Acclimatization and Application of Biofilters for Nitrogen Removal in Marine Recirculating Shrimp Culture System

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การบ่มเชื้อและการประยุกต์ใช้ตัวกรองชีวภาพสำหรับกำจัดไนโตรเจนในระบบการเลี้ยงกุ้งน้ำเค็มแบบปิด

น.ส.เพ็ญพิชา สท้านวัตร

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เพ็ญพิชา สท้านวัตร : การบ่มเชื้อและการประยุกต์ใช้ตัวกรองชีวภาพสำหรับกำจัดไนโตรเจนในระบบการเลี้ยงกุ้งน้ำเค็มแบบปิด. (Acclimatization and Application of Biofilters for Nitrogen Removal in Marine Recirculating Shrimp Culture System) อ.ที่ปรึกษาหลัก : รศ. ดร.วิบูลย์ลักษณ์ พึ่งรัศมี, อ.ที่ปรึกษาร่วม : ดร.สรวิศ เผ่าทองศุข

งานวิจัยนี้เป็นการศึกษาการบำบัดไนโตรเจนในระบบเลี้ยงสัตว์น้ำเค็มแบบปิดผ่านกระบวนการไนทริฟิเคชันและดีไนทริฟิเคชันแบบต่อเนื่องโดยอาศัยตัวกรองชีวภาพแบบติดตั้งภายในบ่อเลี้ยง แบ่งการทดลองออกเป็น 2 ช่วง ช่วงแรกเป็นการประเมินผลกระทบของค่าความเค็ม (5, 15 และ 25 พีเอสยู) และความหนาแน่นสัตว์น้ำ (50 และ 100 ตัว/ตร.ม.) ต่อประสิทธิภาพไนทริฟิเคชัน ดีไนทริฟิเคชัน และความหลากหลายของจุลชีพบนฟิล์มชีวภาพ นอกจากนี้ยังทำการเปรียบเทียบประสิทธิภาพการบำบัดไนโตรเจนของตัวกรองชีวภาพแบบเส้นใยและแบบแผ่นใย จากการทดลองพบว่ากระบวนการไนทริฟิเคชันถูกกระตุ้นในระบบความเค็มต่ำ (5 พีเอสยู) ที่มีการเลี้ยงกุ้งแบบหนาแน่น (100 ตัว/ตร.ม.) โดยมีอัตราไนทริฟิเคชันสูงสุดเท่ากับ 100.42±5.97 มก.-ไนโตรเจน/ตร.ม./วัน (สำหรับตัวกรองแบบเส้นใย) และ 145.43±1.17 มก.-ไนโตรเจน/ตร.ม./วัน (สำหรับตัวกรองแบบแผ่นใย) ตามลำดับ ในขณะที่อัตราดีไนทริฟิเคชันสูงสุดเกิดขึ้นในระบบการเลี้ยงสัตว์น้ำแบบหนาแน่น เท่ากับ 81.86±4.40 มก.-ไนโตรเจน/ตร.ม./วัน ที่ความเค็ม 25 พีเอสยู (สำหรับตัวกรองแบบเส้นใย) และ 165.80±50.17 มก.-ไนโตรเจน/ตร.ม./วัน ที่ความเค็ม 5 พีเอสยู (สำหรับตัวกรองแบบแผ่นใย) ตามลำดับ ผลจากการถอดรหัสพันธุกรรมด้วยเทคนิค Illumina MiSeq แสดงให้เห็นว่า *Proteobacteria* และ *Bacteroidetes* เป็นกลุ่มประชากรแบคทีเรียที่โดดเด่นในทุกชุดการทดลอง ในขณะที่กลุ่มไนทริฟายเออร์และดีไนทริฟายเออร์ที่พบในระบบความเค็มต่ำมีความแตกต่างจากกลุ่มที่พบในระบบที่มีค่าความเค็มปานกลาง (15 พีเอสยู) และค่าความเค็มสูง (25 พีเอสยู) สำหรับการทดลองช่วงที่ 2 เป็นการประเมินประสิทธิภาพของตัวกรองชีวภาพแบบเส้นใย และติดตามการเปลี่ยนแปลงของกลุ่มประชากรจุลชีพระหว่างการเดินระบบเลี้ยงสัตว์น้ำเค็มแบบปิดระยะยาวเป็นเวลา 210 วัน ที่ค่าความเค็ม 25 พีเอสยู และความหนาแน่นกุ้งเริ่มต้น 1 กก./ลบ.ม. จากการทดลองพบว่ากระบวนการไนทริฟิเคชันเกิดขึ้นอย่างสมบูรณ์หลังจากการบ่มเชื้อบนตัวกรองชีวภาพควบคู่กับการเลี้ยงกุ้งเป็นเวลาประมาณ 2 เดือน โดยตลอดระยะเวลาการเดินระบบไนทริฟิเคชันและดีไนทริฟิเคชันแบบต่อเนื่อง 2 รอบ ปริมาณแอมโมเนียและไนไทรต์ถูกควบคุมให้อยู่ในระดับมาตรฐาน ในขณะที่ไนเทรตถูกบำบัดอย่างสมบูรณ์หลังจากการเก็บเกี่ยวผลผลิตกุ้ง ภายใต้สภาวะแอนอ็อกซิกที่มีการเติมเมทานอลที่อัตราส่วนซีโอดี:ไนเทรต-ไนโตรเจน เท่ากับ 5:1 ผลของจุลชีพแสดงให้เห็นว่า Uncultured bacterium clone PI1AB88 และ Uncultured bacterium clone SF_NOB_Cd08 เป็นกลุ่มประชากรหลักที่มีบทบาทในการออกซิไดซ์แอมโมเนียและไนไทรต์ตามลำดับ ในขณะที่ *Methylophaga* และ *Methylotenera* เป็นกลุ่มดีไนทริฟายอิงแบคทีเรียที่พบระหว่างการบำบัดดีไนทริฟิเคชันภายใต้สภาวะแอนอ็อกซิก

