emulsions and time on L-carnitine content in rotifers *B. plicatilis* (B0) and (B10).

Experiment 3:

Effect of L-carnitine on Growth, Survival and L-carnitine Content of Larval Seabass (*Lates calcarifer*)

Fish Culture

Seabass (*Lates calcarifer*) were stocked at density 100 fish larvae l^{-1} in 18 rearing tanks (130 l) (Figure 3.1). Photoperiod was 14L:10D and light intensity approximatly 1000 lux. At first feeding (2 days post hatch-DPH) larvae were reared in green water culture using *T. suecica* and fed enriched rotifers until 13 DPH. Live feed density in larval rearing tanks was maintained adequately to avoid starvation and cannibalism. Densities varied between 10,000-20,000 rotifer ml^{-1}. Fish larvae were fed twice daily at 09:00 and 16:00. Seawater was exchanged daily at 40% before

feeding. Water temperature was kept between 30 and 32 ºC with 25 ‰ salinity under continuous aeration. Ammonia, nitrite and nitrate were less than 0.1, 1 and 10 mg l^{-1}, respectively.

Figure 3.1 Photograph of seabass larvae rearing tanks used in experiment 3.

Fish Treatments

Fish larvae were cultured with six treatments and three replicates per treatment (refer result from experiment 1, Trial 1 and 2 which gave the highest content of L-carnitine in the treatments).

This experimental design is randomized factorials (2 × 3):

	No emulsion	emulsion without L-carnitine	emulsion with L-carnitine
T. suecica without L-carnitine	1	2	3
T. suecica with L-carnitine	4	5	6

Treatment 1: Rotifers fed *T. suecica* without L-carnitine enrichment and no oil emulsion (control)

Treatment 2: Rotifers fed *T. suecica* without L-carnitine enrichment and fed an emulsion without L-carnitine at time 4 h

Treatment 3: Rotifers fed *T. suecica* without L-carnitine enrichment and fed an emulsion enriched with 10 mg l^{-1} L-carnitine at time 4 h

Treatment 4: Rotifers fed *T. suecica* with 10 mg l^{-1} L-carnitine enrichment and no oil emulsion

Treatment 5: Rotifers fed *T. suecica* with 10 mg l^{-1} L-carnitine enrichment and fed an oil emulsion without L-carnitine at time 4 h

Treatment 6: Rotifers fed *T. suecica* with 10 mg l^{-1} L-carnitine enrichment and fed an oil emulsion enriched with 10 mg l^{-1} L-carnitine at time 4 h

A total of 50 larvae from each triplicate of all the treatments were sampled at 0 DPH and 13 DPH for measurement of total length (TL) (Figure 3.2) and wet weight. Sampling was performed early in the morning before feeding. Samples of larvae were anaesthetized in 100 ppm 2-phenoxyethanol solution and TL was measured with an ocular micrometer under a calibrated microscope. Weight was taken using an electronic digital balance after blotting the larvae with water absorbent paper. Total length was defined as the length in millimeters from the tip of the lower mandible to the end of the caudal fin. Larval measurements were completed within 30 min of death to minimize variability due to water loss. Survival rate was determined by counting all larvae from each tank at the end of the experiment on 13 DPH. Larvae

from each treatment were washed with 0.5 M ammonium formate to remove salt and then freeze-dried before analyzed by HPLC method and L-carnitine analysis as for experiment 1.

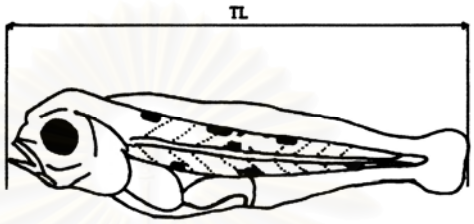

Figure 3.2 Morphometric mearsurement of total length (TL) of seabass (*L. calcarifer*) larvae.

Data Analysis

One-way ANOVAs with the Duncan multiple comparison test were used to compare differences of treatment on total length, weight and L-carnitine content of seabass larvae. Differences were considered significant at the $p < 0.05$ level.

3.3 Results

Experiment 1:
Effect of L-carnitine on Microalga (*Tetraselmis suecica*)

Cell densities on day 3 (exponential growth phase), maximum cell densities and specific growth rates of *T. suecica* obtained from each levels of L-carnitine are shown in Figure 3.3-3.4.

All *T. suecica* enriched with all levels of L-carnitine grew until reaching the exponential growth phase on day 3 with average cell densities ranging from 54.26 to 83.81×10^4 cells ml^{-1} (Appendix A, Table A-1). *T. suecica* enriched with 0.1, 1 and 10 mg l^{-1} L-carnitine exhibited significantly higher specific growth rate than the control. However, there were no significant differences among those cultures with the three levels of L-carnitine. The highest and lowest specific growth rate was 0.94 and 1.11 day^{-1} at L-carnitine levels of 10 and 0 mg l^{-1}, respectively. The maximum cell density of three levels of L-carnitne (0.1, 1 and 10) was on day 9 which ranged from 213-235×10^4 cells ml^{-1}, except for 0 mg l^{-1} L-carnitine, on day 8 (196×10^4 cells ml^{-1}).

Figure 3.5 shows that L-carnitine content in *T. suecica* increased with increasing levels of L-carnitine concentration in culture medium. Significant differences were found at each level. L-carnitine content in *T. suecica* ranged from 259 to 1813 μg g^{-1} dw, the highest and lowest were at L-carnitine levels of 10 and 0 mg l^{-1}.

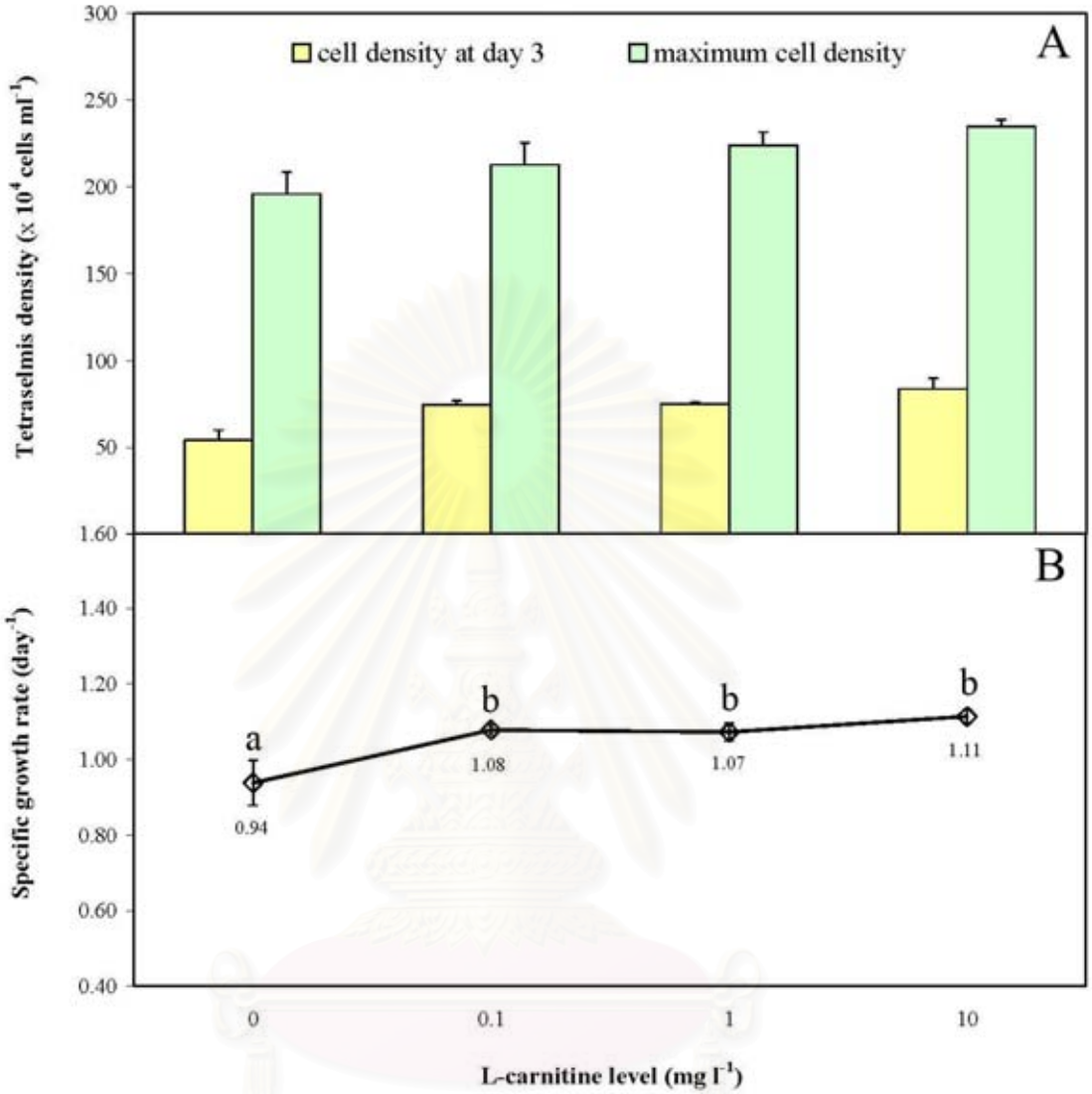

Figure 3.3 (A) Cell density at day 3 and maximum cell density and (B) specific growth rate (day 0-3) (day^{-1}) of *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) are shown as mean ± SD (n=3). Different upper case letters indicate significant differences at $p<0.05$ (ANOVA, Duncan).

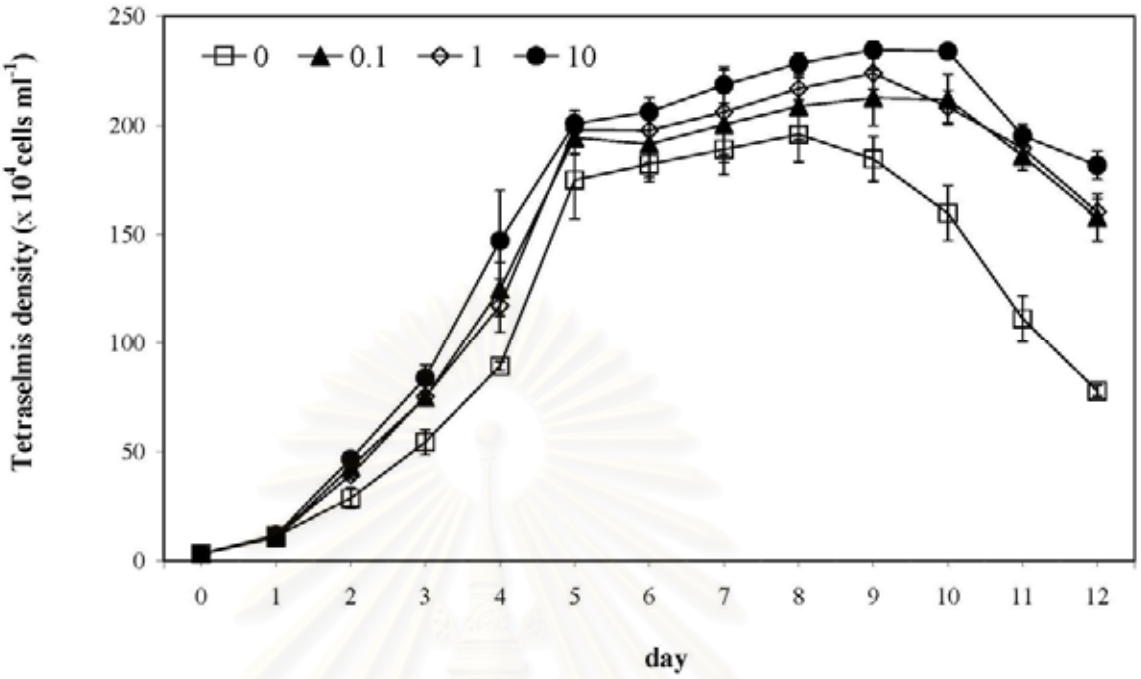

Figure 3.4 Growth curve of cell density of *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 12 days (mean ± SD, n=3).

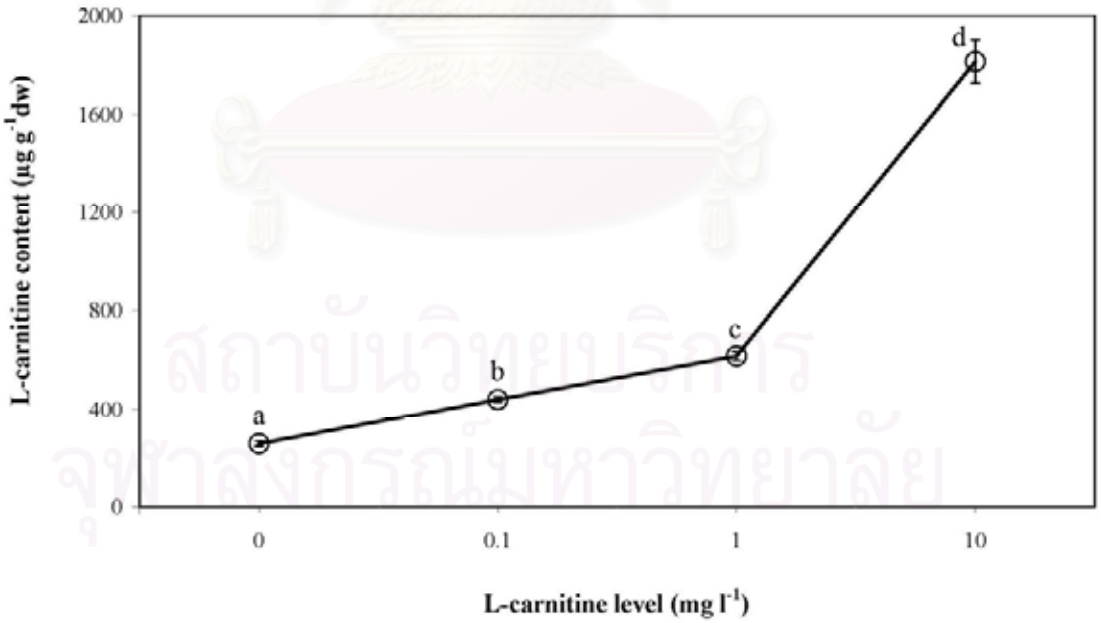

Figure 3.5 L-carnitine content of *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) are shown as mean ± SD (n=3). Different upper case letters indicate significant differences at $p<0.05$ (ANOVA, Duncan).

Experiment 2:

Effect of L-carnitine on Rotifer (*Brachionus plicatilis*)

Trial 1. Indirect Method (L-carnitine uptake from microalgae)

A. Growth, egg ratio and body size

Growth

Population densities of rotifers fed *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1, and 10 mg l^{-1}) during 22 day culture are presented in Figure 3.6. During the culture period day 0 to day 8, there was no significant difference ($P>0.05$) in density between L-carnitine treatments and the control. Significant densities in rotifer densities ($P<0.05$) occurred on day 9 when density of rotifers fed *T. suecica* enriched with 1 mg l^{-1} L-carnitine was greater than other treatments. The highest density of 696 ind.ml^{-1} was observed on day 15. At 0, 0.1 and 10 mg l^{-1}, rotifer population densities were greatest on days 18, 16 and 14 with 429, 328 and 616 ind.ml^{-1}, respectively. Average specific growth rate (SGR) of all treatment during 22 days showed an increasing trend followed by an increase in levels of L-carnitine while the control was lower (Appendix B, Table B-4). L-carnitine at 1 mg l^{-1} resulted in the highest average SGR of 0.0370 day^{-1} (Figure 3.7).

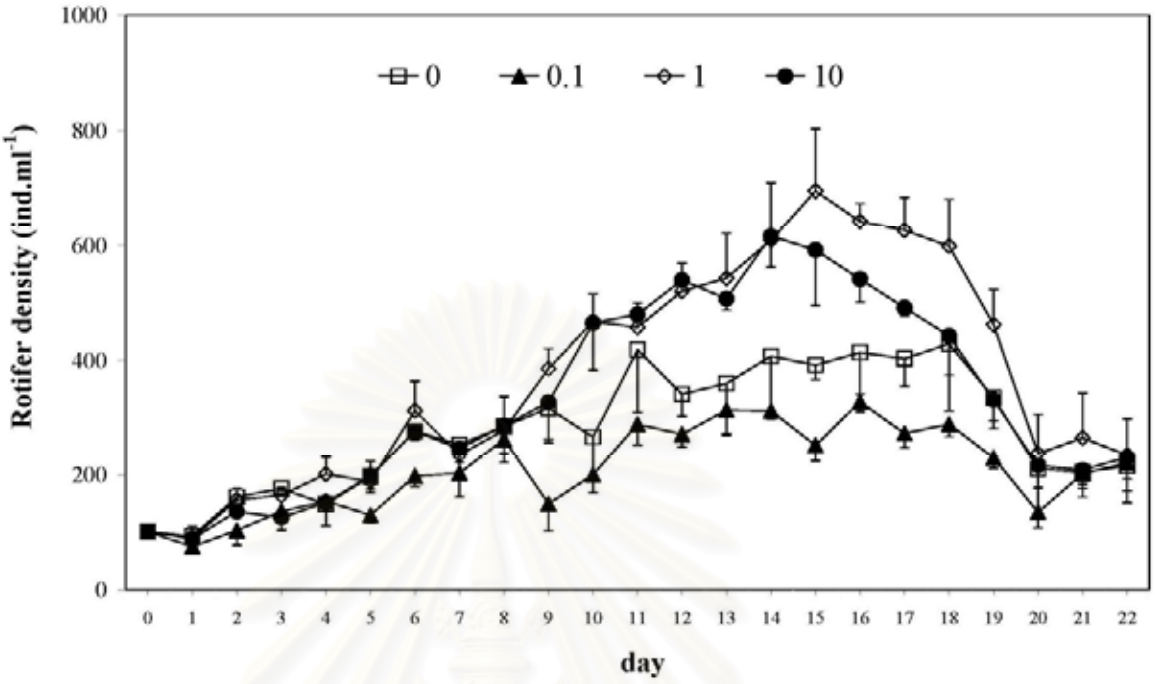

Figure 3.6 Mean density with SD (n=3) of rotifer (*B. plicatilis*) fed with microalgae (*T. suecica*) enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 22 days experiment.

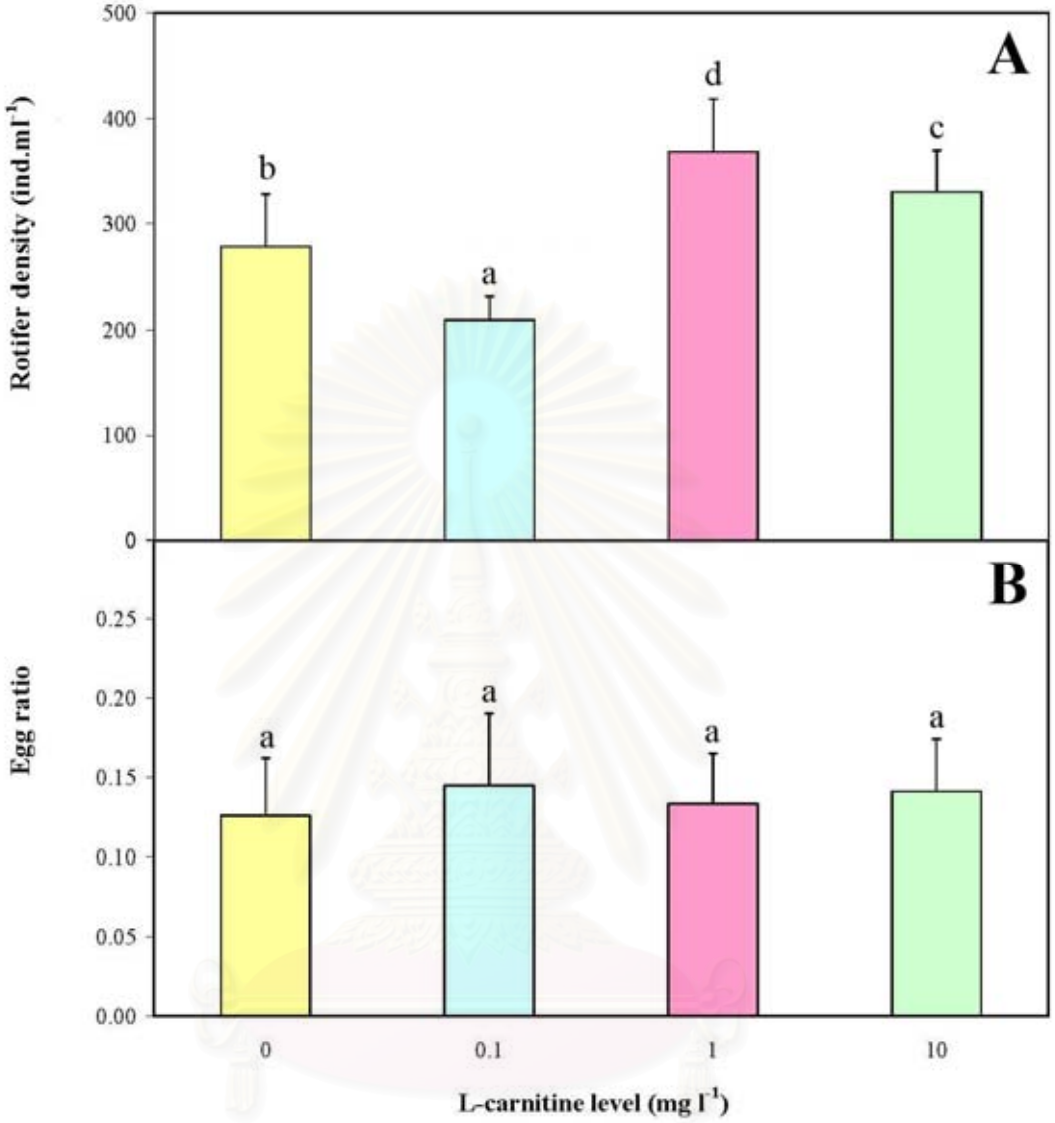

Figure 3.7 (A) Average population density and (B) Average egg ratio of rotifer (*B. plicatilis*) fed with microalgae (*T. suecica*) enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 22 days are shown as mean ± SD (n=3). Different upper case letters indicate significant differences at $p<0.05$ (ANOVA, Duncan).