สาขาวิชา วิศวกรรมสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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Penpicha Satanwat : Acclimatization and Application of Biofilters for Nitrogen Removal in Marine Recirculating Shrimp Culture System. Advisor: Assoc. Prof. Wiboonluk Pungrasmi, Ph.D. Co-advisor: Sorawit Powtongsook, Ph.D.

This research involved in the complete nitrogen removal in marine recirculating aquaculture system (RAS) through sequential nitrification and denitrification processes using internal biofilter within a single tank. The study was divided into two experimental parts. The first study was to estimate the effects of salinity (5, 15 and 25 PSU) and stocking density (50 and 100 shrimp m^{-2}) on nitrification and denitrification efficiencies, as well as on microbial diversity in the biofilm. Also, the nitrogen removal efficiencies of fibrous Biocord^{TM} biofilter and Japanese filter mat were compared. Results showed that the nitrification was stimulated in low-salinity (5 PSU) system with intensive (100 shrimp m^{-2}) shrimp cultivation at the maximum ammonia removal rates of 100.42±5.97 mg-N m^{-2} day^{-1} for fibrous biofilter and 145.43±1.17 mg-N m^{-2} day^{-1} for filter mat, respectively. While the highest denitrification efficiencies were also found in the intensive system as 81.86±4.40 mg-N m^{-2} day^{-1} at 25 PSU for fibrous biofilter and 165.80±50.17 mg-N m^{-2} day^{-1} at 5 PSU for filter mat, respectively. Results from the next-generation sequencing (NGS) on Illumina MiSeq demonstrated that *Proteobacteria* and *Bacteroidetes* were the dominant bacterial groups in all experimental systems. For microorganisms in nitrogen cycle, however, the predominant nitrifiers and denitrifiers observed in low-salinity system was different from that under medium- (15 PSU) and high-salinity (25 PSU) conditions. The second study was to evaluate the performance of fibrous biofilter and to monitor the microbial community dynamics during long-term (210 days) operation of marine (25 PSU) RAS at the initial shrimp density of 1 kg-shrimp m^{-3}. Results showed that the complete nitrification was achieved after approximately 2 months of biofilter acclimation in parallel with shrimp cultivation. Throughout the two rounds replication of aerobic nitrification followed by anoxic denitrification, ammonia and nitrite were controlled within the acceptable condition while nitrate was then remove after shrimp harvest under anoxic condition with methanol supplement at COD:Nitrate-N of 5:1. Microbial results demonstrated that the uncultured bacterium clone PI1AB88 and the uncultured bacterium clone SF_NOB_Cd08 were the main players in ammonia and nitrite oxidation, respectively, while *Methylophaga* and *Methylotenera* were the predominant denitrifying bacteria in anoxic denitrification.

Field of Study:	Environmental Engineering	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

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Introduction

1.1 Introduction

Recently, commercial aquaculture system has grown rapidly in order to support the consumer demand of good quality protein foods. Recirculating aquaculture system (RAS) is utilized in engineered aquatic systems to sustain a water quality in the high-density farming pond (Timmons et al. 2002). The intrinsic problem of such system is the rapid accumulation of toxic inorganic nitrogen species, i.e. ammonia, nitrite and nitrate, which are generated from ammonification of animal excretion and biological degradation of unconsumed feed (Crab et al. 2007). Inorganic nitrogen residues in term of ammonia and nitrite have the deleterious impact on aquatic animals at the levels above 1 mg-N L^{-1} while the concentrations of nitrate over than 10 mg-N L^{-1} can assert the negative effects (El-Shafai et al. 2004; Camargo et al. 2005). Biological filtration is the fundamental water treatment system to eliminate the toxicity of these compounds through nitrification and denitrification processes (Avnimelech 2006). To reuse the water, biofilter acclimation is the primary essential step to establish viable microbial biofilm for nitrogen removal in the RAS. Rapid start-up and stable biofilm formation are among the essential factors positively affecting the overall nitrogen treatment efficiency.

Improper start-up usually causes an incomplete nitrification that remains toxic nitrite accumulation, especially in marine and brackish systems (Manthe and Malone, 1987). The cause for this problem remains unclear and has been attributed to a number of factors, for example, salt concentrations, high organic loadings and other operational parameters (Gutierrez-Wing and Malone 2006). It is a well-known principle that the destabilization of the microbial communities has a significant impact upon a salinity. High salinity has an inhibitory effect on the growth of both *Nitrosomonas* spp. and *Nitrobacter* spp. that involve in ammonia and nitrite oxidation processes, respectively. Generally, the nitrite oxidizing bacteria (NOB) are more affected to salt concentrations than ammonia oxidizing bacteria (AOB) (Cortés-Lorenzo et al. 2015). Inactivation of nitrifying cells under saline environments (over than 30 PSU–practical salinity unit) may able to reduce the nitrification efficiency while a significant decrease of 50% in ammonia removal rate occurs when the salinity levels reach to 43.5 PSU (Magalhães et al. 2005). Notwithstanding, the low salt concentrations can encourage the growth of biological organisms. MacFarlane and Herbert (1984) reported that the ammonia removal rate is stimulated when salinity increases from 0 to 20 PSU while the maximum nitrification activity occurs under the intermediate salinity condition of between 5 and 10 PSU (Jones and Hood, 1980). Contrarily, the activity of denitrifying bacteria is not influenced by the presence of sea salts (Magalhães et al. 2005). This is probably due

to the multiplication of halotolerant denitrifiers under oxygen absent condition within biofilm layers or sediments which protect cells from the external environments.

Nevertheless, the abundance and diversity of microorganisms are not affected by solely salinity, but also by inorganic nitrogen concentration. Normally, nitrogen loading in an aquaculture system refers to the stocking density of aquatic animals. The generation of high ammonia concentrations from biological degradation of unused feed and fecal matters is always found in an intensive aquacuture system (Achuthan et al. 2006). The elevated level of the free ammonia nitrogen (FAN) causes an inhibition on the growth of microorganisms and affects their activities (Yu et al. 2004). Similar to the effects of salinity, it seems like NOB are more sensitive than AOB in ammonia-rich systems. The inhibition of free ammonia on nitrite oxidizers is the major factor for nitrite accumulation (Yun and Kim 2003). The activity and function of *Nitrobacter* spp. are inhibited when ammonia concentrations reach to 1 mg-N L^{-1} (Anthonisen, 1976; Chaarls, 1998), whereas the *Nitrosomonas* spp. can survive under high ammonia conditions as from 5 to 70 µM (Itoi et al. 2006) or from 20 to 100 µM (Foesel et al., 2008). For other nitrifying microorganisms, the ammonia oxidizing archaea (AOA), *Candidatus Nitrosopumilus maritimus*, are abundant in marine environments under low ammonia levels approximately 0.2 µM (Sakami et al. 2012) while *Nitrosospira* spp. are dominant in the terrestrial habitats with ammonia concentrations under 1 µM (Bruns et al., 1999; Kowalchuk and Stephen, 2001).