Egg ratio

Although egg ratio was intended to same as a parameter for egg production and population growth, the results were the same for all levels of L-carnitine (Figure 3.7B). Control, 0.1, 1 and 10 mg l^{-1} L-carnitine treatments all had average egg ratios that were not significantly different as calculated by one-way ANOVA (Appendix B, Table B-3). These results suggest that enrichment of rotifers by L-carnitine prolonged their lifespan, but did not cause increased reproduction rate.

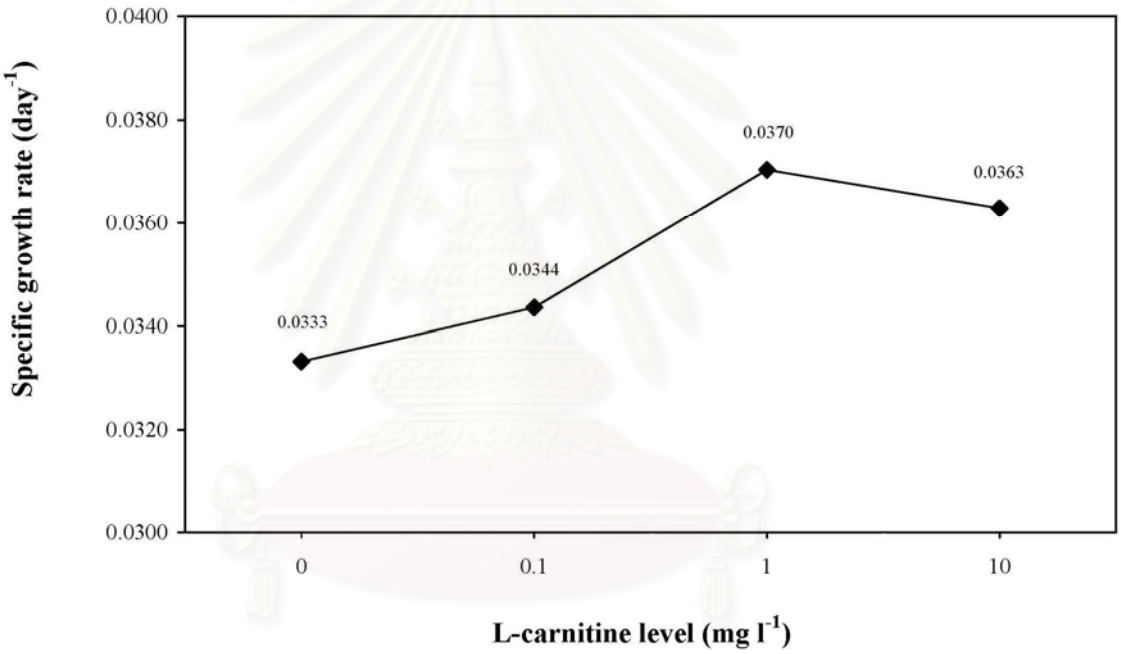

Figure 3.8 Average specific growth rate (SGR) of rotifer (*B. plicatilis*) fed with microalgae (*T. suecica*) enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 22 days.

Body size

Lorica length and width increased with increasing concentration of L-carnitine from 0 to 10 mg l^{-1} (Figure 3.9). The largest average lorica lengths and widths were 176 and 140 µm respectively with 10 mg l^{-1} L-carnitine treatment, which was significantly larger more the other treatments. There were no significant differences in length and width among 0, 0.1 and 1 mg l^{-1} L-carnitine treatments.

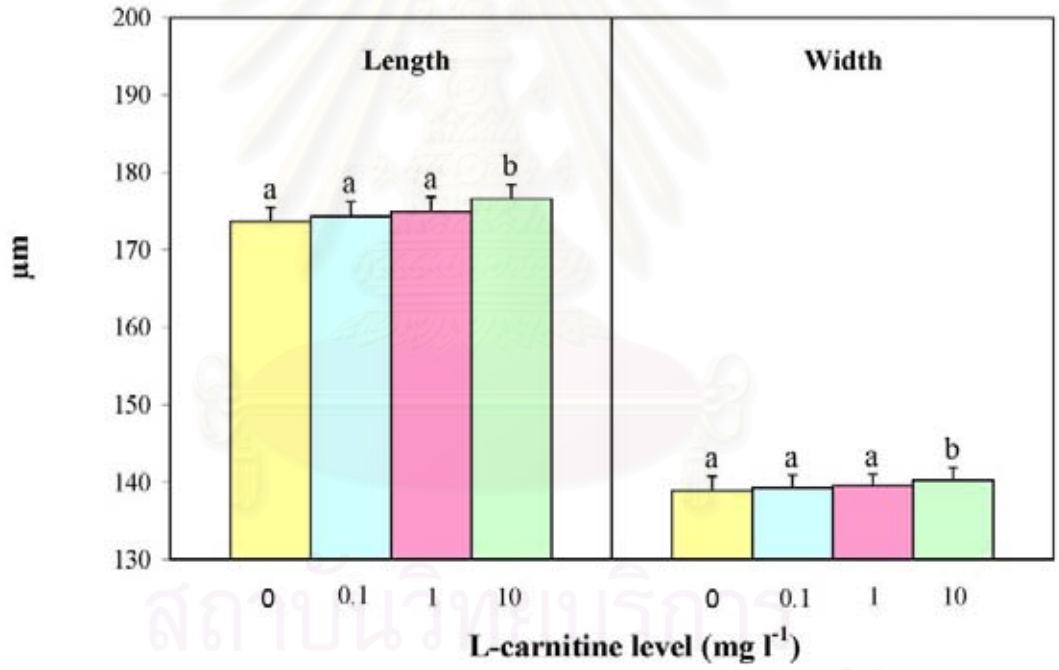

Figure 3.9 Average and SD of lorica length and width (µm) of rotifer (*B. plicatilis*) fed with microalgae (*T. suecica*) enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) for 22 days. Different upper case letters indicate significant differences at $p<0.05$ (ANOVA, Duncan).

L-carnitine content

L-carnitine content of rotifers increased with increasing level of L-carnitine treatments (150-349 μg g^{-1} dw) and were significantly different at all treatments level (Figure 3.9) (Appendix B, Table B-3).

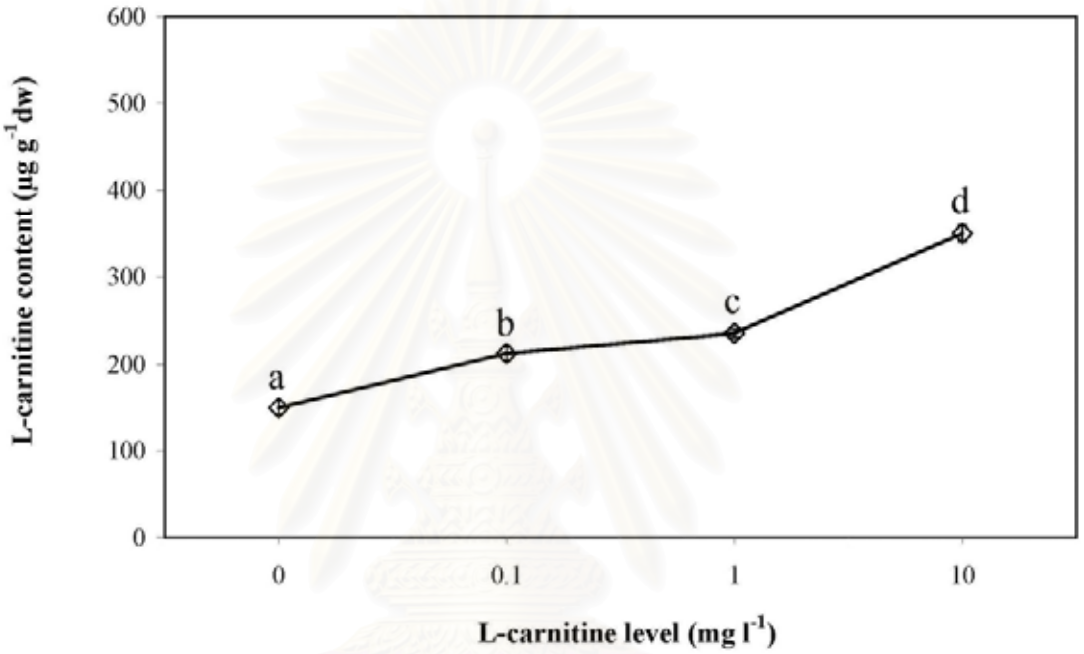

Figure 3.10 Average with SD of L-carnitine content of rotifer (*B. plicatili*s) fed with microalgae (*T. suecica*) enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}). Different upper case letters indicate significant differences at $p<0.05$ (ANOVA, Duncan).

Trial 2. Direct Method (L-carnitine enrichment from oil emulsion)

L-carnitine content of B0 group rotifer enriched with 0 and 0.1 mg l^{-1} L-carnitine emulsions was not significantly different with time of incubation (Figure 3.11). L-carnitine content values in Figure 3.11 were analysed using one-way ANOVA and the Duncan multiple comparison tests for inferring difference among times of incubation in each L-carnitine level. Moreover, two-way ANOVA was performed to determine any interaction between levels of L-carnitine and time in Appendix B, Table B-8 and B-9. Rotifers enriched with 1 mg l^{-1} L-carnitine emulsion indicated less significant difference with time of incubation. Rotifers enriched with 10 mg l^{-1} L-carnitine emulsion exhibited significant difference in L-carnitine content among the four incubation times. The longer incubation time, the greatest content of L-carnitine, which was 491 μg g^{-1} dw with enrichment 4 h (Figure 3.11, B0).

For B10 group rotifer, L-carnitine content did not tend to increase with time on 0, 0.1 and 1 mg l^{-1} L-carnitine emulsion. The increase is more significant when the level of L-carnitine was 10 mg l^{-1} along with time of incubation (1-4 h). The greatest L-carnitine content was 518 μg g^{-1} dw with 4 h enrichment (Figure 3.11, B10).

The results from trial 2 suggest that rotifers enriched on 10 mg l^{-1} L-carnitine emulsion in B10 had greater L-carnitine content than 10 mg l^{-1} L-carnitine emulsion in B0 with the same time of incubation (4 h). Two-way ANOVA analysis (Appendix B, Table B-8 and B-9) showed the interaction between levels of L-carnitine and times. At any levels of L-carnitine and times affect to L-carnitine content in rotifer enrichment.

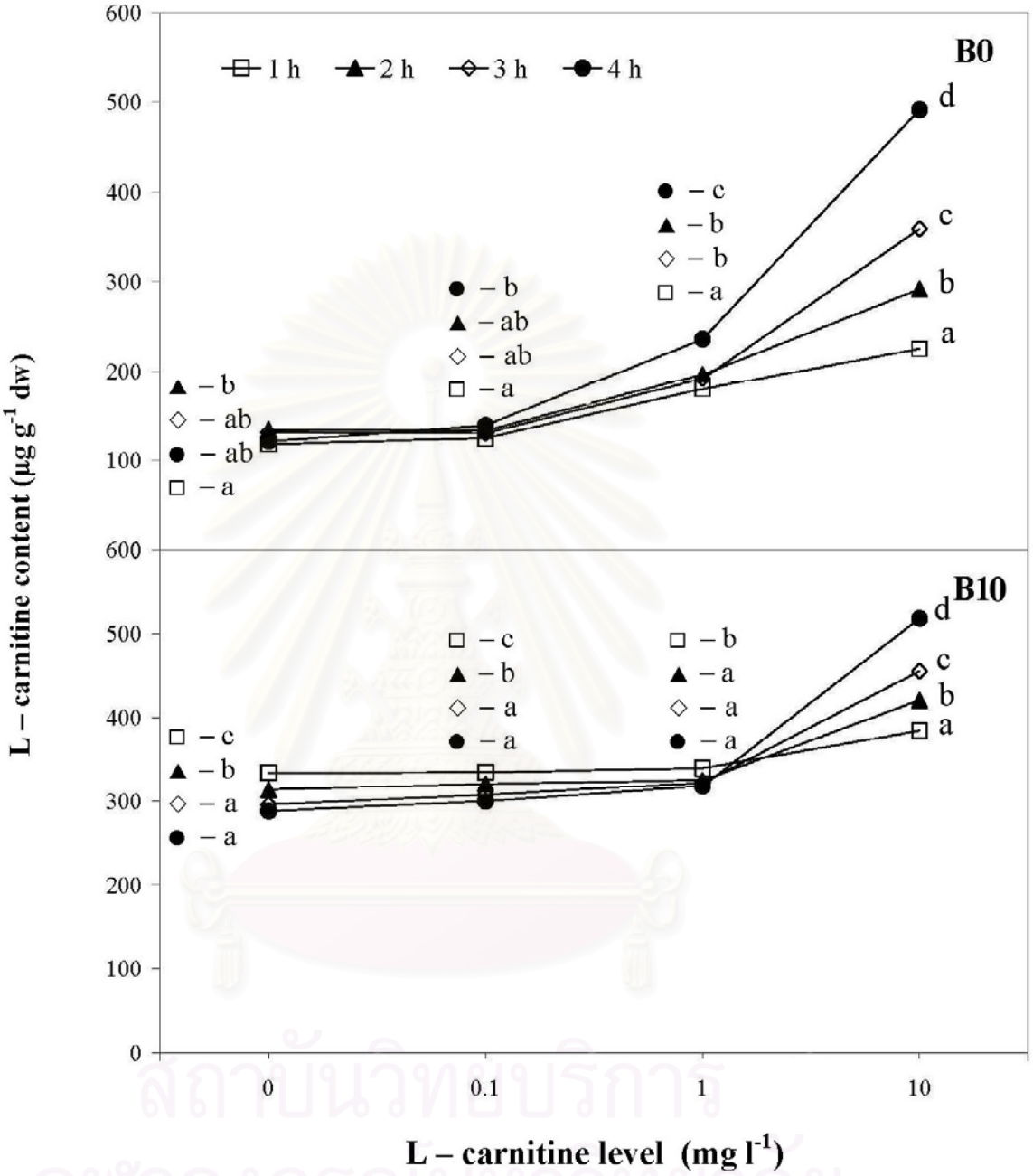

Figure 3.11 L-carnitine contents in rotifers enriched with four levels of L-carnitine emulsions (0, 0.1, 1 and 10 mg l^{-1}) at four incubation times (1, 2, 3 and 4 h). Values (mean ± SD, n=3) in the same column (from bottom to top) with different upper case letters indicate significant differences at $p<0.05$ (ANOVA, Duncan). (B0 are rotifers fed *T. suecica* without L-carnitine, and B10 are rotifers fed *T. suecica* acclimated in culture medium enriched with 10 mg l^{-1} L-carnitine).

Experiment 3:

Effect of L-carnitine on Growth, Survival and L-carnitine Content of Larval Seabass (*Lates calcarifer*)

Feeding different L-carnitine enriched rotifers to seabass (*L. calcarifer*) larvae during their first 13 days of feeding resulted in differences in total length (Figure 3.12A), weight (Figure 3.12B), survival (Figure 3.12C) and L-carnitine content (Figure 3.12D) (Appendix C).

By 13 DPH, larvae in treatment 5 and 6 had significantly greater total length than other treatments while control treatment fish were significantly smaller. There was no significant difference in total length between larvae from the treatment 1, 2, 3 and 4. Largest total length was 3.85 mm in treatment 6 and the smallest total length was 3.15 mm with control treatment fish. L-carnitine enrichment had slight effects on weight of seabass larvae. At 13 DPH, larvae in treatment 3, 4, 5 and 6 were significantly heavier than control treatment. Treatment 2 fish were not significant different from fish in the control treatment. The largest weight was 1.08 mg in treatment 6, and the smallest weight was 0.53 mg in the control treatment.

There were significant differences in survival of fish in several treatments. Larval fish in treatment 5 had greater survival (39%) while those in the control treatment were lowest (29%). Fish from treatments 2, 3, 4 and 6 had similar survivals of 32, 33, 36 and 36%, respectively.

L-carnitine content was not detected in control treatment and treatment 2. However, there was a significant difference in L-carnitine content of larvae at 13 DPH (29.56-78.51 μg g^{-1} dw) among treatment 3, 4, 5 and 6. There were no significant differences between treatment 4 and 5 and between treatment 3 and 6.

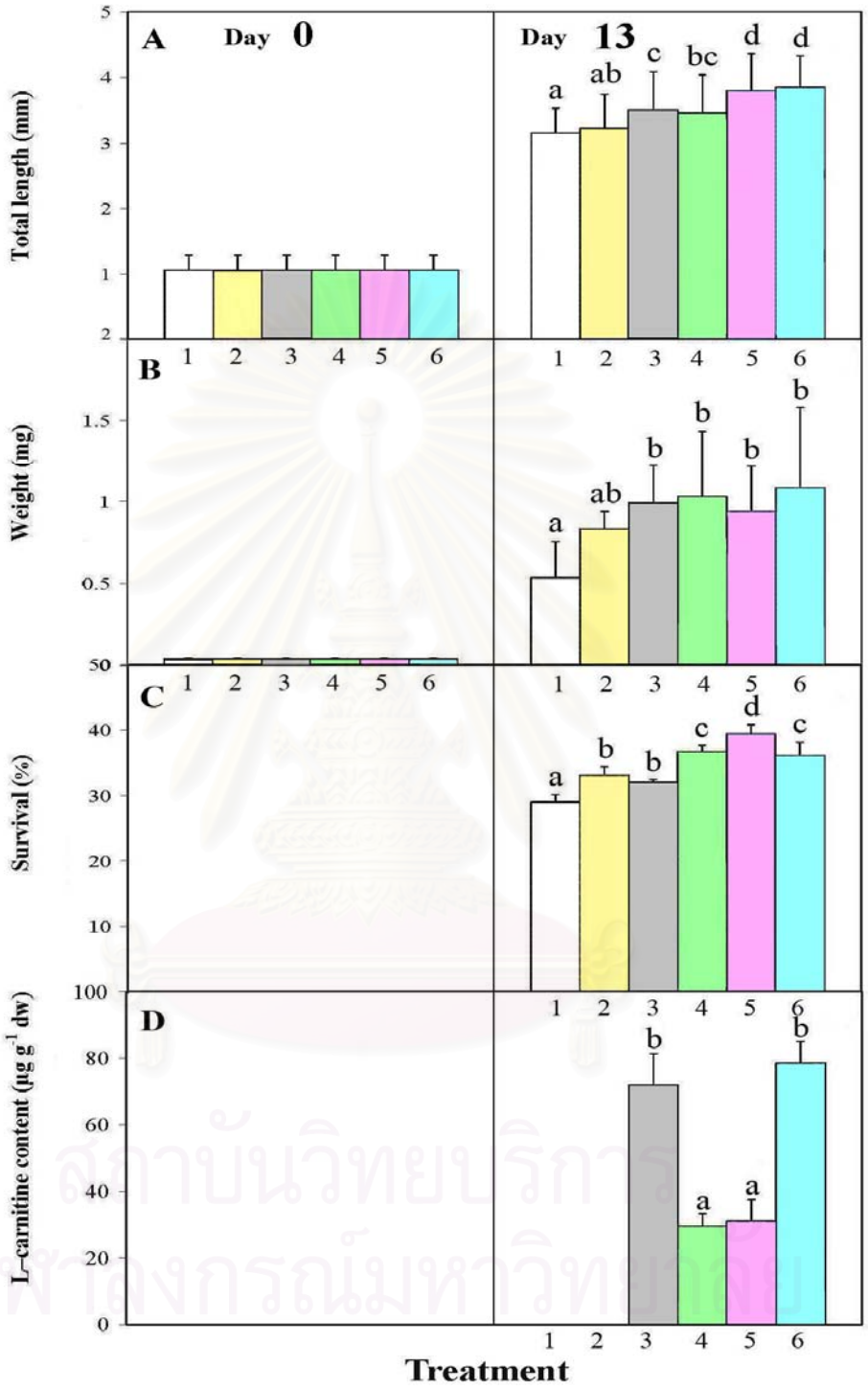

Figure 3.12 Mean ± SD of total length (A), weight (B), survival (C) and L-carnitine content (D). Different upper case letters indicate significant differences at $p<0.05$ (ANOVA, Duncan). At day 0 of (D), L-carnitine content was not detected. Hence data were not shown.

3.4 Discussion and Conclusions

Experiment 1:

Effect of L-carnitine on Microalga (*Tetraselmis suecica*)

This experiment clearly showed that microalgae (*T. suecica*) could uptake L-carnitine from the culture medium into cells (Figure 3.5). Our trials showed that *T. suecica* enriched with 100 mg l^{-1} L-carnitine in culture medium had slower growth and lower density than other L-carnitine concentration. Algal growth and cell density were enhanced by enrichment at optimum levels of L-carnitine culture medium. Similar results were also documented by other authors (Luyen et al. 2007; Nieves et al. 2005). Our findings agree with Rebouche and Seim (1998) who reported that carnitine can stimulate growth and metabolism of microorganisms, varying depending on species and culture condition such as. salinity, oxygenation or anaerobic growth. This implies that supplemental L-carnitine might have an impact on microbial environment in different treatments, in which case enriched microalgae might be indirectly affected by the microbial environment.

Our finding demonstrated that *T. suecica* effect encapsulated L-carnitine thus provides living food capsules for transferring L-carnitine to rotifer and then to fish larvae. Higher concentrations of L-carnitine that we used probably can further affect growth algae rate and L-carnitine accumulation in *T. suecica*.