Biological filters are devices to establish biofilm by providing the space for microbial adsorption and multiplication. The primary concern for selecting appropriate biofilters is the specific surface area (SSA) for attachment of microorganisms. The fibrous Biocord™ biofilter is used various as biomedia in wastewater treatment system (Zhang et al. 2012), suspended solid capture device (Yuan et al. 2012) and nitrifying biofilter in RAS (Sesuk et al. 2009), due to the large SSA of 1,760 m^2 m^{-3}. According to the polypropylene fabric in a helical structure, the complex structure of Biocord™ gives microorganisms the best conditions to multiply at both surface and interior of the cord, which is possible for the living together of aerobes and anaerobes. Similarly, the curled polyester fibers of the Japanese filter mat can also make an ideal environment and dwelling for many different kinds of microorganisms. The filter mat can be applied in the fixed-bed reactors as well as in the filtration unit for solid particle removal (Khammi et al. 2015). Although the SSA of Japanese mat (300 m^2 m^{-3}) is six times lower than the fibrous Biocord™ surface, the submerge ability of filter mat maintains the sufficient amount of biomass by reducing the effects of flow rate dynamics and water shear strength. Both types of materials can be utilized for biological nitrogen removal through the nitrification and denitrification co-processes. Nitrifiers can multiply on the biofiler surface under high oxygen availability while the intricate fibers prevent oxygen from reaching the cored center where denitrifiers prevail. These co-processes contribute to reduce chemical supply for maintaining alkalinity in the system (Daniel et al. 2009). Also, the solid debris preserved inside material structure can be

used as the internal carbon source for heterotrophic denitrifiers without the addition of external substrates (van Rijn et al. 2006).

Biofilter acclimation by natural colonization with starter animals is the method to develop the stable and viable microbial biofilm on material surface. The natural microorganisms are introduced to aquaculture system by the small numbers of aquatic organisms (Dennis and Thomas, 2012). Within the system, the growth of aquatic animals proceeds in parallel with biofilter immobilization. Both aerobes and anaerobes can multiply inside filter media, depending on nutrient sources and condition settings. Nitrogen sources for microorganisms are derived from protein in artificial feed supplied during the cultivation period. Ammonia from organic matter mineralization is an energy source for nitrifying microorganisms while the facultative anaerobes consume nitrate which is generated by nitrification as nitrogen source. The advantage of using organisms entered with starter animals is the attribute that allows an appropriate microorganism for each system. Rapid biofilm establishment obtained from natural colonization with starter animals is desirable to reduce the biofilter activation time and the toxic ammonia and nitrite accumulations (Keuter 2011; Keuter et al. 2017).

Consequently, this research aimed to study the effects of salinity and nitrogen loading on nitrification and denitrification efficiencies, as well as microbial diversity in biofilms during biofilter acclimation in RAS. The effects of salinity were investigated by varying the salt concentrations at 5, 15 and 25 PSU while the effects of nitrogen loading were estimated by adjusting the stocking densities of shrimps at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) levels. Also, the nitrogen removal efficiencies of fibrous Biocord^{TM} biofilter and Japanese filter mat were compared. Thereafter, the performance of using biofilter in long-term operation of marine RAS for 210 days was evaluated while the next-generation DNA sequencing method (MiSeq) was applied to monitor the microbial diversity and community dynamics. The findings of this study provided the suitable conditions for biofilter immobilization in parallel with aquatic animal cultivation, which contributed to minimize biofilter activation time as well as reduce space for reactor installation. Moreover, the evaluation of nitrogen removal performance of biofilter provided the stability of biological filtration system which could be apply for long-term operation of pilot scale marine RAS.

1.2 Objectives

1) To evaluate the effects of salinity, nitrogen loading, and acclimation period on nitrification and denitrification efficiencies, and microbial diversity in biofilms during biofilter acclimation in parallel with aquatic animal cultivation.
2) To compare the efficiencies of nitrification and denitrification between fibrous Biocord^{TM} biofilter and Japanese filter mat.

3) To evaluate the performance of biofilter for long-term operation in marine recirculating aquaculture system.
4) To study the microbial diversity and community dynamics during long-term operation of aquaculture system.

1.3 Hypotheses

1) Biofilter media acclimated at low salinity levels (5 and 15 PSU) have higher nitrification and denitrification efficiencies than in seawater salinity (25 PSU).
2) Low shrimp density (50 shrimp m^{-2}) with low nitrogen input can introduce the natural microorganisms which are suitable for complete nitrification without the effects of elevated ammonia concentrations.
3) Biofilter acclimation in RAS for more than 4 weeks can achieve complete nitrification without nitrite accumulation.
4) Fibrous Biocord^{TM} biofilter can achieve higher nitrogen removal efficiency than Japanese filter mat, without the hydrogen sulfide production inside the material structure.
5) Fibrous Biocord^{TM} biofilter can be applied in marine RAS for long-term operation of nitrification-denitrification co-processes for 7 months with high operational stability.
6) There is no change in microbial community and dominant species of nitrifying microorganisms during long-term operation of aquaculture system.

1.4 Scopes of study

The scopes of this study are listed below:

1) In study 1, the optimum conditions of salinity, stocking density and acclimation period for nitrification and denitrification co-processes were investigated in a 240 L aquaculture tank by varying the salinity levels at 5, 15 and 25 PSU, the stocking densities at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}), and the experimental period of 80 days. While in study 2, the performance of biofilter for long-term operation of 210 days was tested in a 2000 L marine recirculating shrimp culture system at 25 PSU with the initial shrimp density of 1 kg m^{-3}.
2) The estimation of nitrification and denitrification rates of biofilters was test in 3 L test chamber with the saline synthetic wastewater containing ammonium chloride and sodium nitrate as nitrogen sources for nitrifying and denitrifying microorganisms, respectively.
3) Fibrous Biocord^{TM} biofilter (SSA 1,760 m^2 m^{-3}) and Japanese filter mat (SSA 300 m^2 m^{-3}) were applied as material in biological filter unit and used for the attachment of microorganisms.

4) Pacific white shrimp, *Litopenaeus vannamei*, was cultured as the experimental aquatic animal in indoor RAS and fed daily at 3% feeding rate of the total weight, with an artificial feed contained more than 36% of protein.
5) Water quality parameters, i.e. ammonia, nitrite, nitrate, dissolved oxygen (DO), pH, oxidation reduction potential (ORP), temp, alkalinity and chemical oxidation demand (COD), were monitored according to the standard methods.
6) Microbial communities on biofilters in study 1 were investigated by Illumina MiSeq system using universal primers (Bakt_341F and Bakt_805R) for bacteria while the universal primers (Univ515F and Univ806R) for archaea and bacteria were used in study 2.

1.5 Outcomes

1) This study provided the appropriate conditions of salinity and stocking density for biofilter immobilization in parallel with aquatic animal cultivation, which led to the development of improved nitrogen removal efficiency. The biofilter acclimation integrated in an aquaculture system contributed to minimize the biofilter activation time as well as to reduce the space for reactor installation. In addition, the evaluation of nitrogen removal performance provided the stability of biological filtration system, which led to the application of biofilter for long-term operation in the pilot scale marine RAS.
2) The genetic information provided a better understanding of microbial diversity and community dynamics during the biofilter acclimation in parallel with the aquatic animal cultivation, and during the long-term operation of nitrification followed by denitrification in marine RAS.

Chapter 2

Background and literature review

2.1 Aquaculture system

Nowadays, the commercial aquaculture system becomes more important due to the decreases in the volume of aquatic animals from the nature. An aquaculture system has developed rapidly as a response to the demand of good quality protein food instead of natural protein sources. Types of aquaculture system are classified based on water quality management, the physical characteristic of aquaculture pond, and stocking density of aquatic animals, as follows:

2.1.1 Aquaculture system classified by water quality management

- **Open system**

Open cage aquaculture refers to the rearing of aquatic animals within natural environments. The open aquaculture system is performed in freshwater rivers, brackish estuaries, and also coastal marine regions (Lawson 1995). Floating mesh cages are located in natural waterways by varying in size, depending on the operation scale and the cultured species. The natural water is applied in an aquaculture system by flowing through cages directly and then discharging the wastewater into environments without any treatment process. Hence, this method affects the water quality in environments due to the accumulation of organic loading wastes from large volume aquatic animals which is higher than the natural self-regeneration capacity. Another significant issue is increased disease and parasite transmission from other aquaculture systems that are located in the same waterway. The cultured animals in cages have a high-risk infection because this system allows unchecked interactions between the farmed fish and the surrounding environments, which leads to the free exchange of disease, parasites, and fecal matter (Aquaculture Methods, 2016). Also, the cultured animals in open cage have the risk of escape and interbreeding with wild populations. The Center for Food Safety (2012) reported that there were 25 million of fish escapes worldwide and the majority occurred when the netting system was damaged during severe weather.

- **Semi-closed system**

Semi-closed aquaculture refers to the land-based production of aquatic species, in which water is exchanged between the farm and a natural waterway. This system is developed from the traditional open cage system by decreasing volume of water exchange or discharging wastewater at some period for maintaining water quality in aquaculture ponds. Wastewater is released from the ponds into local waterways and

replace with fresh water; therefore, the semi-closed aquaculture can decrease effects of sudden death caused by pathogen infection, which is the main issue in the flow-through system (Lawson 1995). According to the feature of the semi-closed system, the aquaculturists can monitor and control water quality parameters in aquaculture pond during the operation period. At the end of cultivation, moreover, the operating system can be stopped, and then cleaning or removing the small particles of organic waste at the pond bottom before beginning new crop. For these reasons, the animal production from semi-closed aquaculture is higher when compared to the open cage system. However, the effects of wastewater effluent discharge on environments have still occurred because the constant outflow of wastewater may also reduce water quality due to inadequate treatment.

- **Closed or recirculating system**

Closed aquaculture system refers to the land-based rearing of aquatic species in raceways, tanks, and ponds, while the recirculation technology is installed to circulate wastewater to the treatment system and return water back to aquaculture pond without discharging any wastewater. The RAS is developed to decrease the impact on natural environments and to reduce the volume of water used in aquaculture (Timmons et al. 2002). Recently, the RAS has become more popular due to the ability to maintain a sustainable water quality in farming pond. The engineered aquatic systems, for example, biological filtration and biofloc technology (BFT), utilized to remove an inorganic nitrogen compound and total suspended solids generated by aquatic species. Hence, a higher stocking density for commercial aquaculture can completely be achieved within RAS due to the proper water quality management. Careful design and management are the basis for a successful waste management. The operating RAS under well-controlled culture conditions contribute to an efficient feed utilization and low waste production (van Rijn 2013). However, the recirculating aquaculture limitation is the complicated operation. Both aquaculture and wastewater treatment process must be operated in parallel, which is difficult for aquaculturists to perform solely. Also, high operation and maintenance costs are the disadvantages that are not recommended for small farms.

2.1.2 Aquaculture system classified by physical characteristic of aquaculture pond

- **Outdoor earthen pond**

Aquaculture in outdoor soil pond refers to the land-based production of aquatic animals in soil pond. This aquaculture is easy to operate and requires low operation cost due to the natural nitrogen removal as well as oxygen regeneration capacity (van Rijn 2013). Nitrogen removal which occurred by algae and natural microorganisms

cooperation in a farming pond. Photosynthesis in algae and plant plankton contributes to raising the dissolved oxygen concentration in an outdoor pond, which required for nitrogen mineralization and nitrification processes. The biological degradation plays an important role in the conversion of organic nitrogen from unused feed and fecal matters to inorganic nitrogen (Achuthan et al. 2006). Thereafter, an inorganic nitrogen in term of ammonia is converted by nitrifying organisms to nitrite and finally nitrate, which is consumed as nitrogen nutrient for the growth of algae. At the same time, under oxygen absent condition at the bottom of aquaculture pond, nitrate is removed by heterotrophic microorganisms through denitrification process (van Rijn et al. 2006). Consequently, an aquaculture in the soil pond also reduces the chemical feeding for maintaining alkalinity related to the balancing of bicarbonate by simultaneous nitrification and denitrification. Nevertheless, according to the natural nitrogen removal, the efficiency of this process is uncontrollable and fluctuated, depending on the rates of oxygen generation by algae and nitrogen removal by microorganisms. Furthermore, another issue of an outdoor earthen pond is the high organic waste accumulation at the bottom of aquaculture pond, which brings about the infectious disease caused by the presence of the pathogen in a soil, and the hydrogen sulfide generation due to poor oxygen condition. Hence, to disinfect dried soil pond requires after harvesting of animal products.