Experiment 2:

Effect of L-carnitine on Rotifer (*Brachionus plicatilis*)

Trial 1. Indirect Method (L-carnitine uptake from microalgae)

We found that enrichment of rotifer with L-carnitine through their algal food source resulted in significant responses in population growth and body size. Furthermore, we found that L-carnitine effects were significant in the second generation during 10 days culture period. L-carnitine probably prolonged lifespans of rotifer, in the 1 and 10 mg l^{-1} L-carnitine treatments that had greater population densities. If this occurred it would explain lowers egg ratio than 0.1 mg l^{-1} L-carnitine treatment. Our study agrees with previous research where the population growth of rotifer had a typical sigmoid curve. The first 7 days was an exponential growth phase and after that it was a post exponential and stationary growth phase (Yoshinaga et al. 2001). The egg ratio indicated birth rate and relative population growth at the same time. However, the egg ratio on 0.1 mg l^{-1} L-carnitine treatment was higher than other treatment but it had lower population density.

Clement et al.(1983) reported that most rotifer mechno- and chemoreceptors sensitive to environmental stimulation are in direct contact with external medium, it is therefore possible that the effect of L-carnitine by these method are direct. In support of previous research (Gallardo et al. 1997; 2000; Yoshimatsu et al. 2006; Hayashi et al. 2007), we study found that rotifer population growth increased by additional chemical compound both direct and indirect methods. As observed results of individual growth in this study, increased body size of 0.1-10 mg l^{-1} L-carnitine enriched rotifers may be the response to the protein sparing action produced by exogenous L-carnitine stimulation. It had a possible effect on lorica size followed by an increase in level of L-carnitine compare with control. This finding is similar to result from previous investigations by enzymatic reaction method (Zhang et al. 2006).

In conclusion, the results of our work indicate that L-carnitine enrichment of rotifer by indirect method caused significant responses in rotifers population growth, body size and L-carnitine content. The best results in terms of population growth were

obtained with 1 mg l^{-1} L-carnitine but body size and L-carnitine content were greater with 10 mg l^{-1} L-carnitine.

Trial 2. Direct Method (L-carnitine enrichment from oil emulsion)

The kinetics of changing nutritional quality of the rotifers by feeding is a two step process: first filling of the gut in about 30 min, and secondly incorporation into rotifer tissue (Olsen et al. 1989). This proven is normally designated short-term enrichment (less than 24 h), and is done to improve nutritional quality of rotifers for live food presentation for fish or other organisms (Watanabe et al. 1983; Lubzens, 1987; Reitan et al.1997) In our study, L-carnitine enriched rotifers were produced using short-term enrichment with oil-based emulsions. This enrichment does not result in significant rotifer growth responses since it in a simple filling of the rotifer's gut with dietary oils. However, this L-carnitine is then easily transferred to fish larvae when they eat the rotifers.

In order to obtain increased L-carnitine content in rotifers, it is more effective to enrich the rotifer by emulsion (direct method). Our best results in terms of L-carnitine content was obtained with 10 mg l^{-1} L-carnitine emulsion at 4 h of incubation in B10 trials. B10 trials included rotifers fed with *T. suecica* acclimated in culture medium enriched with 10 mg l^{-1} L-carnitine.

Experiment 3:

Effect of L-carnitine on Growth, Survival and L-carnitine Content of Larval Seabass (*Lates calcarifer*)

L-carnitine content of seabass larval diet is a possibly indicator of potential fish growth. Although other studies evaluated L-carnitine supplementation in various fish species (from fingerlings to juveniles, reviewed by Harpaz, 2005 as well as L-carnitine enrichment in rotifer, our study was the first trial using L-carnitine in fish larvae. Our present study found that seabass larvae exhibited greater growth as a result of L-carnitine supplementation.

Seabass larvae fed diets of rotifers that were enhanced with L-carnitine grow larger and had greater survival than fish larvae fed diets not fortified with L-carnitine. L-carnitine content of rotifers were enhanced by either feeding microalgae with greater L-carnitine content, and/or by enhancing L-carnitine content of rotifers using an oil emulsion method. Greatest fish growth occurred when rotifer were enriched with L-carnitine using both L-carnitine enhanced microalgae and oil emulsion. Moreover, survival rate of fish larvae is dependent on factors such as nutrients stored in the yolk sac, temperature, time of initial feeding, feed density and rearing conditions (Kailasam et al. 2007; Barlow et al. 1995; Bagarinao, 1986).

Our result suggests that rotifers fed an oil emulsion enriched with 10 mg l^{-1} L-carnitine for 4 h incubation produced greatest seabass larvae growth.

CHAPTER IV

USE OF L-CARNITINE TO ENHANCE GROWTH OF ROTIFER (*B. plicatilis*) IN A RECIRCULATION CULTURE SYSTEM

4.1 Introduction

Larval culture of most fish and crustaceans require motile prey organisms of certain taste and palatability, that are usually lacking with accept inert/dry diets. Development of dry diets for fish larvae is an important good for aquaculturists. Before this is achieved, however live food (zooplanktons and phytoplanktons) will remain the food items of choice for the early larval stages.

Live food use in larviculture has many advantages namely; motility, suitable prey size, source of essential fatty acids (EFAs) needed by marine fish larvae but not present in dry diets; source of exogenous enzymes; delivery agents (enrichment); less pollution of culture medium, among others. One of the important live feeds used in larviculture is the marine rotifer *Brachionus plicatilis*. Successful development of commercial farms in the Mediterranean area was made possible by several improvements in the production techniques of this live food (Dehasque et al. 1997; Candreva et al. 1996). This success is not only attributable to use of the brine shrimp *Artemia* spp. but also to use of the brackish water zooplankton *B. plicatilis* that is universally used as starter diet (Fukusho, 1989). In Europe, marine hatcheries still prefer to use rearing techniques based on batch or continuous systems in which algae, or a mixture of algae and baker's yeast or the formulated feed Culture Selco® are used as food. In these systems, rotifer cultures are subjected to repeated water renewals to maintain an acceptable water quality, but these stressful rearing conditions seldom allow rotifer densities to exceed 600 ind. ml^{-1} (Morizane, 1991; Fukusho, 1983).

Several methods to enhance rotifer population growth by environmental manipulations and chemical treatments have been investigated by a number of researchers (Yoshimura et al. 1997; Dhert et al. 2001; Hagiwara et al. 2001). Nutritional manipulations of enhancement with vitamins (B_{12}, C, A, D and E) and n-3 HUFA (EPA and DHA) have been developed as methods of secondary culture for practical use in rotifer mass culture (Yoshimatsu et al. 1997; Lubzens et al. 2001). Approaches for manipulating biological characteristics in rotifers with chemical treatments have achieved effective results, including use of some vertebrate and invertebrate hormones, γ-aminobutyric acid (GABA) and growth hormone (GH) manipulations as techniques to stabilize rotifer cultures (Gallardo et al. 1999, 2000; Hagiwara et al. 2001).

Our best results from Chapter III (Experiment 2) demonstrated greatest SGR of rotifer when rotifers were fed on microalgae enriched with 1 mg l^{-1} L-carnitine. However, the use of L-carnitine in a recirculation culture system is cost effective and to save time for long term enrichment. According to chemical property of their chemical agent, L-carnitine could be transported through the membrane (Fritz et al. 1963). The application of L-carnitine is now widely used in cosmetics, pharmacy and so on. Therefore, this experiment was designed to find out the possibility of direct accumulation of L-carnitine into rotifer with the short time exposure to certain concentration of this chemical.

4.2 Materials and Methods

These experiments were carried out at the Laboratory of Aquaculture and Artemia Reference Center (ARC), University of Ghent, Belgium.

The Culture Stock

The stock culture of rotifer provided from ARC were always maintained in 50 ml conical centrifuge tubes and kept at 28±2°C in a culture room, following the culture procedure described in Dhert (1996).

Preparation of Rotifer Culture for Experiment

The 50 ml conical centrifuge tubes were used as culture for initial rotifer culture. Each culture tube was filled with 20 ml seawater at salinity of 25‰ and autoclaved prior to use. A portion of rotifer culture stock was inoculated into the culture tube at initial rotifer density of 2 ind.ml^{-1}. All culture tube were kept in the culture room and placed on a rotating shaft evolving at 4 rpm. Light was provided by two fluorescent lamps at light intensity of 20 µmol photon m^{-2} s^{-1} with 24:0 h (Light:Dark cycle). Two milliliter of *Tetraselmis* sp. (1.6×10^6 cells ml^{-1}) was added into each culture tube daily as food for rotifer. After one week, rotifer densities reached about 200 ind.ml^{-1} and were then harvested using a set of two filter screens. The larger mesh filter screen was 200 µm and the smaller one was 50 µm mesh size. Waste particles remained on the larger screen while rotifers were collected on the smaller screen. A small portion of collected rotifers was kept as source stock and the remained rotifers were used for up scaling the culture (Dhert, 1996; Suantika et al. 2000, 2001).

Experimental Conditions

Starter cultures were kept at 28±2°C in the culture room. In an Erlenmeyer flask, rotifers were stocked at a density of 50 ind.ml^{-1} and fed approximately 50 ml of microalgae (*Tetraselmis* sp. 1.6×10^6 cells ml^{-1}) daily. Rotifers density reached about 200-300 ind.ml^{-1}, after about 3 days and were then harvested and inoculated in 15 l

glass bottles for further mass production. Two liters inoculums of concentrated rotifers (50 ind.ml^{-1}) were placed in the 15 l glass bottles with tube aeration and fed microalgae (*Tetraselmis* sp. 1.6×10^{6} cells ml^{-1}) daily. Every other day cultures were cleaned (by using a set of two filter screens) and restocked at rotifer densities of 500 ind.ml^{-1} until a total volume of 15 l was achieved (about 6-7 days). These rotifer cultures were used for mass culture. The mass cultures were performed in 100 l aerated, cylindro-conical tanks with 50 l culture water volume. Temperature and salinity were kept at a constant level of 25±1°C and 25 ‰. Figures 4.1 present overview diagram and photograph of this recirculation system.

Rotifers were maintained in rearing tanks that were equipped with a central nylon screen (50 µm mesh size) (Figure 4.2). An aeration collar enclosed on the outer bottom part of the filter provided soft aeration. This ensured good oxygenation and uniform distribution and mixing of rotifers and their diet. Each culture tank was equipped with spongy material used to remove organic waste material. A 300 watt immersion heater was used to maintain constant temperature at 25±1°C. Effluent water exited the six replicated culture tanks and flowed by gravity to a 100 l settlement tank before being treated with a protein skimmer. Water was then pumped into a protein skimmer using a 50 watt water pump. The protein skimmer (DBPr. 3525861 Aquarien Technik Klaes, Germany) had a 55 l capacity and a maximum water flow rate of 1200 l h^{-1}. Suspended organic matter such as excess food, floccules and ciliates from the effluent water were trapped in foam from the protein skimmer and removed daily. After physical separation in the protein skimmer, effluent water underwent a biological filtration in submerged biofilters with 90 l capacity. Media used in the submerged biofilters was gravel (size = 3-8 mm) filled to 60 l capacity. Biofilters were inoculated with an enriched culture of nitrifying bacteria. After biological filtration, treated water was pumped using a 50 watt water pump and reinjected into the rotifer culture a daily water renewal rate of 500% day^{-1} (Suantika et al. 2003).

Culture Treatment

Rotifers were sequentially concentrated in each 100 l cylindo-conical tanks at rotifer density of 500 ind.ml^{-1} and expose to 1 mg l^{-1} L-carnitine for 48 h in the L-carnitine treatments, the following order (Figure 4.1): D1 (L-carnitine), D2 (control), D3 (L-carnitine), D4 (control), D5 (L-carnitine), and D6 (control). Rotifers were fed *Tetraselmis* sp. (1.6×10^6 cells ml^{-1}) on day 1 and *Tetraselmis* sp. plus artificial food (Culture Selco), on day 2 at a ratio of 1:1. After 2 days, in each tank was cleaned and disinfected with chlorine and HCl, then rinsed with clean seawater before inoculating with rotifer at densities of 500 ind.ml^{-1} in the same tanks.

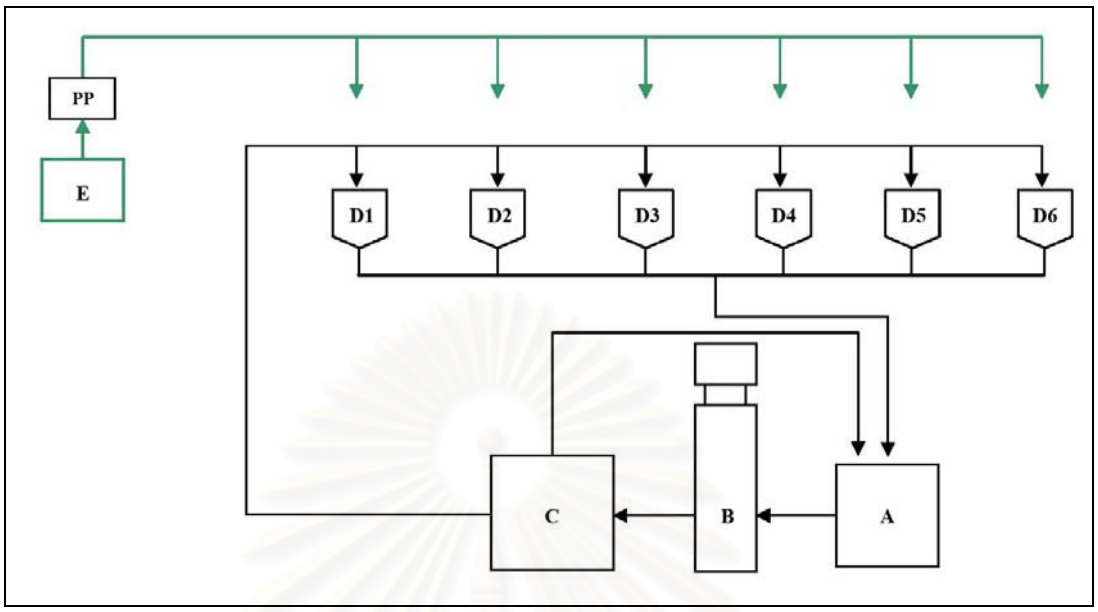

Figure 4.1 Diagram (top) and photograph (bottom) of the recirculation system. A = Settlement tank, B = Protein skimmer, C = Biofilter tank, D1-6 = Rotifer culture tanks, E = Food storage, PP = Peristatic pump, ⟶ Direction of flow.

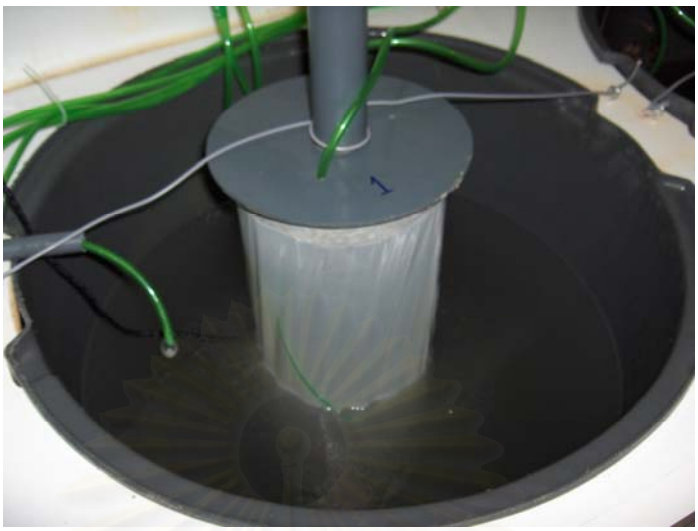

Figure 4.2 A central nylon screen (mesh size of 50 µm)

Figure 4.3 Refrigerated feed suspension distributed to the individual rotifer tanks by mean of a peristaltic pump.

Sample collection and growth measurement

Rotifer densities were determined daily before feeding. Three sub-samples of 250 µl were taken from all tanks using an automatic micropipette. Three drops of Lugol's Iodine were added to the samples to immobilize and kill the rotifers before counting. Rotifer densities and egg ratios were counted. Empty and transparent loricas belonging to dead rotifers were not counted. Specific growth rate was calculated using the equation 5. Egg ratio was calculated using the equation 6.

Rotifer Diet

In all the experiments, the experimental rotifer diet Culture Selco® (CS) formulated by INVE N.V., Belgium was used. This dry food was suspended in 800 ml water and mixed vigorously with a kitchen blender. This suspension containing the daily food ration was kept in cold storage (4°C) for 24 hours. From the cold storage tanks, food was administered every hour automatically by means of a peristaltic pump to the individual rotifer culture tanks (Figure 4.3). A standard feeding regime was optimized from Lavens et al. (1993) and derived from the following equation.

$$CSH = 0.0168 \times D^{0.415} \times V$$

Where CSH = the experimental diet (g)
D = rotifer density (ind. ml^{-1})
V = culture water volume (l)

A timer connected to the peristaltic pump regulated the food distribution at a rate of 800 ml food suspension per day.

Physico-Chemical Parameters

NH_4^+, NO_2^-, NO_3^-, and DO (Dissolved oxygen) of the water were measured daily early morning. NH_4^+, NO_2^- and NO_3^- measurements were measured from culture water of rotifer tanks after filtered through a 30 µm filter. The parameters were determined as follows: NH_4^+ (using ammonium test kit of Merck® with a scale of 0, 0.5, 1, 2, 3, 5 and 10 mg l^{-1}), NO_2^- (using nitrite test kit of Merck® with a scale of 0,

0.05, 0.15, 0.25, 0.5, and 1 mg l^{-1}), and NO_3^- (using nitrate test kit of Visocolor Eco® with a scale of 0, 4, 10, 20, 30, 50, 70, 90 and 120 mg l^{-1}). Dissolved oxygen (DO) was measured using DO meter.

Statistical Analysis

One-way ANOVAs with the Duncan multiple comparison test were used to compare differences of treatment on density and egg ratio of rotifer. Differences were considered significant at the $p < 0.05$ level.

4.3 Results

Average density and specific growth rate (SGR) of rotifers reared in the recirculation system exposed to L-carnitine treatment and the control are presented in Table 4.1 and Figure 4.4. Significant increase in rotifer densities ($p<0.05$) occured with L-carnitine exposure. During the 9 day culture period, rotifer densities of 7,247 ind.ml^{-1} and 4,431 ind.ml^{-1} were observed with L-carnitine exposure and the control, respectively. However, during the 9 days culture period, there was no significant differences ($P>0.05$) in SGR between L-carnitine exposure and control (Table 4.1).

Egg ratio was used as a parameter for egg formation. The results indicate that egg ratio followed more or less the same trend in the two treatments (Table 4.2). Highest egg ratios in L-carnitine and control treatments were 0.42 and 0.46, respectively. There were no significant differences in egg ratio between the two treatments.

Ammonium concentrations (NH_4^+) in the recirculation system during 9 days culture period ranged from 0.5 to 7.5 mg l^{-1}. For the system exposed to L-carnitine the ammonium concentration ranged from 0.5 to 7.5 mg l^{-1} and stable at 7.5 mg l^{-1} on day 6. In the control treatment ammonium ranged from 0.5 to 5 mg l^{-1} and table at 5 mg l^{-1} on day 4. Nitrite concentration increased slightly in both treatments and followed the same trend as ammonium ranging from 0 to 1.83 mg l^{-1}. Nitrate increased slightly in

both treatments and followed the same trends ranging from 10 to 30 mg l^{-1}. Dissolve oxygen (DO) concentration decreased slightly and varied during 9 day of culture period in both treatments (Appendix E).

Table 4.1 Density and specific growth rate (SGR) of rotifer (*B. plicatilis*) (ind.ml^{-1}) obtained in a recirculation system with 1 mg l^{-1} L-carnitine exposure treatment and the control treatment.

Day	Control	L-carnitine Treatment
0	500 ± 0	500 ± 0
1	512 ± 63[a] (0.02)	615 ± 60[a] (0.21)
2	807 ± 67[a] (0.46)	1,003 ± 119[a] (0.49)
3	1,235 ± 138[a] (0.43)	1,726 ± 77[b] (0.54)
4	1,512 ± 154[a] (0.20)	2,147 ± 215[b] (0.22)
5	1,816 ± 233[a] (0.18)	2,399 ± 150[b] (0.11)
6	2,344 ± 210[a] (0.26)	3,411 ± 299[b] (0.35)
7	3,460 ± 433[a] (0.39)	4,934 ± 234[b] (0.37)
8	4,018 ± 477[a] (0.15)	6,747 ± 765[b] (0.31)
9	4,431 ± 1,141[a] (0.10)	7,247 ± 617[b] (0.07)

Note: - Values (Mean ± SD of three replicate)
- Mean with different superscript letter is significantly different ($p<0.05$), (ANOVA, Duncan's multiple comparison).
- Specific growth rate (SGR) values are in parenthesis

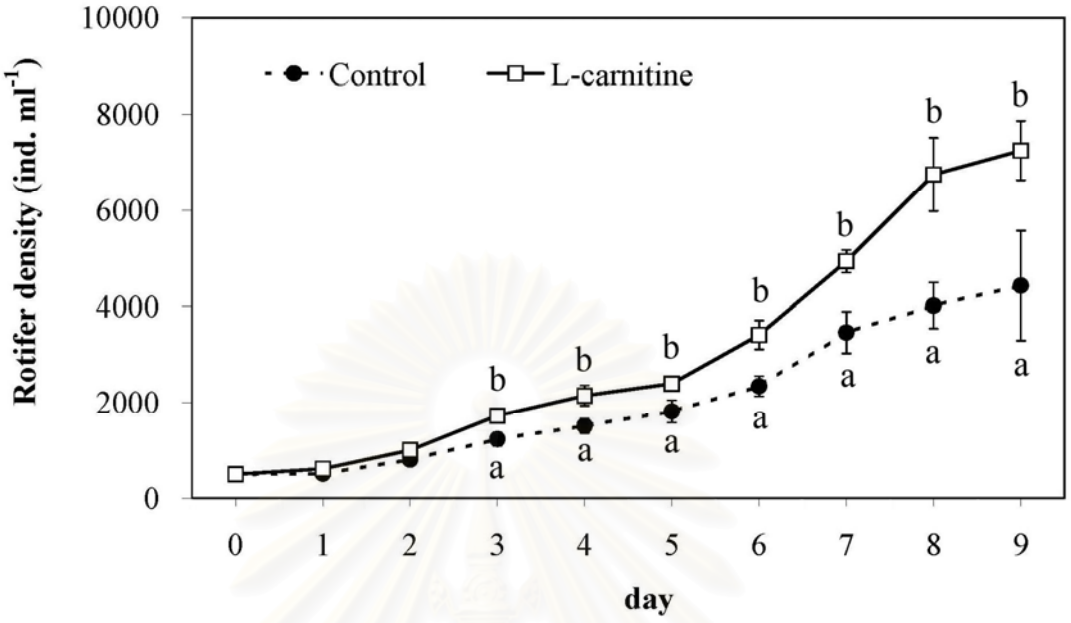

Figure 4.4 Rotifer density of rotifer (*B. plicatilis*) (ind. ml^{-1}) obtained in a recirculation system with 1 mg l^{-1} L-carnitine exposure treatment and control treatment.

Table 4.2 Egg ratio of rotifer (*B. plicatilis*) obtained in a recirculation system with 1 mg l^{-1} L-carnitine exposure treatment and control treatment.

Day	Control	L-carnitine Treatment
0	0.15 ± 0	0.15 ± 0
1	0.46 ± 0.06[a]	0.39 ± 0.03[a]
2	0.41 ± 0.02[a]	0.42 ± 0.04[a]
3	0.27 ± 0.03[a]	0.25 ± 0.01[a]
4	0.29 ± 0.03[a]	0.27 ± 0.02[a]
5	0.35 ± 0.02[a]	0.30 ± 0.04[a]
6	0.26 ± 0.02[a]	0.24 ± 0.02[a]
7	0.17 ± 0.03[a]	0.17 ± 0.02[a]
8	0.20 ± 0.02[a]	0.18 ± 0.01[a]
9	0.21 ± 0.01[a]	0.20 ± 0[a]

Note: - Values (Mean ± SD of three replicate)

- Mean with different superscript letter is significantly different ($p<0.05$), (ANOVA, Duncan's multiple comparison).

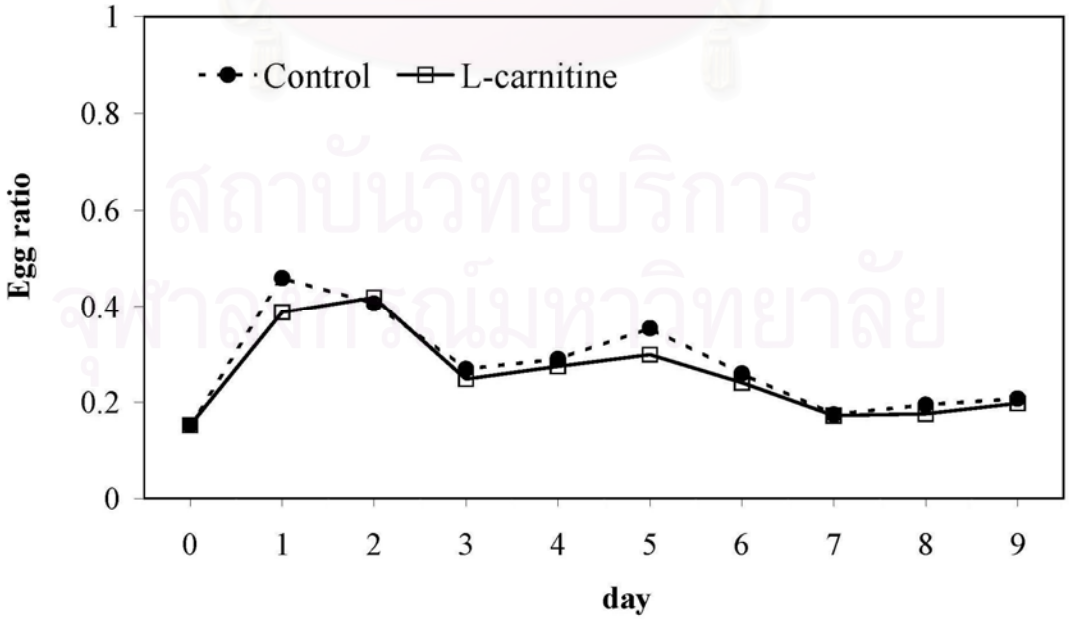

Figure 4.5 Egg ratio of rotifer (*B. plicatilis*) obtained in a recirculation system with 1 mg l^{-1} L-carnitine exposure treatment and control treatment.

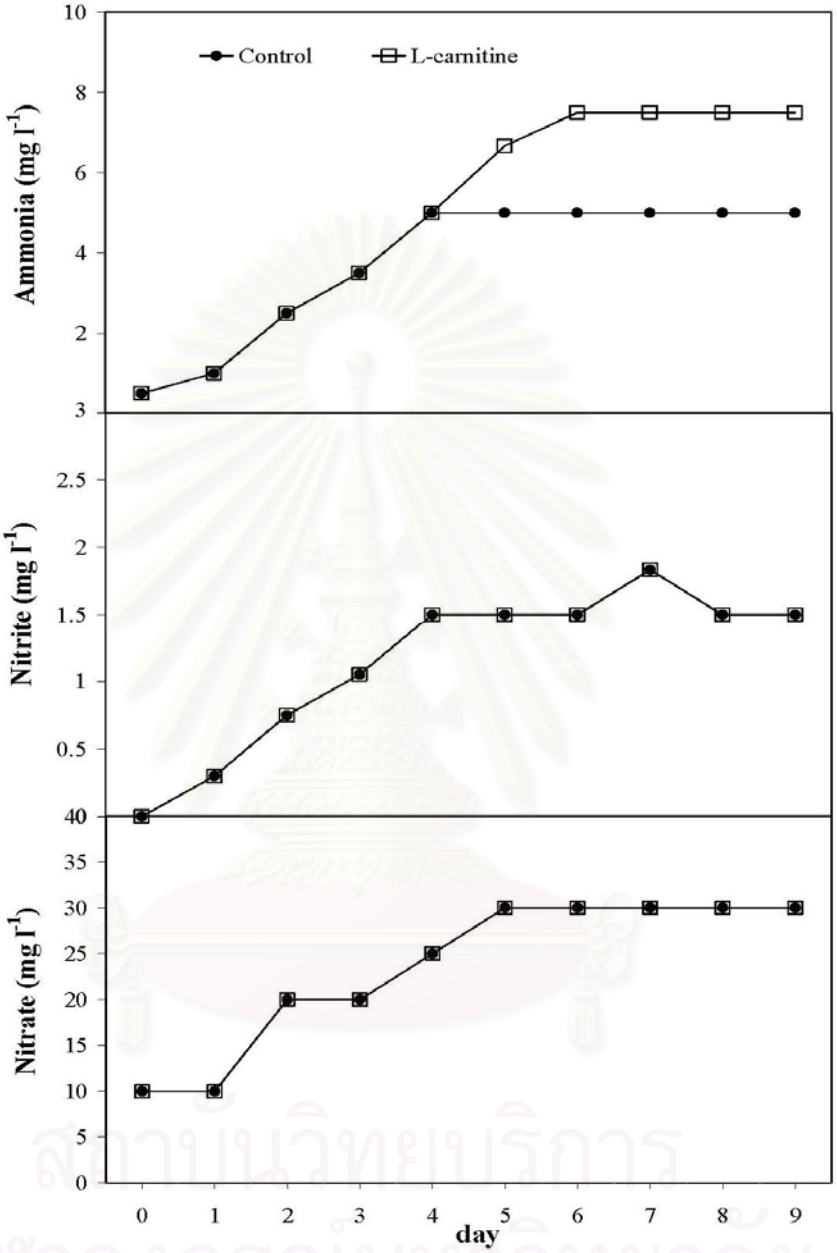

Figure 4.7 Ammonia, nitrite and nitrate concentrations (mg l^{-1}) in a recirculation system with 1 mg l^{-1} L-carnitine exposure treatment and control treatment. (The term "ammonia" refers throughout to the total of NH_3 and NH_4^+)

4.4 Discussion and Conclusion

In the present study, rotifer density was used as a parameter to evaluate rotifer production performance in our recirculation system. L-carnitine applications produced 1.2-1.7 times greater densities than control treatments during 9 days culture.

Although high densities of rotifers were obtained in small-scale (100 1) culture system, it may be risky to run this production close to its maximum carrying capacity. At maximum carrying capacity, the rotifers stopped growing exponentially and could suffer a crash. It would also be less attractive to hatcheries that need to have continuous, adequate, and clean rotifer production to feed fish or crustacean larvae. Continuous production of rotifers in a larger recirculation system (1000 l), seems like a better alternation.

Egg ratio is an important parameter that relates to rotifer population growth. It can be used as an early warning of culture collapse (Snell et al. 1987). In our experiments, there was no clear difference between treatments on egg ratios (Table 4.2 and Figure 4.5). Greatest egg productions in both treatments were observed on days 1 and 2 (38-45 %), which decreased to 17 % on day 7. This decrease can be attributed to water quality deterioration. At the beginning of the culture period, water quality was still optimal that resulted in high egg ratios. This agrees with observations of Hagiwara et al. (1993) and Fukusho (1989) who stated that the egg ratio is influenced by quality of the water medium.

Tremblay and Bradley (1992) reported that L-carnitine provided protection against toxic levels of ammonia in fish culture. Therefore, the addition of L-carnitine in our recirculation system may have protected rotifers from ammonia toxicity. Our previous experiments (Chapter III; experiment 2) have shown that L-carnitine significantly increased density population of rotifers in batch culture. It is possible that L-carnitine prolonged lifespan of rotifers. In this L-carnitine treatment had greater rotifer population densities as a result. Egg ratio reflects both birth rate and relatively population growth at the same time. However, the egg ratio with 1 mg l^{-1} L-carnitine treatment was greater than the control treatment, but it had lower population density (Figure 3.7). Since Clement et al. (1983) reported that most rotifer mechno- and

chemoreceptors sensitive to environmental stimulation were in direct contact with external medium, it was therefore possible that the effect of L-carnitine by these methods were direct. There were several disadvantage of the rotifer recirculation culture system at ARC included settling of excess food on nylon screen and the wall of culture tank (Table 4.3).

The application of L-carnitine in the recirculation system resulted in a higher production density of rotifers than without L-carnitine. It also allowed a prolongation lifespan of rotifer, but not increased egg ratio. High levels of ammonium (NH_4^+) and nitrite were observed in the culture supplemented with L-carnitne. This was attributed to biofilter efficiency.

The use of L-carnitine in commercial aquaculture will not be prohibitive since L-carnitine costs only about 6,000 BHT per kilogram (Lonza Biotec s.r.o., Czech Republic), dosage was low (1 mg l^{-1}), and rotifers were concentrated at high density during L-carnitine treatment. Production reliability and practical experience of staff are the critical factors for this culture system. In Europe, North America and Japan, manpower cost is one of the main concerns, while equipment cost is a main concerns in countries like Thailand.

Table 4.3 Advantages and disadvantage of the rotifer recirculation culture system at ARC, Belgium.

Advantage	**Disadvantage**
-high rotifer production	- a lot of routine maintenance work (harvest and clean nylon screen) - easy crash by water quality and protozoa - use a lot of seawater - high investment and operational cost - expensive artificial diet - need expert labor (for counting rotifer density, measuring water quality, rotifers harvesting, filter rinsing and feeding)

CHAPTER V

DEVELOPMENT OF A CLOSED-RECIRCULATING, CONTINUOUS CULTURE SYSTEM FOR MICROALGA (*T. suecica*) AND ROTIFER (*B. plicatilis*)

5.1 Introduction

Live rotifers, *Brachionus plicatilis* are one of the most important and widely used foods for larviculture of fish and other aquatic animals that require live food of small size with high nutritional value. Three economically important, cultured fish species in Thailand requiring live rotifers during their larviculture are seabass (*Lates calcarifer*), grouper (*Epinephelus coioides*), and mullet (*Liza subviridis*) (Pechmanee, 1997). Rotifers are cultured using a wide variety of culture systems, including batch, semi-continuous, and continuous culture (Lubzens, 1987). In Thai hatcheries, rotifers are most often cultured using open, batch culture systems in tanks or ponds with microalgae and/or yeast as food sources for rotifers (Kongkeo, 1991). Although these batch culture systems are relatively simple, rotifer production is often unpredictable and requires considerable labor for operation and maintenance. Rotifer production is therefore often insufficient to meet hatchery needs during critical larviculture stages.

Continuous, mass culture systems for microalgae and rotifers are generally much smaller than batch culture systems, but require more intensive management. Chemostats are the most advanced type of continuous culture used in aquaculture for microalga production (James and Abu-Rezeq, 1989; James and Al-Khars, 1990). Alga production is the critical first step in production of rotifers and other live, larval fish foods. Considerable effort has gone into development of substitutes for live microalgae, but as yet these substitutes have not proven satisfactory (Fu et al. 1997; Navarro, 1999; Suantika et al. 2001; Yoshimura et al. 1997). Microalga substitutes often lack adequate nutritional value, are difficult to maintain, or have other

functional problems, especially in tropical places like Thailand. Consequently, live culture of *Chlorella* spp., *Nannochloropsis* spp., and *Tetraselmis* spp. microalgae still provide the basis for rotifer culture in Asia (Kongkeo, 1991).

Batch culture of microalgae typically involves a multi-step process using backups for each step, and where each step is used to inoculate the next step leading to rotifer food production. Although this process is conceptually simple, it does require considerable labor, equipment, materials, and space resources. Furthermore, it is also susceptible to unpredictable "crashes" caused by protozoans, alga contamination, and/or other causes (De Pauw et al. 1984). In addition, it is often difficult to operate batch culture systems for marine species at inland locations, or at locations without a reliable source of high quality, seawater. Inland hatcheries in Thailand typically must import brine (150-200‰ salinity) at considerable expense from salt farms located in coastal areas. Effective re-use of this seawater is therefore desirable (Suantika et al. 2001; 2003). These needs generated considerable interest in development of closed-recirculation systems for larviculture food production as an alternative to open, batch culture systems (James and Al-Khars, 1990; Laing, 1991; Rusch and Christensen, 2003; Walz et al. 1997). To date, however, most of these closed systems are either too costly, and/or are too technically complex and difficult to operate in most commercial hatchery settings.

We know of no continuous, closed-recirculation system for algae and rotifers that is appropriate for widespread use in Thai hatcheries. Our motivation, therefore, was to develop such a system using microalga *T. suecica* and rotifer *B. plicatilis* that would overcome the problems identified above. Our aim was to develop an efficient but simple culture system for rotifers that could produce sufficient quantities of rotifers on demand, while at the same time reducing labor, risks, and resource costs. The culture system we describe herein met our objectives for both small and large-scale rotifer production needs.

5.2 Materials and Methods

5.2.1 Microalga and Rotifer Culture

Microalga (*T. suecica*) and rotifers (*B. plicatilis*) were obtained from the Department of Marine Science, Chulalongkorn University and Bangsaen Institute of Marine Science, Burapha University, Thailand respectively. Stock cultures of these organisms were maintained using batch culture techniques at Angsila Marine Station, Chulalongkorn University. *T. suecica* were maintained using 25‰ Conway medium (Walne, 1966) at 28 ± 2°C, 24:0 h Dark/Light cycle, with white fluorescent light at the intensity of 40 µmol photon m^{-2} s^{-1} under continuous aeration. Batch cultures were scaled-up to 20 to 30 l and cultured until reaching exponential growth before transfer to the microalga culture tank of the continuous culture system. Batch cultures of *B. plicatilis* were maintained in 25‰ seawater at 28±2°C with continuous aeration and fed *T. suecica* daily. Batch cultures were scaled-up to 200 l at 150 to 200 rotifers ml^{-1} before transfer to the rotifer culture tank of the continuous culture system.

5.2.2 Experimental Setup

5.2.2.1 Description of Continuous Culture System

Our continuous culture system consisted of three sub-components; microalga culture component, rotifers culture and harvest component, and water treatment and re-use component (Figure 5.1 and 5.2). The microalga culture component included a nutrient source, culture medium tank, and microalga culture tank. Flow of concentrated nutrient solution from the nutrient source reservoir (Figure 5.1; 4) to the culture medium tank was controlled by an electronic on-off switch (1) that regulated a small water pump (2). A magnetic stirrer (3) kept nutrients well mixed, starting one minute before pumping to the culture medium tank. At the same time nutrients were pumped, seawater was also pumped from the water re-use tanks into the culture medium tank. The culture medium tank's water volume was 150 l when full. Water flow from the recycled/new water tanks was regulated by a liquid level control (LLC)

switch (8) that sensed water levels inside the culture medium tank. When water volume in the culture medium tank was reduced to 20 l, re-use water was automatically and synchronously pumped into the culture medium tank along with concentrated nutrients at a ratio of 750:1. When water volume reached 10 l in the culture medium tank, pumps were turned off. All LLC switches were from Shin Tung Electronics Industry Co., Ltd., Taiwan, model ST-65AB.

Refill water passed through a 20 µm filter before entering the culture medium tank, and was then recycled through an ultraviolet (UV) sterilizer (Figure 5.1; 6) below the tank by a separate pump (7). The culture medium tank had two LLC switches. One LLC switch (8) controlled refill water and nutrient inflows into the culture medium tank as described above, while the second LLC switch (5) controlled ozonation (27) of water in the water re-use tanks. The second LLC switch (5) in the culture medium tank turned off ozonation in the water re-use tanks when water volume fell to 40 l in the culture medium tank. When water volume increased to 150 l, ozonation resumed in the water re-use tanks. Discontinuous ozonation was necessary to prevent ozone from entering the microalga culture tank where it could kill algae. The ozonator was a Hailea model HLO-810, 10 watt capacity that operated using airflow of 3.5 l min^{-1} and air stone injection. This model produced 200 mg O_3 hr^{-1}. All tanks in the system were made of polyethylene, while hard pipes were of blue PVC plastic, and flexible pipes and tubes were of soft PVC.

The microalga culture tank was 0.72 m diameter, 1 m height with 260 l water volume. The light energy source for alga growth consisted of three, white florescent lamps (Figure 5.1; 12; 36 watts capacity each) inside three layers of concentric, clear acrylic tubes (11). Water temperature in the microalga culture tank was maintained using a cooling system that consisted of water exchange between the first space created by the concentric acrylic tubes (11) and a cooling system tank next to the microalga culture tank. Mechanical cooling was not provided in the cooling water tank, but water was circulated between the cooling water tank and the center of the microalga tank in order to remove heat generated by the light source. Water temperatures in the cooling water tank was 30ºC and 27-28ºC in microalga culture water. Room temperature was maintained at 28 ± 2ºC using air conditioning. In addition, two small ventilation fans (10, 13) circulated air through the top of the

culture tank. One fan pumped outside air into the culture tank, while the other fan pumped air out. Each fan was rated at 30 watts. Culture medium water was pumped at a constant rate from the culture medium tank into the microalga culture tank using a metering pump, while continuous aeration was provided at 4 to 5 l min^{-1}.

The rotifer culture tank was 55 cm dia., 88 cm height and with 200 l water volume (Figure 5.1). Water flows into the rotifer culture tank were by gravity from the micoalga culture tank, which in turn equaled the constant rate of water inflows from the culture medium tank. The amount of microalga food thus supplied to rotifers was related to water flow rates (l min^{-1}) and alga densities (cells l^{-1}) in the microalga culture tank. Rotifers were harvested in the 200 l rotifer harvest tank using a conical harvest net (14) with 0.6 m diameter, 0.6 m height, and 58 μm mesh size. Water flowed by gravity from the rotifer culture tank into the harvest net. During rotifer harvest, the net was raised using a rope and pulley arrangement and rotifers were concentrated in a small, plastic container at the cod-end of the net. Rotifers adhering to the net were washed into this container using a hand held sprayer (15) that operated on a separate water pump that drew water from the first compartment of the water treatment tank. Effluent from the rotifer harvest tank then flowed into the water treatment tank.