- **Outdoor lining pond**

Aquaculture in outdoor lining pond refers to the land-based rearing of aquatic species in soil pond lined with plastic sheets, and cement or plastic pond, without soil at the bottom. The lining pond is developed to solve the infection disease problem caused by pathogens in soil. Similar to outdoor soil pond, the phototrophic organisms, such as plants, algae and autotrophic organisms contribute to control the water quality in aquaculture ponds (van Rijn 2013). However, the nitrate removal under anoxic condition occurs lower compared to the earthen pond because of a lack of soil. Accordingly, the nitrate accumulation at high concentrations is always found in this system. The long-time exposure to elevated levels causes the toxicity of nitrate on aquatic organisms by conversion of oxygen-carrying pigments (Camargo et al. 2005). Also, the nitrate accumulation stimulates the growth rate of phototrophic organisms which increase the number of the population rapidly. The main problem is the decrease in dissolved oxygen concentration in aquaculture ponds due to the respiration of algae and aquatic animals, especially at nighttime. These algae also increase water turbidity and interfere with sunlight penetration. Besides, the dead algae release nitrogen back to aquaculture system, leading to increasing dissolved nitrogen compound, as well as the dead cell accumulation at the bottom can be digested by anaerobic organisms, as a result in hydrogen sulfide production (Boyd, 2014). For these negative reasons, the outdoor lining pond should be operated with recirculating system to control water

quality and maintain nitrogen nutrient in aquaculture ponds to prevent the effects of algae growth.

- **Indoor pond**

Similar to the outdoor lining pond, the aquaculture in an indoor pond refers to the land-based production of aquatic organisms in cement and plastic ponds which are located inside the building or covered by the roof. The advantage of this aquaculture is the controllable ability to external and internal environmental factors during the cultivation period. The indoor pond can prevent rain, sunlight and temperature variation. During the rainy season, the rainwater causes the sudden drop in dissolved oxygen concentration, alkalinity and salinity, while the acid rain can also decrease the pH in aquaculture pond. The sunlight contributes to the difference between day and night-time temperatures. Aquatic animals, such as fish and amphibians, are a cold-blooded species in which their body temperature can only change in a limited range. Hence, when the environmental factors change dramatically and exceed the permitted limits, the animals will be weak, shocked, and possibly dead (Gutierrez-Wing and Malone 2006). According to the lack of sunlight, the condition within the indoor pond is not suitable for the growth of phototrophic organisms; thus, the mechanical aeration is strongly required to maintain the levels of dissolved oxygen. Generally, the indoor pond always operates with the recirculating system to allow aquaculturists complete year round-control over all growing conditions (van Rijn 2013). The strength of well-designed and well-executed indoor RAS is the ability to allow water quality of the rearing environment to be controlled while minimizing the entry of pathogens.

2.1.3 Aquaculture system classified by stocking density

- **Extensive farming**

Extensive aquaculture refers to the original aquaculture system in large areas of natural environments, for example, freshwater rivers, brackish estuaries, coastal marine regions and outdoor earthen pond, using the traditional methods of tidal exchange of water and natural feed supply. The stocking density in an extensive farm is too low which is less than 1 ton ha^{-1} per year, and requires the water approximately 20 to 40 m^3 kg^{-1} of aquatic animals. According to the report from Food and Agriculture Organization of the United Nations (2015), the stocking density in an extensive shrimp farming was 1,000 to 3,000 juvenile ha^{-1}, while the production per cycle ranged from 0.5 to 1 ton ha^{-1}. The natural foods are used for culturing the aquatic species in aquaculture system, without a supply of artificial feed. Also, the life-support systems, such as aerators and pumps, are not employed. Both organic and inorganic fertilization promote the growth of simple plants, phytoplankton and zooplankton, which are the base of the food chain for stocked animals. Thus, the animal production of this system

is uncontrollable and fluctuated based on the quality and quantity of natural foods. Increasing the effectiveness of this system, an extensive aquaculture can be integrated with other types of crop or livestock production, using animal manure and agricultural by-products as sources to stimulate the primary production.