The water treatment tank measured 1.2×0.6 m with 65 cm height with 300 l water volume (Figure 5.1). It contained several sub-components designed to restore water quality by removing both solid and dissolved wastes and contaminants. These sub-components were contained within five compartments of the water treatment tank. Water flows into compartment-1 of the water treatment tank were gravity flows from the rotifer harvest tank into a PVC pipe (18) containing a 38 μm mesh net that removed most rotifers and other debris. After passing through this net, water was pumped through an UV sterilizer (19) into five additional PVC pipes (20), each measuring 10.2 cm dia. with 55 cm height. All six PVC pipes contained three layers of charcoal (150 g) on the bottom, oyster-shells (1.5 kg) in the middle layer, and spun-fiberglass filter material on top. Water flow was nearly equal between the five pipes with total flow of 23 l min^{-1}. The UV sterilizer (19) operated 3 hrs ON and 1 hr OFF, while the protein skimmer (16) operated continuously with of 33 l min^{-1} water flow. The protein skimmer (16) operated independent of the pump providing

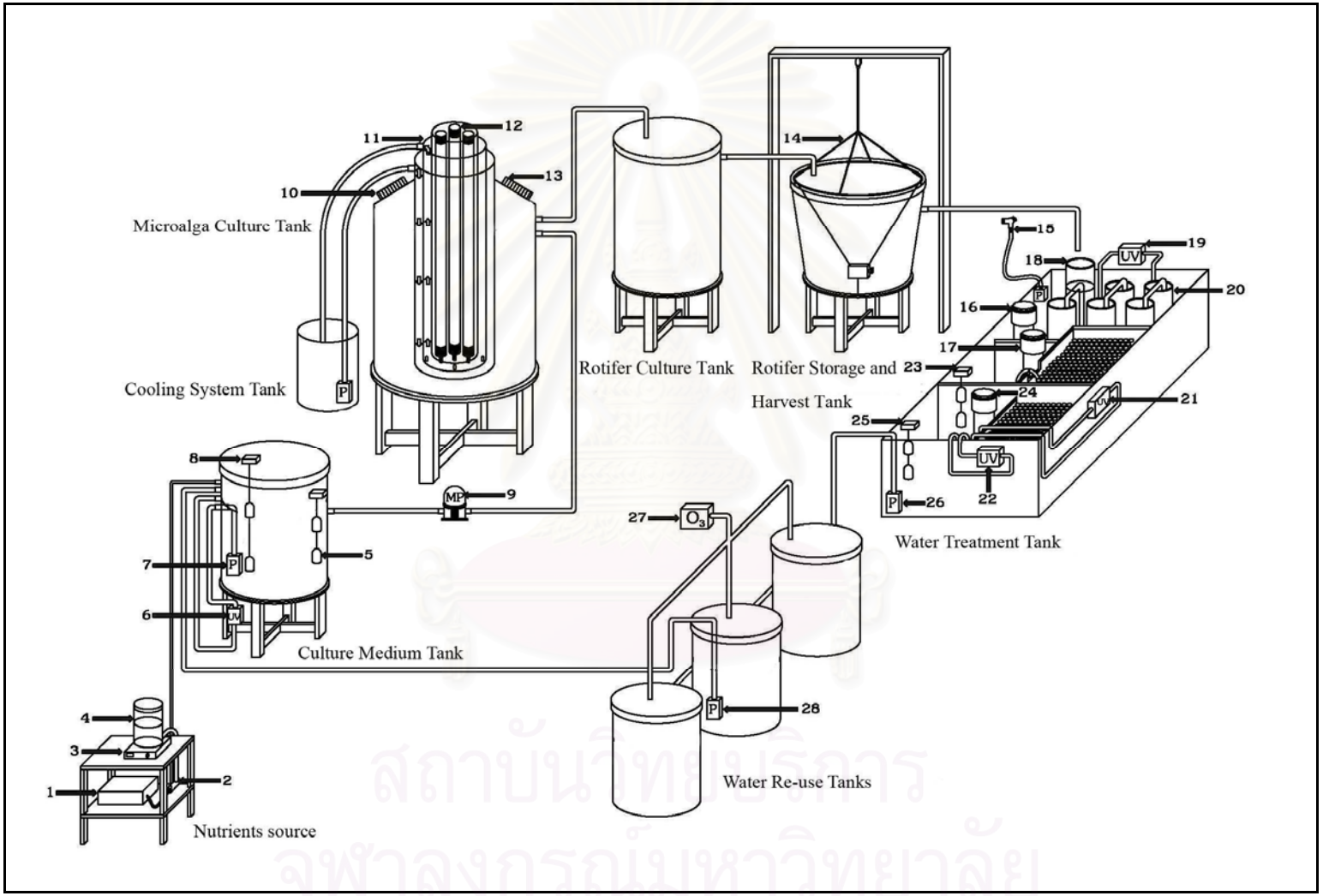

Figure 5.1 Diagram of a closed-recirculating, continuous culture system for microalga (*T. suecica*) and rotifer (*B. plicatilis*). See text for description of numbered labels on system components.

Figure 5.2 Photograph of a closed-recirculating, continuous culture system for microalga (*T. suecica*) and rotifer (*B. plicatilis*).

water to the six pipes and to the UV sterilizer (19) in compartment-1 of the water treatment tank. Intermittent operation of all UV sterilizers was necessary to prevent damage to their lamps. All UV sterilizers were Atman, 11 watt capacity; while all protein skimmers were Queen Turbo-Skimmers model TS-2000. Wastes from protein skimmers were discharged to drain.

Water from compartment-1 of the water treatment tank flowed by gravity into compartment-2. Compartment 2 contained a protein skimmer (Figure 5.1; 17) for removing suspended solids and remaining dissolved proteins. Compartment-2 also had an LLC switch (23) that actuated when water levels reached a pre-set level, pumping water into compartment-3. The LLC switch turned off the pump when water levels dropped to the lower pre-set level. Compartments-3 and 4 of the water treatment tank contained biofilter media for nitrification. Water flowed from compartment-3 into section-4 by gravity flow. When compartment-4 was full, water flowed by gravity compartment-5. Compartment-5 contained two UV sterilizers (21, 22) on water re-circulation pumps and a protein skimmer (24) that also recirculated water between the UV sterilizers and the compartment. The protein skimmer removed bacterial floc from the biofilters. The UV sterilizers were on a timer switch with 3 hrs ON and 1 hr OFF. Water from compartment-5 of the water treatment tank was then pumped (26) into three water re-use tanks with 400 l total water volume. Water flows were controlled by a LLC switch (25).

A control board (Figure 5.3) contained protection and electrical control for the continuous culture system. This board included a ground-fault control for all circuits to protect system operators, timers for the UV sterilizers, and circuit breakers for pumps and other electrical components.

5.2.2.2 Operation and Performance of Continuous Culture System

We assessed performance of the continuous culture system during on a 28 day trial at a water flow-through rate of 121 l d^{-1} and dilution rates (Dilution rate (D) = Medium Flow Rate (F)/Tank Volume (V), according to Richmond, 1986) in the microalga culture tank and rotifer culture tank of 0.47 and 0.61 respectively (Table 4.2). We then observed the effects of these dilution rates on microalga and rotifer

densities and production, and on water quality. The system operated at 28±2°C with continuous aeration, as described above.

Figure 5.3 Control panel for continuous culture system of Figure 5.1, where: A = SENSE Model 540SR, ground-fault control for all circuits; B = timer for UV sterilizer (6); C = timer for UV sterilizer (22); D = timer for UV sterilizer (19 & 21). E through K = circuit breakers, where E-G were for timers B-D, H was for LLC switches (5 & 8) and ozonator (27), I was for LLC switches (8) and nutrient source and pump (28), J was for microalga culture tank, cooling system pump and MP (9), and K was for water treatment tank equipment.

Water used in our continuous culture system was from seawater diluted to 25‰ and treated using ozone injection for 1 day, then aerated for 4 hrs to remove and residual ozone. Treated seawater was filtered through 20 μm nylon netting before transfer to the culture medium tank, microalga culture tank, rotifer culture tank, and the water re-use tanks. Several months before our trials began, we preconditioned nitrifying bacteria in the continuous culture system using continuous aeration and NH_4Cl at 2 mg l^{-1} with trace elements added. The nutrient solution for *T. suecica* in the continuous culture system consisted of four types of cost-effective agricultural-grade fertilizers (modified from Okauchi and Kawamura, 1997) (Table 5.1).

Table 5.1 Composition of culture media used in this work (All for one litre seawater)

Chemical	Conway medium (mg)	Agricultural-grade fertilizer (mg)
$FeCl_3.6H_2O$	2.60	
$MnCl_2.4H_2O$	0.72	0.36
H_3BO_3	67.20	
Na_2-EDTA	90	
Fe-EDTA		15
$Na_2HPO_4.2H_2O$	40	7
$NaNO_3$	200	105
$ZnCl_2$	42	
$CoCl_2.6H_2O$	40	
$(NH_4)_6Mo_7O_{24}.4H_2O$	18	
$CuSO_4.5H_2O$	40	
Vitamin B_1	0.10	
Vitamin B_{12}	0.005	

We initially stocked the microalga culture tank with 26 l of *T. suecica* innoculum to achieve 1-2×10^5 cells ml^{-1}, while the rotifer culture tank was initially stocked with 150-200 ind. ml^{-1}. After stocking, the metering pump from the culture

medium tank was turned on and continuous flow-through began at 121 l d^{-1} and remained constant during the 28 day trial.

5.2.2.3 Sampling and Counting

Microalga and rotifer densities were measured daily using a haemacytometer and Sedgewick-Rafter slide under light microscope with triplicate samples preserved using Lugol's Iodine solution. Microalgae were counted in both the microalga culture tank and rotifer culture tank, while rotifers were counted in the rotifer culture tank only.

5.2.2.4 Physico-Chemical Parameter

Inorganic nitrogen (ammonia-N, nitrite-N, nitrate-N) and total phosphorus (P) were measured every five days at five locations in the continuous culture system according to Strickland and Parsons (1972) and APHA et al. (1992) (Appendix F). Temperature and salinity were measured daily, while freshwater and/or seawater was added to maintain salinity at 25‰, and to compensate for evaporation and other water losses.

5.3 Results

Daily microalga densities in the microalga culture tank ranged from 10.1-16.8×10^4 cells ml^{-1} (Figure 5.4A), while average density for the culture period was 13.4×10^4 cells ml^{-1} (Table 5.2). Estimated, average microalga yield from the microalga culture tank was 1.6×10^{10} cells day^{-1}. These alga yields provided food for rotifers in the rotifer culture tank, and to a lesser extent for rotifers in the rotifer storage and harvest tank. Microalga densities in the rotifer culture tank averaged 0.4×10^4 cells ml^{-1}, or about 3% of alga densities in the microalga culture tank. This reduction in alga density was the result of grazing by rotifers on algae and reduced light intensities in the rotifer culture tank. It is noteworthy that daily fluctuations in alga densities in the microalga culture tank were relatively small. Standard deviation

for daily alga densities was 6% of mean densities, indicating rather stable alga production under water flow rates, nutrient concentrations and light intensities used.

Rotifer densities in the rotifer culture tank during the 28 day culture period were more variable than alga densities, ranging from 17 to 64 ind. ml^{-1}, with peak values between days 9 and 13, and with lowest values on days 19 and 26-28 (Figure 5.4B). Average rotifer densities in the rotifer culture tank were 35 ind. ml^{-1} and in rotifer storage and harvest tank were 119 ind. ml^{-1} for the culture period (Table 5.2). There were no clear responses in rotifer densities in the rotifer culture tank related to alga densities in influent waters. Alga densities in influent waters and in the rotifer culture tank were relatively stable, as noted above. Estimated rotifer yields from the rotifer culture tank ranged from 1.7-6.4$\times 10^6$ ind. day^{-1}, with a mean estimate daily yield of 4.24$\times 10^6$ ind. day^{-1} (Table 5.2). These estimated yields based on rotifer densities in the rotifer culture tank and water flow rates agree well with mean observed rotifer harvests of 4.66$\times 10^6$ ind. day^{-1} in the rotifer storage and harvest net. Average estimated and observed values differed by only 10%.

Ammonia at five locations in the continuous culture system during 28 days culture ranged from 0.0 to 0.86 mg-N l^{-1}, with maximum values in the rotifer storage and harvest tank on days 9 (Figure 5.5). Nitrite ranged from 0.03 to 3.09 mg-N l^{-1}, with maximum values in the rotifer culture tank. Nitrate ranged from 1.1 to 60 mg-N l^{-1} in all tanks on day 28. Phosphate ranged from 0.0 to 3.5 mg-P l^{-1}, with continuous increases at all locations during the culture period.

Table 5.2 Water volumes in tanks, water flow and dilution rates, and resultant densities and production of microalga (*T. suecica*) and rotifers (*B. plicatilis*) in the continuous culture system during a 28 day culture trial.

	Microalga culture tank	Rotifer culture tank	Rotifer storage and harvest tank
volume (l)	260	200	39 (in a harvest net)
flow rate (l day^{-1})	121	121	121
dilution rate (day^{-1})	0.47	0.61	-
microalga daily densities ($\times 10^4$ cells ml^{-1})	10.1 ± 0.5-16.8± 1.1	0.2 ± 0.1-0.8 ± 0.2	-
rotifer daily densities & yields (ind. ml^{-1}) & ($x10^6$ day^{-1})	-	16.7 ± 2.1-64.0 ± 5	1.1 ± 0.4-13.5 ± 0.7
mean microalga-28 day density ($\times 10^4$ cells ml^{-1})	13.4 ± 0.8	0.4 ± 0.1	-
mean rotifer-28 day density (ind. ml^{-1})	-	35 ± 5	119 ± 10
mean daily microalga yield[1] ($\times 10^{10}$ cells day^{-1})	1.63 ± 0.1		
mean daily rotifer yield[2] ($x10^6$ day^{-1})	-	4.24 ± 0.55	4.66 ± 0.40

[1]Microalga yields from the microalga culture tank were calculated as the water flow rate from the mircoalga culture tank times average microalga density in this tank on that date.

[2]Rotifer yields from the rotifer culture tank (stock) were calculated as the water flow rate from the rotifer culture tank times average rotifer density in this tank on that date, while rotifer yields for the rotifer storage and harvest tank were actual numbers of rotifers harvested from the net in that tank on that date.

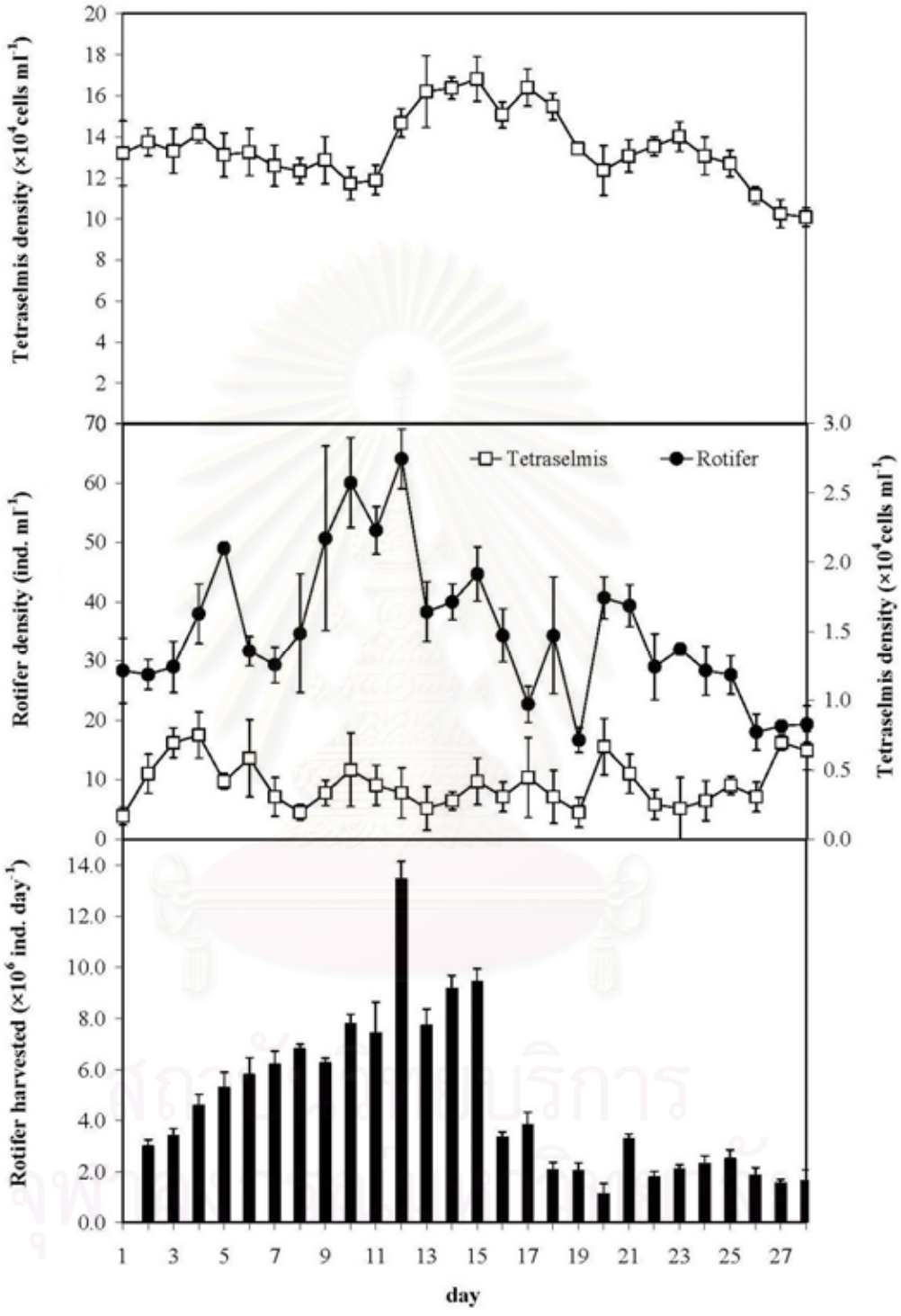

Figure 5.4 (A). Microalga (*T. suecica*) densities in the microalga culture tank of the continuous culture system during 28 days, (B) Microalga and rotifer (*B. plicatilis*) densities in the rotifer culture tank and (C) Rotifer harvested from the collection net of the rotifer storage and harvest tank. Standard deviation bars are shown for each daily value.

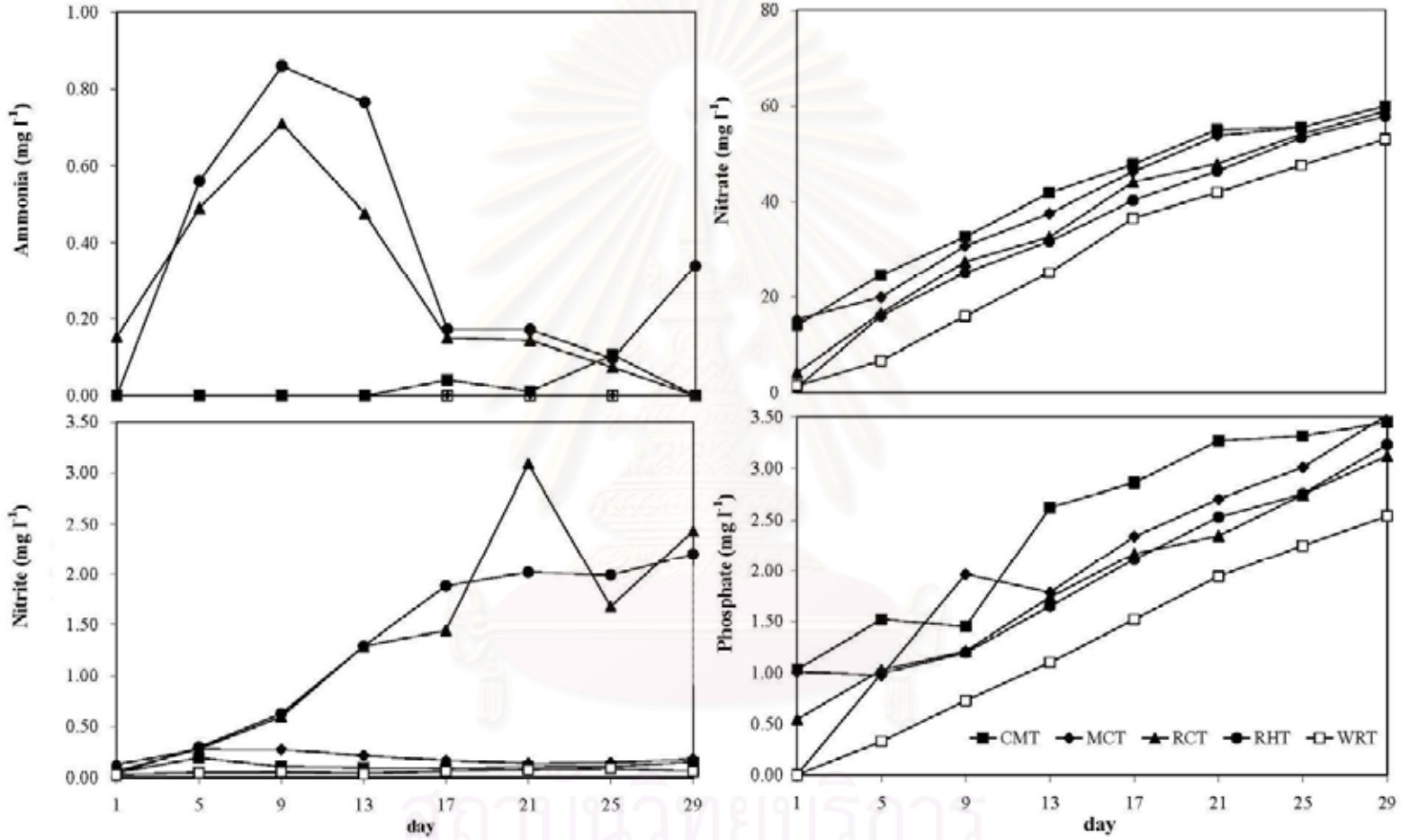

Figure 5.5 Ammonia, nitrite, nitrate and phosphate concentrations (mg-N-P l^{-1}) in the continuous culture system during a 28 day culture trial, where; CMT = Culture Medium Tank, MCT = Microalga Culture Tank, RCT = Rotifer Culture Tank, RHT = Rotifer Storage and Harvest Tank, and WRT = Water Re-use Tanks.

5.4 Discussion and Conclusion

Our trials with the closed-recirculating, continuous culture system demonstrated that this culture system is capable of sustained and acceptable levels of rotifer production for at least 28 days. Microalga densities and yields were quite stable, while rotifer yield varied more during our 28 day culture period. Rotifer variability seemed unrelated to alga densities and yields, but rotifer production was still entirely acceptable. We did not observe serious problems with either alga or rotifer contamination with this system. We did, however, find that algae became attached to the acrylic plastic around the light source in the microalga culture tank by day 10. This required daily scrubbing to remove the algae.

Water quality values were within an acceptable range for all parameters measured. Ammonia-N is known to cause cessation of rotifer reproduction when concentrations approach 3-5 mg l^{-1}, and rotifer mortality within two days at >5 mg l^{-1} (Schlüter and Groeneweg, 1985). However, we did not observe more than 0.86-mg l^{-1} ammonia-N at any time or location with our continuous culture system (Figure 5.5). Nitrite-N toxicity for rotifers is said to begin in the range of 90-140 mg l^{-1} (Lubzens, 1987). Again, we did not observe nitrite-N concentrations much greater than 2.2-mg l^{-1} at any time. Nitrate-N and phosphate did increase during our 28 day culture trial, but neither of these appeared to cause rotifer mortality or problems with rotifer production. Both nitrates and phosphates continued to increase throughout the culture period. Nitrates generally are not toxic in fish culture at concentrations of less than 1,000 mg l^{-1} (Lorsordo et al. 1992). These observations suggest that at the production rates we achieved, nitrate concentrations would not negatively impact the system's operation under most anticipated operating scenarios.

Most alga culture systems used in batch and other culture systems typically use a light source suspended 0.5 to 1.5 m above the alga culture tank (Donaldson 1991), or next to polyethylene culture bags (Sato, 1991). These light sources are outside the alga culture vessel (James and Al-Khars, 1990), and much of the light is therefore not directly available for alga growth. With our continuous culture system, the light source was inside the alga culture tank. We therefore expect much greater

photosynthetic efficiencies per unit of light energy. We did not, however, measure this efficiency during our initial trials.

If we compare our closed-recirculating, continuous culture system with batch or semi-continuous culture practices in Thailand, we see that our system has many advantages. These advantage for microalga culture with our closed-recirculating, continuous culture system include a shorter start-up time, less complicated start-up procedures, greater cell densities, lower light power requirements, no labor, fewer problems that could lead to alga culture “crashes”, and much reduced need for seawater imports (Table 5.3). Likewise, our closed-recirculating, continuous culture system has similar advantages over batch or semi-continuous culture practices for rotifers. These advantages for rotifer production included substantially shorter start-up time, less complicated and shorter start-up procedures, smaller tanks, more stable culture temperatures, simplified food sources for the rotifers, more consistent harvest densities, simple and continuous harvest procedures, greater production per unit labor and tank space, less contamination, greater control over the culture process, and much reduced need for seawater imports (Table 5.3).

For our closed-recirculating, continuous culture system, the total investment cost is about 162,376 THB, the most of the cost (37%) is spent for investment on fixed assets and 63% is consumed by electricity, chemicals and fertilizers and seawater and tap water (Table 5.4). Total annual cycle running cost for our closed-recirculating, continuous culture system rotifer feed is also for 864 THB in feed cost (chemicals and fertilizers) and water consumption around 1,560 l lower than the experiment of Suantika et al. (2003) that needed 11,154 Euro (535,000 THB) in feed cost and 7,840 l for water consumption. It is easy to operate our systems for marine species at inland locations or at locations without a reliable source of high quality seawater.

Our closed-recirculating, continuous culture system clearly demonstrated successful and continuous culture of microalgae and rotifers. The system opens new perspective in term of automated production of rotifer without labor cost, can easily harvest rotifer daily by conical harvest net and no routine maintenance work. Our continuous culture system met our design criteria of system simplicity, minimal contamination of culture organisms, low cost, and system stability and reliability. We

Table 5.3 Comparison of batch and semi-continuous culture practices for microalgae and rotifers in Thailand (Kongkeo, 1991; Fulks and Main, 1991) with the continuous culture system developed during our present study.

production	Batch/Semi-continuous cultures	Continuous culture (present study)
Microalgae		
Start-up time (days)	6	3-5
Size/shape/construction materials	1,000 and 4,000 l 1,000 l: round fiberglass 4,000 l: square or rectangular concrete	260 l round plastic tank
Start-up procedure (stock to mass)	To start with, 20 l of the microalgae stock is added to a l,000 l transparent fiberglass tank with 15‰ enriched seawater. After three day, the culture is expanded to a 10,000 l concrete tank for another three days. Every two to three days, fertilizer is added to prolong the stationary phase of growth. Ten percent of the culture is then added to another 10,000 l concrete tank and used as feed for rotifers.	To start with, the stock culture of *T. suecica* is added with approx. 26 l to microalga culture tank until two days, then the continuous culture system will begin operation and used to feed for rotifer daily.
Density at harvest (cells ml^{-1})	$2\text{-}4\times10^4$	$1\text{-}2\times10^5$
Land/space (m^2) /location	500/outdoor	16/indoor
Typical causes of microalgae crashes	1. rain (low light intensity, cloud cover, wind, temperature) 2. contamination (protozoa, inhibitory or pathogenic bacteria, other alga species, rotifers) 3. poor quality of starter microalgae	1. protozoa 2. other alga species 3. settling of *T. suecica* cells on the wall of the clear acrylic plastic tube and tank
rotifers		
Start-up time (days)	17	3-5
Start-up procedure	Indoors start at 1 l; then 20 l; then 1,000 l (12 days). Then outdoor start at 10,000 l (5 days)	Stocking density 150-200 ind. ml^{-1} and only a few days to first harvest
size/shape/construction materials	10,000 - 20,000 l square and rectangular concrete	200 l round plastic tank
temperature	20-25°C (shaded) or 28-32°C (without shade)	28 ± 2 °C (controlled)
feed	*N. oculata* or *Tetraselmis* + baker's or marine yeast	*T. suecica*
harvest procedure	25% of the culture is utilized to start another tank (stock), the remaining 75% is harvested for larval feed by gravity fed into a 50 μm bag that is 40 cm dia. and 1 m deep	daily harvest all rotifers from a 58 μm conical harvest net at rotifer harvest tank
causes of rotifer crashes	1. predators (protozoa) 2. quantity of algae 3. metabolic waste	water quality
location	outdoor	indoor
Labor requirements (person)	3-4	1

anticipate that as culturists gain experience using this system under different operating conditions that it will become even more applicable to culture needs. We anticipate changes in system size and capacity, use of additional culture species, and perhaps microprocessor control of the continuous culture system in order to achieve even greater control over production results.

Table 5.4 Economic annual analysis for the rotifer production of 4×10^6 rotifers day^{-1} in a closed-recirculating, continuous culture system.

Item	Description	Total cost (THB)
1. Durable materials and equipments	metering pumps, water pumps, air blower, tanks, acrylic tube, biofilter media, electrical equipments, UV sterilizers, ozonators, protein skimmers, PVC pipes, nylon net, etc.	60,000
2. Electricity[1]	system unit (300 × 12 kWh)[2]	14,400
	air supply/blower (269 × 12 kWh)[2]	12,912
3. Chemicals and fertilizers[1]		864
4. Seawater and tapwater[1]	seawater in system replace per 28 days	1,200
5. Labor	1 person (200 BHT/day)	73,000
Total investment cost		**162,376**
Total running cost excluding durable materials		**102,376**

[1]cost and waste materials

[2]electrical cost, 4 THB per kWh

A designed, developed and operated a closed-recirculating, continuous culture system to produce microalgae and rotifers in seawater (25‰) for larval fish culture. This new, automated system had three sub-components, including an alga culture component, a rotifer culture and storage with harvest component, and a water treatment and re-use component. After treatment using mechanical filtration, biofiltration, UV sterilization, ozonation and protein skimmers, water was re-used, thus nearly eliminating the need for seawater imports to the system. Our trials with the closed-recirculating, continuous culture system demonstrated that this culture system is capable of sustained and acceptable levels of microalgae and rotifer production for at least 28 days. During a continuous culture trial, microalga (*T. suecica*) production averaged 1.63×10^{10} cells day^{-1} while rotifer (*B. plicatilis*) production averaged 4.6×10^{6} $ind.day^{-1}$. Nitrogenous waste compounds were stable and well within acceptable levels, although nitrate and phosphate concentrations increased throughout the culture period, but did not negatively impact rotifer production. This 28 day culture trial demonstrated the benefits and advantages of this culture system compared with more commonly used batch or semi-continuous culture system for microalgae and rotifers.

CHAPTER VI

CONCLUSIONS

This study consisted of three experiments. For all topics related to this thesis, detailed literature reviews were performed and appropriate experiment were designed to achieve our research objectives. The first experiment (Chapter III) involved the study of L-carnitine effects on microalga (*T. suecica*), rotifer (*B. plicatilis*) and larval seabass (*L. calcarifer*). The second experiment (Chapter IV) was evaluated use of L-carnitine to enhance population growth of rotifer (*B. plicatilis*) in a recirculation culture system. The final experiment (Chapter V) included development of a closed-recirculating, continuous culture system for microalga (*T. suecica*) and rotifer (*B. plicatilis*).

Major findings from this work can be summarized as follows.

Chapter III

1. L-carnitine enrichment significantly increased the growth rates and accumulation of L-carnitine in *T. suecica*.

2. L-carnitine enrichment of rotifer by indirect method (emulsion emersion) caused significant increases in population growth, body size and L-carnitine content of rotifers. The greatest referred in terms of population growth was obtained with 1 mg l^{-1} L-carnitine, but body size and L-carnitine content were obtained with 10 mg l^{-1} L-carnitine.

L-carnitine content increased in rotifers, was most effectively achieved by emulsion emersion (direct method). Greatest increase in L-carnitine content was obtained with 10 mg l^{-1} L-carnitine emulsion for 4 h incubation with rotifers fed *T. suecica* acclimated in culture medium enriched with 10 mg l^{-1} L-carnitine (B10).

3. Rotifers enriched with emulsion at 10 mg l^{-1} L-carnitine for 4 h could be used for feeding seabass larvae.

Chapter IV

Use of L-carnitine in the recirculation system resulted in greater rotifers density than without L-carnitine. Use of L-carnitine in commercial aquaculture will not be very expensive since L-carnitine costs only about 6,000 BHT per kilogram (Lonza Biotec s.r.o., Czech Republic), dosage rate was low (1 mg l^{-1}), and rotifers were concentrated at high density during L-carnitine treatment. However, production reliability and practical experience of staff are the main factors limiting use of this culture system in Thailand. In Europe, North America and Japan, manpower cost is one of the main concerns, while cost of equipment is a main concern in countries such as Thailand.

Chapter IV

We designed, developed, and operated a closed-recirculating, continuous culture system to produce microalgae and rotifers in seawater (25‰) for larval fish culture. This new, automated system had three sub-components, including an alga culture component, a rotifer culture and storage with harvest component, and a water treatment and re-use component. After treatment using mechanical filtration, biofiltration, UV sterilization, ozonation and protein skimmers, water was re-used, thus nearly eliminating the need for seawater imports to the system. Our trials with the closed-recirculating, continuous culture system demonstrated that this culture system is capable of sustained and acceptable levels of microalgae and rotifer production for at least 28 days. During a continuous culture trial, microalga (*T. suecica*) production averaged 1.63×10^{10} cells day^{-1} while rotifer (*B. plicatilis*) production averaged 4.6×10^{6} ind.day^{-1}. Nitrogenous waste compounds were stable and well within acceptable levels, although nitrate and phosphate concentrations increased throughout the culture period, but did not negatively impact rotifer production. This 28 day culture trial demonstrated the benefits and advantages of this culture system compared with more commonly used batch or semi-continuous culture system for microalgae and rotifers.

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APPENDICES

APPENDIX A

ORIGINAL DATA FROM EXPERIMENT 1 IN CHAPTER III

Table A-1 Cell density of *T. suecica* ($\times 10^4$ cells ml^{-1}) enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}).

day	0	sd	0.1	sd	1	sd	10	sd
0	3.20	0.10	3.26	0.17	3.22	0.22	3.21	0.08
1	11.54	0.52	10.78	0.10	11.02	0.16	11.91	0.31
2	28.52	4.59	42.78	1.06	39.24	1.84	46.46	1.23
3	54.26	5.75	74.83	2.24	75.24	0.75	83.81	6.04
4	89.33	2.10	124.87	12.26	117.30	12.30	147.20	23.00
5	175.02	17.80	194.37	7.45	198.17	4.99	200.78	6.13
6	182.24	8.11	191.48	15.09	197.81	14.95	206.26	2.61
7	189.10	11.70	200.41	17.42	206.10	19.44	218.60	8.29
8	195.91	12.66	208.71	15.22	216.92	5.20	228.49	4.60
9	184.62	10.41	212.70	12.79	223.96	7.56	234.72	3.86
10	159.83	12.64	211.93	11.47	208.49	7.55	234.00	3.21
11	111.35	10.61	186.09	6.71	189.67	3.88	195.33	5.00
12	77.92	2.57	157.84	10.79	160.43	6.13	181.78	6.44

Table A-2 Specific growth rate and L-carnitine content in *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}).

	levels of L-carnitine (mg l^{-1})			
	0	0.1	1	10
Specific growth rate (day^{-1})	0.94 ± 0.06^{a}	1.08 ± 0.01^{b}	1.07 ± 0.02^{b}	1.11 ± 0.02^{b}
L-carnitine content (μg g^{-1} dw)	258.64 ± 11.47^{a}	440.03 ± 10.93^{b}	617.78 ± 17.88^{c}	1813.32 ± 88.52^{d}

Values (mean ± s.d. of three replicate) in the same row not sharing superscript are significantly different ($p<0.05$).

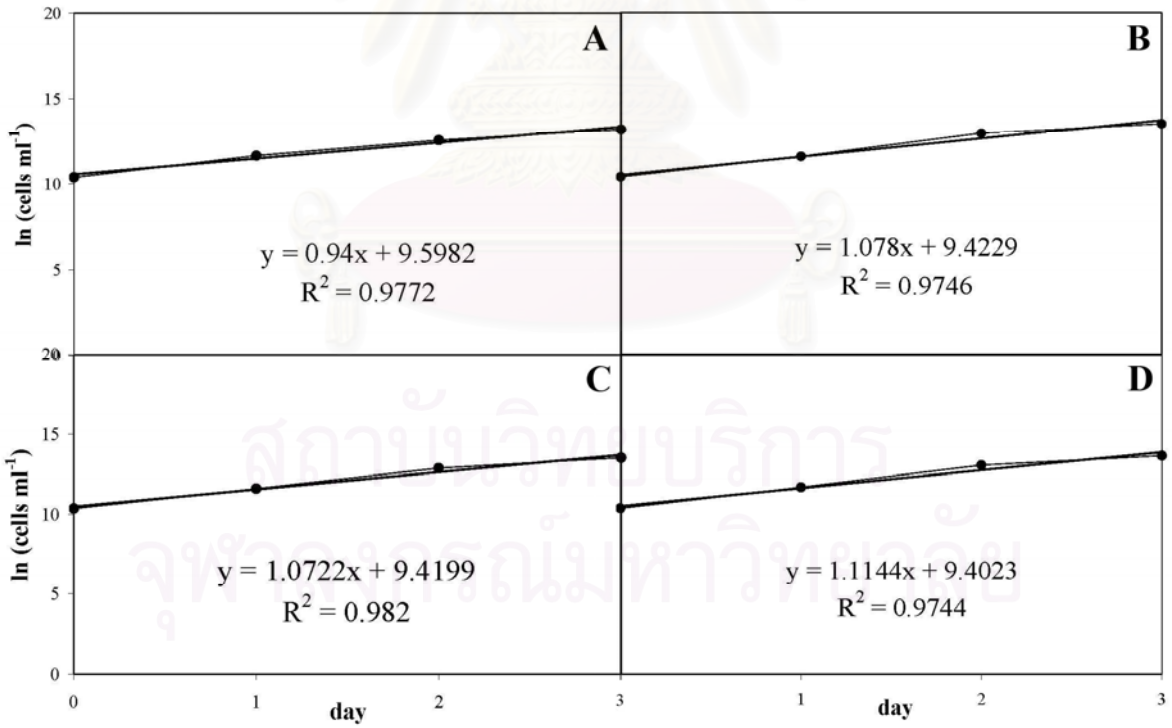


Figure A-1 Slope of ln N vs. time with linear regression value for estimated specific growth rate in *T. suecica* enriched with four levels of L-carnitine (A) 0 (B) 0.1 (C) 1 and (D) 10 mg l^{-1},(Values; mean of three replicate).

APPENDIX B

ORIGINAL DATA FROM EXPERIMENT 2 IN CHAPTER III

Table B-1 Density of *B. plicatilis* fed on *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 22 days.

day	**0**	**sd**	**0.1**	**sd**	**1**	**sd**	**10**	**sd**
0	100	0	100	0	100	0	100	0
1	92	14	74	11	88	21	88	12
2	161	36	103	26	156	19	136	8
3	175	10	135	17	164	13	125	22
4	148	38	154	17	201	31	151	7
5	196	28	129	13	188	36	198	23
6	277	16	198	19	313	51	277	10
7	253	31	202	5	235	17	246	85
8	285	64	262	26	278	59	286	34
9	317	55	149	47	386	34	327	71
10	266	70	200	32	467	48	464	82
11	418	109	289	36	457	43	479	9
12	341	39	271	23	520	49	539	20
13	360	90	314	41	543	78	507	20
14	408	95	312	14	609	100	616	55
15	392	25	252	27	696	108	592	97
16	414	73	328	17	642	31	541	41
17	403	48	273	26	626	58	491	14
18	429	54	289	22	599	82	442	130
19	335	54	230	19	462	61	332	37
20	210	103	134	11	237	69	216	39
21	204	21	202	41	265	78	208	33
22	215	65	220	27	234	64	230	59
Mean	**278**	49	**209**	23	**368**	50	**330**	39

Table B-2 Egg ratio of *B. plicatilis* fed on *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 22 days.

day	**0**	sd	**0.1**	sd	**1**	sd	**10**	sd
0	0.10	0.00	0.10	0.00	0.10	0.00	0.10	0.00
1	0.17	0.04	0.04	0.03	0.27	0.06	0.12	0.04
2	0.11	0.03	0.33	0.09	0.21	0.03	0.23	0.07
3	0.12	0.01	0.18	0.04	0.12	0.01	0.25	0.08
4	0.09	0.05	0.20	0.04	0.11	0.03	0.13	0.02
5	0.07	0.03	0.12	0.04	0.04	0.02	0.05	0.03
6	0.02	0.02	0.05	0.02	0.02	0.00	0.04	0.01
7	0.14	0.02	0.18	0.05	0.12	0.04	0.14	0.01
8	0.13	0.05	0.18	0.03	0.21	0.05	0.20	0.06
9	0.16	0.01	0.11	0.04	0.21	0.02	0.21	0.02
10	0.31	0.09	0.25	0.08	0.15	0.06	0.15	0.04
11	0.10	0.01	0.09	0.03	0.22	0.02	0.09	0.01
12	0.22	0.09	0.24	0.05	0.32	0.00	0.31	0.02
13	0.12	0.02	0.12	0.03	0.14	0.03	0.18	0.03
14	0.05	0.02	0.08	0.02	0.15	0.06	0.09	0.01
15	0.09	0.03	0.26	0.08	0.07	0.02	0.09	0.00
16	0.18	0.07	0.09	0.01	0.07	0.01	0.12	0.02
17	0.10	0.02	0.04	0.00	0.06	0.02	0.11	0.06
18	0.06	0.02	0.09	0.04	0.04	0.03	0.07	0.04
19	0.09	0.03	0.08	0.02	0.07	0.03	0.10	0.05
20	0.12	0.07	0.24	0.15	0.15	0.10	0.14	0.05
21	0.22	0.06	0.12	0.08	0.12	0.06	0.20	0.07
22	0.12	0.05	0.12	0.06	0.08	0.02	0.12	0.03
Mean	**0.13**	0.04	**0.15**	0.05	**0.13**	0.03	**0.14**	0.03

Table B-3 Average specific growth rate (SGR) and L-carnitine content in *B. plicatilis* fed on *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}).

	levels of L-carnitine (mg l^{-1})			
	0	0.1	1	10
Specific growth rate (day^{-1})	0.0333	0.0344	0.0370	0.0363
L-carnitine content (μg g^{-1} dw)	150.09±7.40[a]	212.54±8.05[b]	235.30±4.86[c]	349.75±9.59[d]

Values (mean ± SD of three replicate) in the same row not sharing superscript are significantly different ($p<0.05$).

Table B-4 Specific growth rate (SGR) of rotifers *B. plicatilis* fed on *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 22 days.

day	0	0.1	1	10
0	0	0	0	0
1	-0.08	-0.30	-0.13	-0.13
2	0.56	0.33	0.57	0.43
3	0.09	0.28	0.05	-0.08
4	-0.17	0.13	0.20	0.19
5	0.28	-0.18	-0.07	0.27
6	0.34	0.43	0.51	0.34
7	-0.09	0.02	-0.29	-0.12
8	0.12	0.26	0.17	0.15
9	0.11	-0.57	0.33	0.13
10	-0.17	0.29	0.19	0.35
11	0.45	0.37	-0.02	0.03
12	-0.20	-0.06	0.13	0.12
13	0.05	0.15	0.04	-0.06
14	0.12	0.00	0.12	0.20
15	-0.04	-0.22	0.13	-0.04
16	0.05	0.27	-0.08	-0.09
17	-0.03	-0.18	-0.03	-0.10
18	0.06	0.06	-0.05	-0.11
19	-0.25	-0.23	-0.26	-0.29
20	-0.47	-0.54	-0.67	-0.43
21	-0.03	0.41	0.11	-0.04
22	0.05	0.09	-0.12	0.10
mean	**0.03332**	**0.03436**	**0.03704**	**0.03629**

Table B-5 Average egg ratio of rotifers *B. plicatilis* fed on *T. suecica* enriched with four levels of L-carnitine (0, 0.1, 1 and 10 mg l^{-1}) during 22 days.

day	0	0.1	1	10
0	0.10	0.10	0.10	0.10
1	0.17	0.04	0.27	0.12
2	0.11	0.33	0.21	0.23
3	0.12	0.18	0.12	0.25
4	0.09	0.20	0.11	0.13
5	0.07	0.12	0.04	0.05
6	0.02	0.05	0.02	0.04
7	0.14	0.18	0.12	0.14
8	0.13	0.18	0.21	0.20
9	0.16	0.11	0.21	0.21
10	0.31	0.25	0.15	0.15
11	0.10	0.09	0.22	0.09
12	0.22	0.24	0.32	0.31
13	0.12	0.12	0.14	0.18
14	0.05	0.08	0.15	0.09
15	0.09	0.26	0.07	0.09
16	0.18	0.09	0.07	0.12
17	0.10	0.04	0.06	0.11
18	0.06	0.09	0.04	0.07
19	0.09	0.08	0.07	0.10
20	0.12	0.24	0.15	0.14
21	0.22	0.12	0.12	0.20
22	0.12	0.12	0.08	0.12
mean	**0.1259**	**0.1450**	**0.1334**	**0.1413**

Table B-6 L-carnitine content in rotifers *B. plicatilis* enriched with four different emulsions in relation to levels of L-carnitine and time.

	Time (h)	L-carnitine content (μg g^{-1} dw)			
		0	**0.1**	**1**	**10**
B0	1	118.05±9.89^{a}	124.54±5.69^{a}	179.57±3.99^{b}	225.15±3.13^{c}
B0	2	134.03±5.61^{a}	132.86±4.12^{a}	196.21±1.51^{b}	291.56±3.17^{c}
B0	3	131.10±7.43^{a}	129.91±4.67^{a}	191.32±3.59^{b}	358.77±2.81^{c}
B0	4	120.71±4.91^{a}	138.75±4.18^{b}	235.93±3.13^{c}	491.59±6.67^{d}
B10	1	333.78±4.64^{a}	334.57±5.35^{a}	339.56±6.46^{a}	384.17±5.65^{b}
B10	2	313.21±6.24^{a}	320.56±4.17^{a}	324.87±6.96^{a}	420.56±18.33^{b}
B10	3	295.32±5.17^{a}	307.19±7.09^{b}	321.95±2.96^{c}	455.37±5.63^{d}
B10	4	287.59±7.42^{a}	299.32±6.54^{a}	317.66±5.60^{b}	518.07±9.26^{c}

Values (mean ± SD of three replicate) in the same row not sharing superscript are significantly different ($p<0.05$).

Table B-7 L-carnitine content in rotifers *B. plicatilis* enriched with four different emulsions in relation to levels of L-carnitine and time.

	Time (h)	L-carnitine content (μg g^{-1} dw)			
		0	**0.1**	**1**	**10**
B0	1	118.05±9.89^{a}	124.54±5.69^{a}	179.57±3.99^{a}	225.15±3.13^{a}
B0	2	134.03±5.61^{b}	132.86±4.12^{ab}	196.21±1.51^{b}	291.56±3.17^{b}
B0	3	131.10±7.43^{ab}	129.91±4.67^{ab}	191.32±3.59^{b}	358.77±2.81^{c}
B0	4	120.71±4.91^{ab}	138.75±4.18^{b}	235.93±3.13^{c}	491.59±6.67^{d}
B10	1	333.78±4.64^{c}	334.57±5.35^{c}	339.56±6.46^{b}	384.17±5.65^{a}
B10	2	313.21±6.24^{b}	320.56±4.17^{b}	324.87±6.96^{a}	420.56±18.33^{b}
B10	3	295.32±5.17^{a}	307.19±7.09^{a}	321.95±2.96^{a}	455.37±5.63^{c}
B10	4	287.59±7.42^{a}	299.32±6.54^{a}	317.66±5.60^{a}	518.07±9.26^{d}

Values (mean ± SD of three replicate) in the same column not sharing superscript are significantly different ($p<0.05$).

Table B-8 Two-way analysis of variance performed on the L-carnitine content in rotifers *B. plicatilis* (B0) enriched with four different emulsions in relation to levels of L-carnitine and time.

Source of variation	df	SS	MS	F
L-carnitine content				
L-carnitine level (A)	3	363226.366	121075.455	4718.636[a]
Time (B)	3	45341.011	15113.670	589.020[a]
(A) × (B)	9	77476.341	8608.482	335.496[a]
Error	32	821.088	25.659	

[a] = $p<0.05$

Table B-9 Two-way analysis of variance performed on the L-carnitine content in rotifers *B. plicatilis* (B10) enriched with four different emulsions in relation to levels of L-carnitine and time.

Source of variation	df	SS	MS	F
L-carnitine content				
L-carnitine level (A)	3	150094.578	50031.526	892.384[a]
Time (B)	3	931.874	310.625	5.540[a]
(A) × (B)	9	35076.287	3897.365	69.515[a]
Error	32	1794.080	56.065	

[a] = $p<0.05$

APPENDIX C

ORIGINAL DATA FROM EXPERIMENT 3 IN CHAPTER III

Table C-1 Total length, weight, survival and L-carnitine content of seabass, *L. calcarifer* larvae in six treatments.

Treatment	O DPH		13 DPH			
	Length (mm)	**Weight (mg)**	**Length (mm)**	**Weight (mg)**	**Survival (%)**	**L-carnitine (μg g^{-1} dw)**
1	1.06±0.23	0.04±0.01	3.15±0.38^{a}	0.53±0.22^{a}	29.02±1.13^{a}	n.d.
2	1.06±0.23	0.04±0.01	3.23±0.52^{ab}	0.84±0.10^{ab}	33.08±1.36^{b}	n.d.
3	1.06±0.23	0.04±0.01	3.50±0.59^{c}	0.99±0.23^{b}	32.06±0.40^{b}	71.92±9.36^{b}
4	1.06±0.23	0.04±0.01	3.46±0.58^{bc}	1.03±0.40^{b}	36.72±0.97^{c}	29.56±3.79^{a}
5	1.06±0.23	0.04±0.01	3.80±0.57^{d}	0.94±0.28^{b}	39.46±1.35^{d}	31.26±6.24^{a}
6	1.06±0.23	0.04±0.01	3.85±0.48^{d}	1.08±0.80^{b}	36.12±2.03^{c}	78.51±6.62^{b}

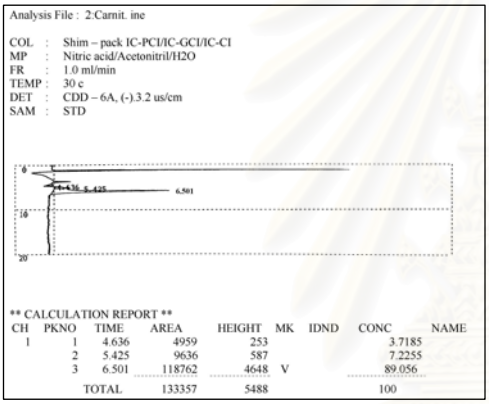
Values (mean±SD of three replicate) in the same row not sharing a common superscript are significantly different ($p<0.05$).

n.d., not detected

APPENDIX D

CHROMATOGRAM FROM HPLC ANALYSIS OF L-CARNITINE

1. Standard



Analysis File : 2:Carnit. ine

COL : Shim – pack IC-PCI/IC-GCI/IC-CI
MP : Nitric acid/Acetonitril/H2O
FR : 1.0 ml/min
TEMP : 30 c
DET : CDD – 6A, (-).3.2 us/cm
SAM : STD

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDND	CONC	NAME
1	1	4.636	4959	253			3.7185	
	2	5.425	9636	587			7.2255	
	3	6.501	118762	4648	V		89.056	
		TOTAL	133357	5488			100	

Figure D-1 HPLC Chromatogram of L-carnitine of Standard

2. Microalgae *T. suecica*

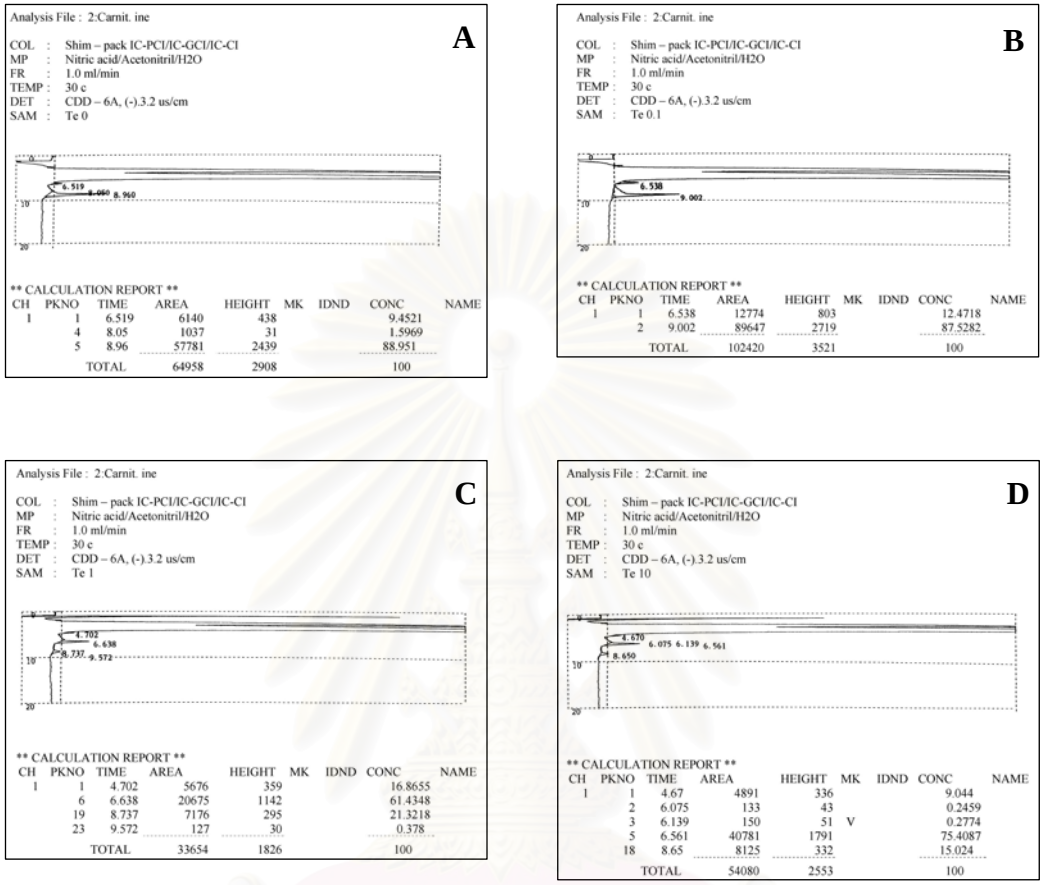


Figure D-2 HPLC Chromatogram of L-carnitine of (A) *T. suecica* without L-carnitine, (B) *T. suecica* enriched with 0.1 mg l^{-1} L-carnitine, (C) *T. suecica* enriched with 1 mg l^{-1} L-carnitine, (D) *T. suecica* enriched with 10 mg l^{-1} L-carnitine.

3. Rotifer *B. plicatilis*

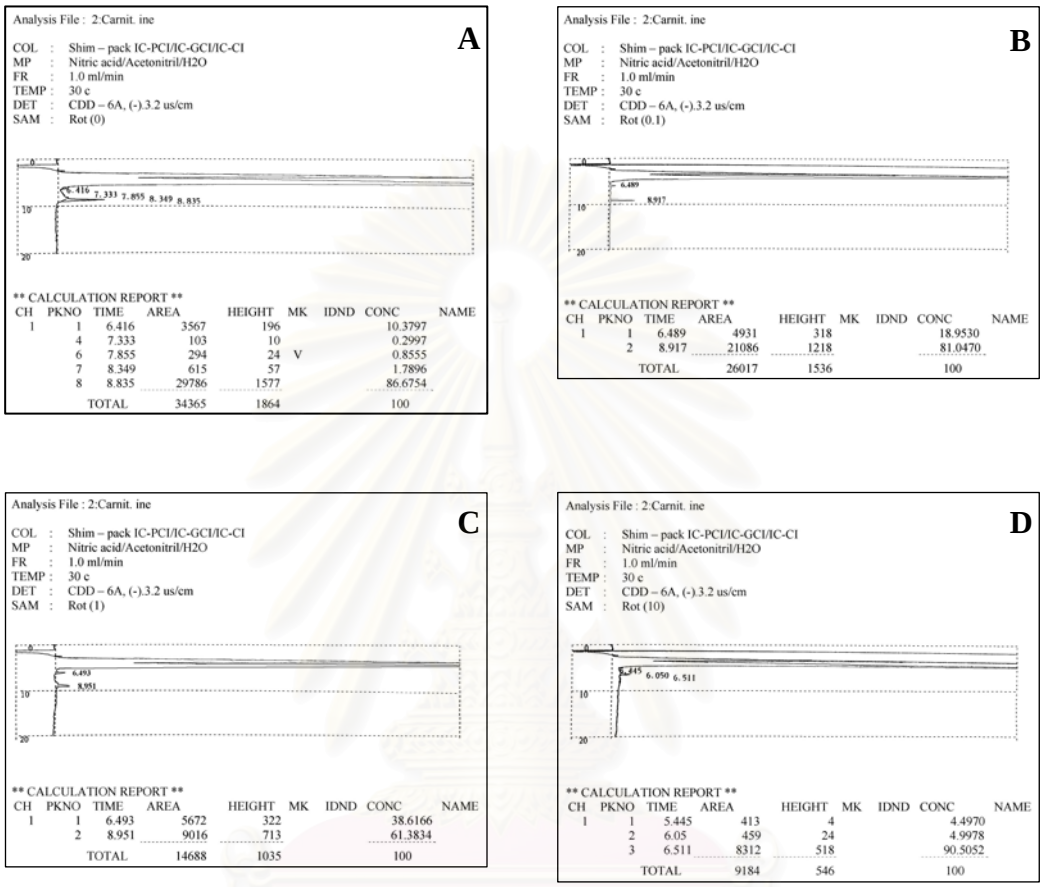


Figure D-3 HPLC Chromatogram of L-carnitine of (A) Rotifer without L-carnitine, (B) Rotifer fed with *T. suecica* enriched with 0.1 mg l^{-1} L-carnitine, (C) Rotifer fed with *T. suecica* enriched with 1 mg l^{-1} L-carnitine and (D) Rotifer fed with *T. suecica* enriched with 10 mg l^{-1} L-carnitine

4. Emulsion (Rotifer fed *T. suecica* without L-carnitine)

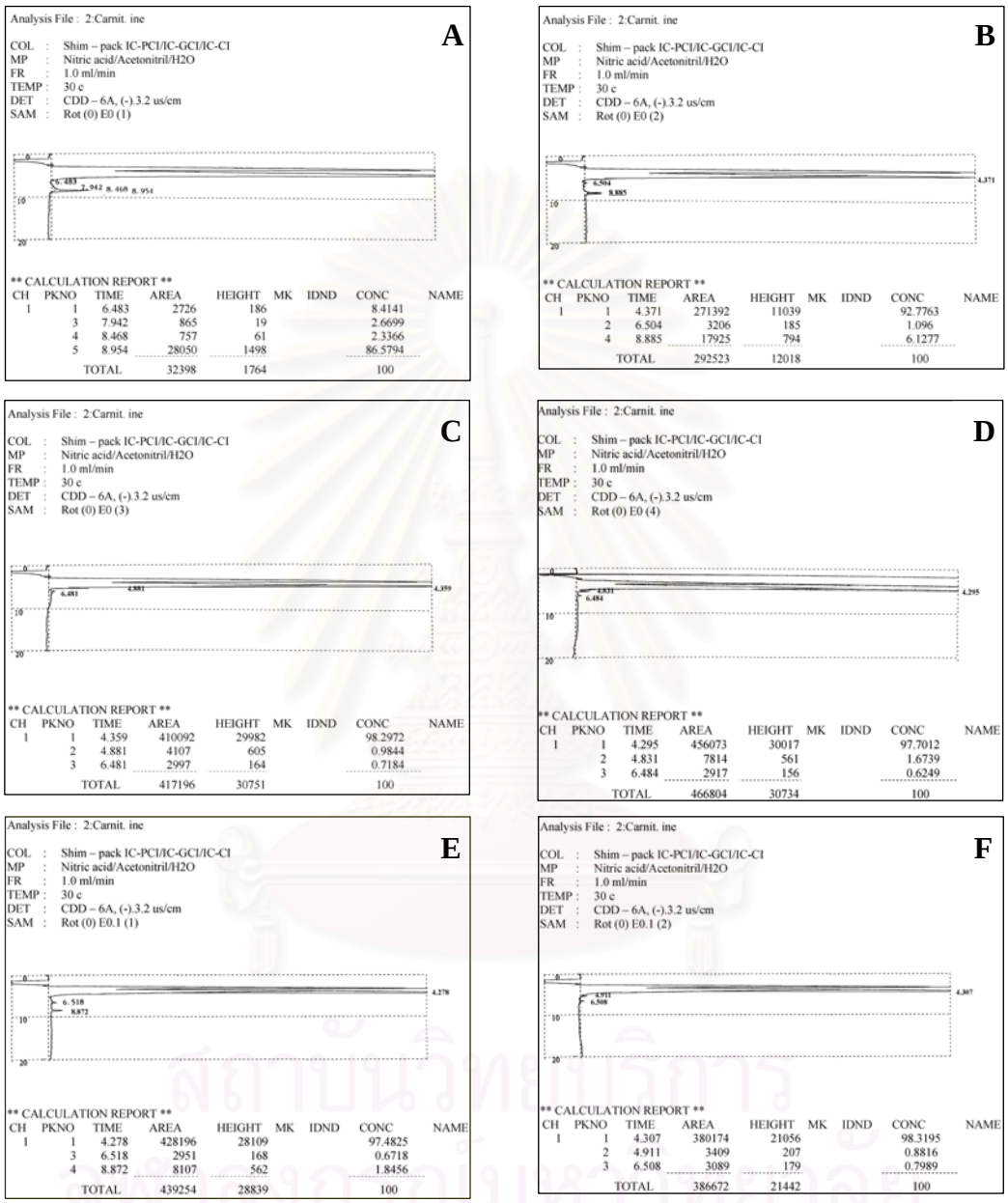


Figure D-4 HPLC Chromatogram of L-carnitine of (A) B0 enriched with emulsion without L-carnitine for 1 h., (B) B0 enriched with emulsion without L-carnitine for 2 h., (C) B0 enriched with emulsion without L-carnitine for 3 h., (D) B0 enriched with emulsion without L-carnitine for 4 h., (E) B0 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 1 h. and (F) B0 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 2 h.

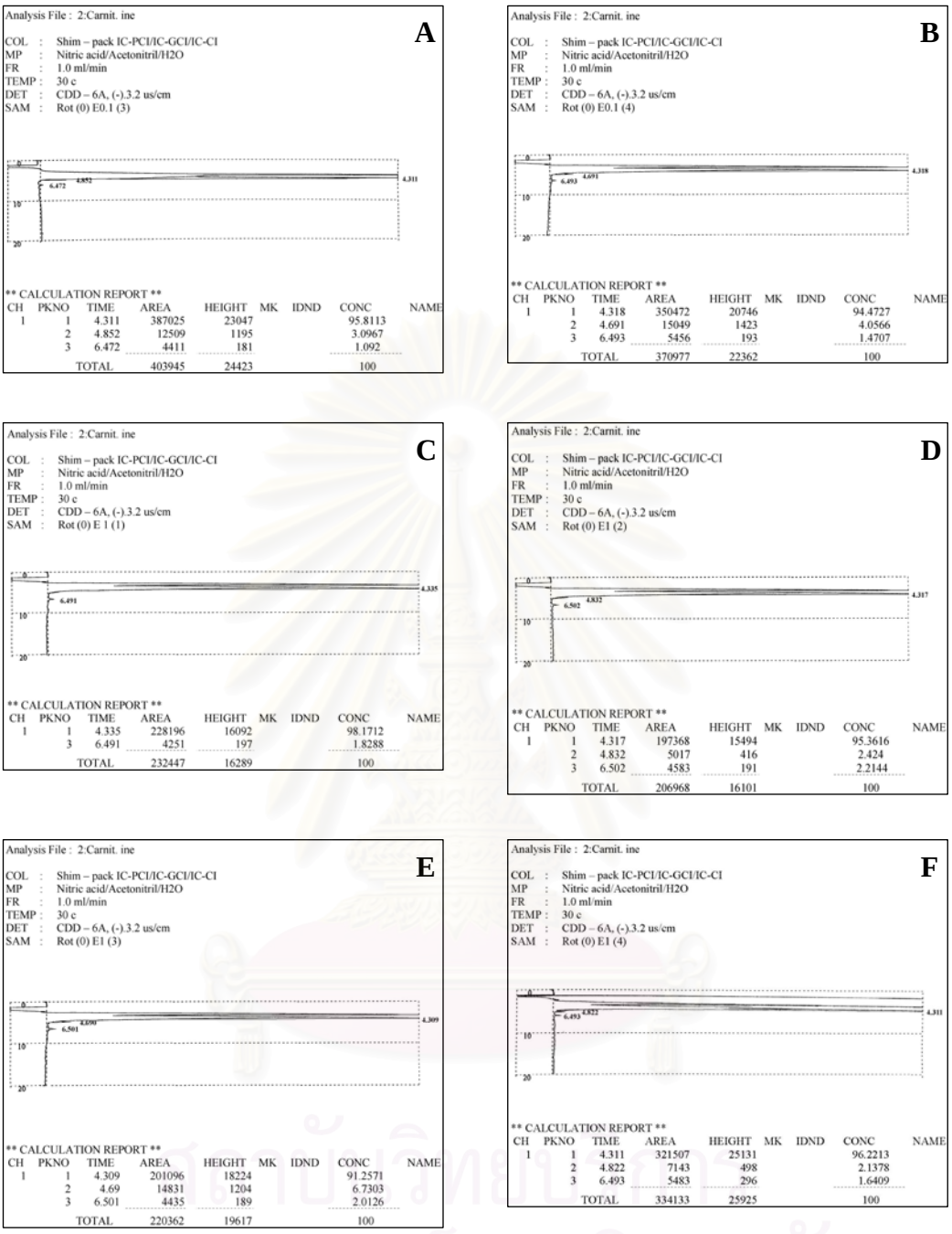


Figure D-5 HPLC Chromatogram of L-carnitine of (A) B0 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 3 h., (B) B0 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 4 h., (C) B0 enriched with emulsion with 1 mg l^{-1} L-carnitine for 1 h., (D) B0 enriched with emulsion with 1 mg l^{-1} L-carnitine for 2 h., (E) B0 enriched with emulsion with 1 mg l^{-1} L-carnitine for 3 h., (F) B0 enriched with emulsion with 1 mg l^{-1} L-carnitine for 4 h.

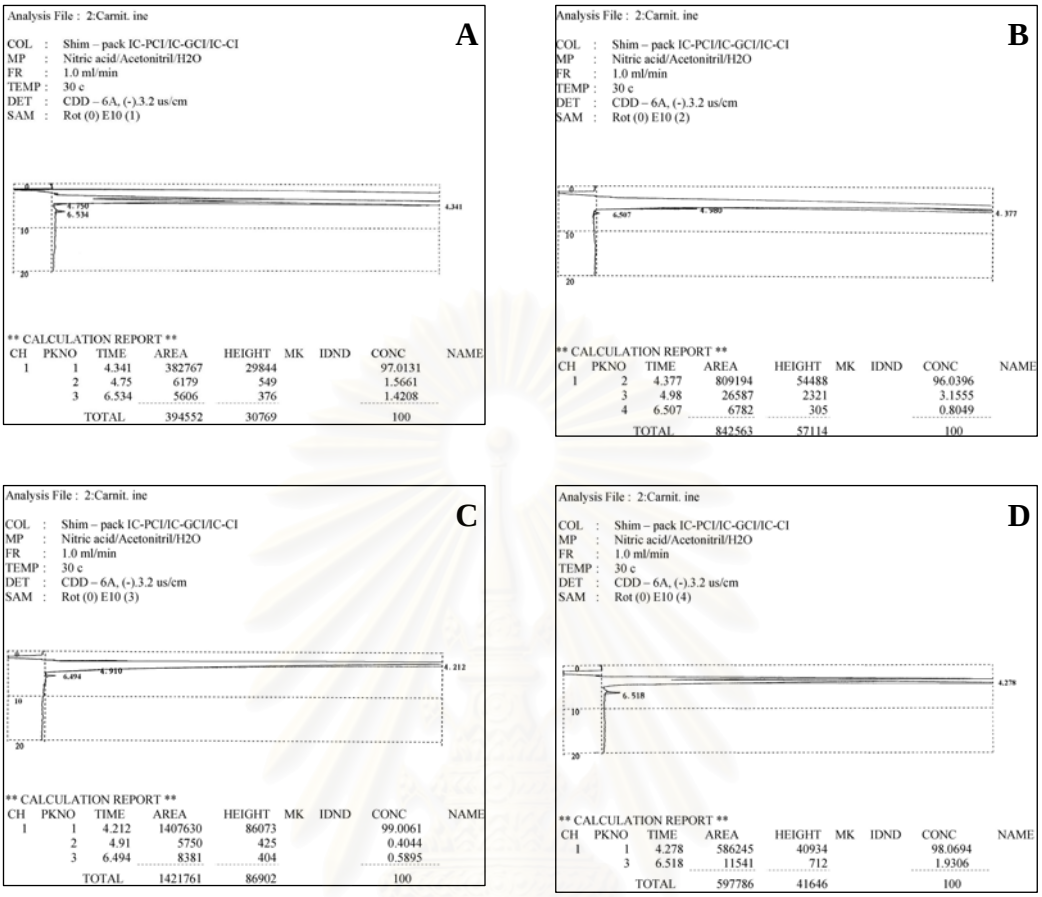


Figure D-6 HPLC Chromatogram of L-carnitine of (A) B0 enriched with emulsion with 10 mg l^{-1} L-carnitine for 1 h., (B) B0 enriched with emulsion with 10 mg l^{-1} L-carnitine for 2 h., (C) B0 enriched with emulsion with 10 mg l^{-1} L-carnitine for 3 h., (D) B0 enriched with emulsion with 10 mg l^{-1} L-carnitine for 4 h.

5. Emulsion (Rotifer fed *T. suecica* with L-carnitine)

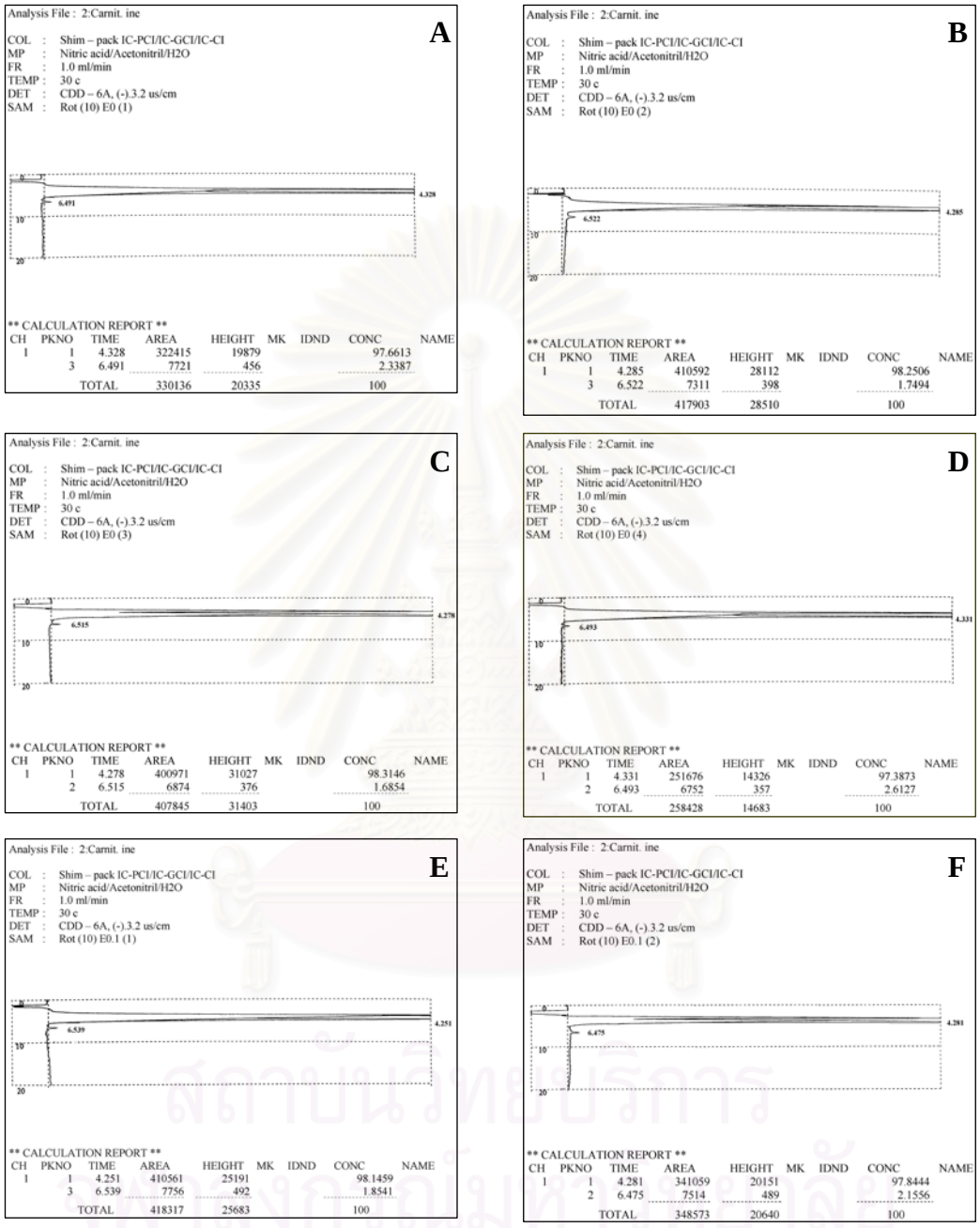


Figure D-7 HPLC Chromatogram of L-carnitine of (A) B10 enriched with emulsion without L-carnitine for 1 h., (B) B10 enriched with emulsion without L-carnitine for 2 h., (C) B10 enriched with emulsion without L-carnitine for 3 h., (D) B10 enriched with emulsion without L-carnitine for 4 h, (E) B10 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 1 h and (F) B10 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 2 h.

Figure D-8 HPLC Chromatogram of L-carnitine of (A) B10 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 3 h, (B) B10 enriched with emulsion with 0.1 mg l^{-1} L-carnitine for 4 h, (C) B10 enriched with emulsion with 1 mg l^{-1} L-carnitine for 1 h, (D) B10 enriched with emulsion with 1 mg l^{-1} L-carnitine for 2 h, (E) B10 enriched with emulsion with 1 mg l^{-1} L-carnitine for 3 h and (F) B10 enriched with emulsion with 1 mg l^{-1} L-carnitine for 4 h.

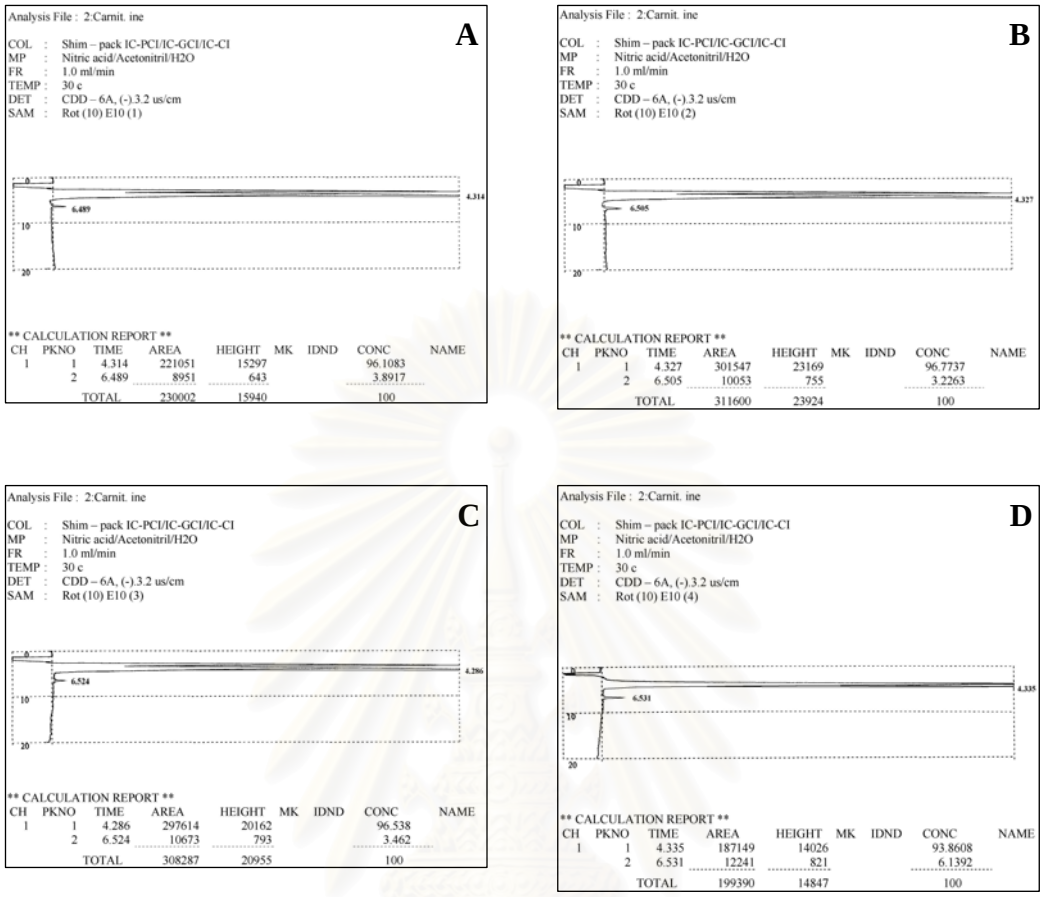


Figure D-9 HPLC Chromatogram of L-carnitine of (A) B10 enriched with emulsion with 10 mg l^{-1} L-carnitine for 1 h., (B) B10 enriched with emulsion with 10 mg l^{-1} L-carnitine for 2 h., (C) B10 enriched with emulsion with 10 mg l^{-1} L-carnitine for 3 h, and (D) B10 enriched with emulsion with 10 mg l^{-1} L-carnitine for 4 h.

APPENDIX E

RAW DATA FROM WATER QUALITY IN CHAPTER IV

Table E-1 Ammonia, nitrite and nitrate concentrations in a recirculation system with 1 mg l^{-1} L-carnitine exposure treatment and control treatment.

Day	Control (mg l^{-1})				L-carnitine Treatment (mg l^{-1})			
	NH_4^+	NO_2^-	NO_3^-	DO.	NH_4^+	NO_2^-	NO_3^-	DO.
0	0.5	0	10	9.11	0.5	0	10	9.07
1	1.0	0.3	10	8.71	1.0	0.3	10	8.74
2	2.5	0.75	20	8.83	2.5	0.75	20	8.88
3	3.5	1.05	20	8.76	3.5	1.05	20	8.54
4	5.0	1.5	25	8.80	5.0	1.5	25	8.59
5	5.0	1.5	30	8.95	6.7	1.5	30	8.73
6	5.0	1.5	30	8.27	7.5	1.5	30	8.51
7	5.0	1.83	30	8.22	7.5	1.83	30	8.18
8	5.0	1.5	30	8.31	7.5	1.5	30	7.97
9	5.0	1.5	30	8.12	7.5	1.5	30	8.00

APPENDIX F

METHOD FOR WATER QUALITY ANALYSIS IN CHAPTER V

1. Determination of Ammonium

(Alternative method, Parson et al. 1989)

1.1 Reagents

1.1.1 De-ionized water: This water should be prepared fresh for use.

1.1.2 Phenol solution: Dissolve 20 g of analytical grade phenol in 200 ml of 95% v/v ethyl alcohol.

1.1.3 Sodium nitroprusside solution: Dissolve 1.0 g of sodium nitroprusside, $Na_2[Fe(CN)_5NO] \cdot 2H_2O$, in 200 ml of de-ionized water. Store in a dark glass bottle; the solution is stable for at least a month.

1.1.4 Alkaline reagent: Dissolve 100 g of sodium citrate and 5 g of sodium hydroxide in 500 ml of de-ionized water. The solution is stable indefinitely.

1.1.5 Sodium hypochlorite solution: Use commercially available hypochlorite (e.g. “Chlorox”) which should be about 1.5 N. The solution decomposes slowly and should be checked periodically.

1.1.6 Oxidizing solution: Mix 100 ml of reagent 4 and 25 ml of reagent 5. Keep stoppered while not in use and prepare fresh every day.

1.2 Experimental procedure

1.2.1 Filter the water sample through GFC filter paper. Pipette 50 ml of filtered sample into a 50 ml Erlenmeyer flask.

1.2.2 Add 2 ml of phenol solution, swirl to mix, and then add in sequence 2 ml of nitroprusside and 5 ml of oxidizing solution (Note c) ; mix after each addition by swirling the flasks.

1.2.3 Allow the flasks to stand to room temperature (20-27 ºC) for 1 hr. The top of the flask should be covered with parafilm during this period. The color is stable for 24 hr after the reaction period.

1.2.4 The blue indophenol color is developed in a water sample and the absorbance of the sample is measured at 640 nm. The concentration of ammonia can be estimated by reference to calibration graph.

1.2.5 A calibration graph must be prepared as described in step 1.2.1 to 1.2.4. The blue indophenol color is developed in solutions with know standard concentrations of ammonium and the intensity of the blue indophenol color in this series of standard solutions is evaluated by spectrophotometer at 640 nm. Absorbance values are plotted on the Y – axis versus their respective concentrations of ammonium on the X – axis to give a calibration graph.

2. Determination of Nitrite

(Parson et al. 1989)

2.1 Reagents

2.1.1 Sulfanilamide solution

2.1.2 N-(1-Naphthyl)–ethylenediamine dihydrochloride solution

2.2 Experimental procedure

2.2.1 Filter the water sample through GFC filter paper. Pipette 50 ml of filtered sample into a 50 ml Erlenmeyer flask.

2.2.2 Add 1.0 ml of sulfanilamide solution from an automatic pipette to each 50-ml sample, mix and allow the reagent to react for more than 2 min but less than 10 min to assure a complete reaction.

2.2.3 Add 1.0 ml of naphthylethylenediamine reagent and mix immediately. Between 10 min and 2 hr afterwards, measure the extinction of the solution in a 10-cm cuvette at a wavelength of 543 nm.

2.2.4 The concentration of nitrite can then be estimated by reference to the calibration graph.

2.2.5 To use the highly colored azo dye to estimate 4 nitrite concentration in water samples, a calibration graph must be prepared as described in step 2.2.1 to 2.2.3 The bright pink color (azo compound) is developed in solutions with know standard concentrations of nitrite and the intensity of the bright pink color in this series of standard solutions is evaluated by spectrophotometer at 543 nm. Absorbance values are plotted on the Y – axis versus their respective concentrations of nitrite on the X – axis to give a calibration graph.

3. Determination of Phosphate

(Parson et al. 1989)

3.1 Reagents

3.1.1 Ammonium molybdate solution: Dissolve 15 g of analytical reagent grade ammonium paramolybdate $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 500 ml of distilled water. Store in plastic bottle away from direct sunlight. The solution is stable.

Sulfuric acid solution: Add 140 ml of concentrated analytical reagent quality sulfuric acid to 900 ml of distilled water. Allow the solution to cool and store it in a glass bottle.

Ascorbic acid solution: Dissolve 27 g of ascorbic acid in 500 ml of distilled water. Store the solution in a plastic bottle frozen solid in the freezer. The solution is stable for many months but should not be kept at room temperature for more than one week.

Potassium antimonyl-tartrate solution: Dissolve 0.34 g of potassium antimonyl-tartrate (tartar emetic) in 250 ml of water, warming if necessary. Store in a glass or plastic bottle. The solution is stable for many months.

Mixed reagent: Mix together 100 ml ammonium molybdate, 250 ml

sulfuric acid, 100 ml ascorbic acid, and 50 ml of potassium antimonyl-tartrate solutions. Prepare this reagent when needed and discard any excess. Do not store, the quantity is suitable for about 50 samples.

3.2 Experimental procedure

3.2.1 Warm the samples to room temperature (15-30 ºC)

3.2.2 To a 100 ml sample, add 10 ml of mixed reagent using a syringe-type pipette and mix at once.

3.2.3 After 5 min and preferably within the first 2-3 hr, measure the absorbance using a wavelength of 885 nm.

3.2.4 The concentration of nitrite can then be estimated by reference to the calibration graph.

3.2.5 Dissolve 0.816 g of anhydrous potassium dihydrogen phosphate in 1 l of distilled water. Store in a dark bottle, the solution is stable for many months. Dilute 10 ml of the standard to 1 l with distilled water. Pipette 5 ml of dilute standard into each of three Erlenmeyer flasks and make up to 100 ml with distilled water. Carry out the procedure in steps 3.2.1 to 3.2.4. Absorbance values are plotted on the Y – axis versus their respective concentrations of nitrite on the X – axis to give a calibration graph.

4. Determination of Nitrate

(APHA et al. 1992)

4.1 Scope and Application

This method is applicable to the analysis of drinking, surface and saline waters, domestic and industrial wastes. Modification can be made to remove or correct for turbidity, color, salinity, or dissolved organic compounds in the sample. The applicable range of concentrations is 0.1 to 2 mg NO_3-N/liter. This method is based upon the reaction of the nitrate ion with brucine sulfate in a 13 N H_2SO_4

solution at a temperature of 100°C. The color of the resulting complex is measured at 410 nm. Temperature control of the color reaction is extremely critical.

4.2 Reagents

4.2.1 Brucine-sulfanilic acid reagent: Dissolve 1 g brucine sulfate [$(C_{23}H_{26}N_2O_4)2\ H_2SO_4\ 7H_2O$] and 0.1 g sulfanilic acid ($NH_2C_6H_4SO_3H\ H_2O$) in 70 ml hot distilled water. Add 3 ml conc. HCl, cool, mix and dilute to 100 ml with distilled water. Store in a dark bottle at 5 °C. This solution is stable for several months; the pink color that develops slowly does not effect its usefulness.

4.2.2 Sulfuric acid solution: Carefully add 500 ml conc. H_2SO_4 to 125 ml distilled water.

4.2.3 Sodium chloride solution (30%): Dissolve 300 g NaCl in distilled water and dilute to 1 liter.

4.2.4 Potassium nitrate stock solution: 1.0 ml = 0.1 mg NO_3-N. Dissolve 0.7218 g anhydrous potassium nitrate (KNO_3) in distilled water and dilute to 1 liter in a volumetric flask. Preserve with 2 ml chloroform per liter. This solution is stable for at least 6 months.

4.2.5 Potassium nitrate standard solution: 1.0 ml = 0.001 mg NO_3-N. Dilute 10 ml of the stock solution (4.2.4) to 1 liter in a volumetric flask. This standard solution should be prepared fresh weekly.

4.3 Experimental procedure

4.3.1 Filter the water sample through GFC filter paper. Pipette 10 ml of standards and samples or an aliquot of the samples diluted to 10.0 ml - into the sample tubes.

4.3.2 Add 2 ml of the 30% sodium chloride solution to the reagent blank, standards and samples. Mix contents of tubes by swirling and place rack in cold water bath (0 – 10 °C).

4.3.3 Pipette 10.0 ml of sulfuric acid solution into each tube and mix by swirling. Allow tubes to come to thermal equilibrium in the cold bath. Be sure that temperatures have equilibrated in all tubes before continuing.

4.3.4 Add 0.5 ml brucine-sulfanilic acid reagent to each tube and carefully mix by swirling, then place the rack of tubes in the 100°C water bath for exactly 25 minutes.

4.3.5 Remove rack of tubes from the hot water bath and immerse in the cold water bath and allow to reach thermal equilibrium (20-25 °C).

4.3.6 Read absorbance against the reagent blank at 410 nm.

4.3.7 Obtain a standard curve by plotting the absorbance of standards run by the above procedure against mg NO_3-N/L. (The color reaction does not always follow Beer's law).

BIOGRAPHY

Miss Chatchadapon Sananurak was born on July 17, 1977 in Chainat Province. She graduated with a Bachelor and Master degree in Marine Science from Department of Marine Science, Faculty of Science, Chulalongkorn University in 1998 and 2002, respectively. In 2003, she started the Ph.D. Program of study at Department of Marine Science, Faculty of science, Chulalongkorn University.