- **Semi-intensive farming**

Semi-intensive aquaculture refers to the land-based production of aquatic species, in which water is exchanged between the main rearing ponds and a reservoir. This system is adapted to increase the production rate over the traditional extensive system, by providing the extra nutrients to complement the other ingredients obtained from nature. During the first month of cultivation period, the aquatic animals are dependent on natural foods, while the supplemental feed is provided from the second month onwards. The stocking density in semi-intensive farming is approximately 0.8 to 1.2 ton ha^{-1}, and requires the low volume of water approximately 5 m^3 kg^{-1} of aquatic animals. The amount of harvested fish is planned by controlling the quality and quantity of artificial feed. The production rate per cycle ranged from 1.5 to 3.5 ton ha^{-1} which is higher than obtained in an extensive aquaculture. However, the animal production can be as high as 15 ton ha^{-1} in the system fed with high-quality artificial feed at least 4 to 5 times a day. Because of the extreme feed, the anaerobic condition caused by organic and inorganic fertilization occurs in aquaculture ponds within 100 days of the grow-out period. Therefore, the aeration by paddle-wheels or oxygen injectors is strongly required to maintain the oxygen levels above 5 ppm.

- **Intensive farming**

Intensive aquaculture relies on the technology to raise the numbers of cultured animals at very high stocking densities within the limited space of artificial tanks. Instead of both extensive and semi-intensive systems, nowadays, the intensive farming becomes more popular due to the ability to maintain suitable conditions in the aquaculture system. The water quality parameters, such as dissolved oxygen, pH, salinity, temperature, stocking densities, quality of artificial feed, and feeding rate are given in the optimal condition to promote growth, reduce stress, prevent disease, and decrease mortality. The stocking density in an intensive aquaculture is approximately 12 to 18 kg m^{-3}, and reached to 50 kg m^{-3} when supplying the aquaculture tanks with liquid oxygen. Therefore, according to the complete control of these factors, the high-yield production of aquatic animals achieved in this system. Although intensive aquaculture is completely mechanized, the intrinsic problem of this farming is related to the rapid accumulation of toxic organic and inorganic nutrients, i.e. carbon, nitrogen and phosphorus, from unconsumed feed and animal excretion (Gutierrez-Wing and Malone 2006; Crab et al. 2007). The frequent water exchange using a pump is traditionally required to maintain desirable water quality. Furthermore, the proper

incorporation of wastewater treatment systems within the recirculating contribute to decreasing the effects of waste production (Timmons et al. 2002; van Rijn 2013).

2.2 Biology of Pacific white shrimp

Pacific white shrimp, *Litope*naeus *vannamei*, also known as white-leg shrimp and king prawn, is native to the Eastern Pacific coast where water temperatures are higher than 20°C throughout the year. This shrimp specie has the translucent body and white legs. The taxonomic tree of white shrimp is as follows:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Suborder: Dendrobranchiata

Family: Penaeidae

Genus: *Litopenaeus*

Species: *Litopenaeus vannamei*

Figure 1. The Pacific white shrimp, *Litope*naeus *vannamei*.

The body structure of white shrimp is divided into two segments as cephalothorax and abdomen. The cephalon or head (five somites) and thorax (eight somites) are fused into the cephalothorax and covered by the carapace which is the helmet-like plate of exoskeleton. The shrimp head consists of two pairs of segmented sensory antennule and antenna, one pair of mandible for cutting food, and two pairs of maxilla for food handling. The carapace protrudes forward is the rostrum, while the compound eyes are behind a beak-like structure for photoreception and movement detection. The thorax consists of three pairs of maxillipeds for food handling and five

pairs of pereiopods. The first and second pereiopods are applied for food gathering, grooming and signaling, whereas the posterior third, fourth and fifth pereiopods are walking legs. In terms of the abdomen section, it consists of six segments which are covered by the band-like plate of the exoskeleton to allow the flexibility and quick movement. There are five pairs of pleopod or swimmeret used for swimming, while the last segment is a pair of uropods (Ruppert et al. 2004).

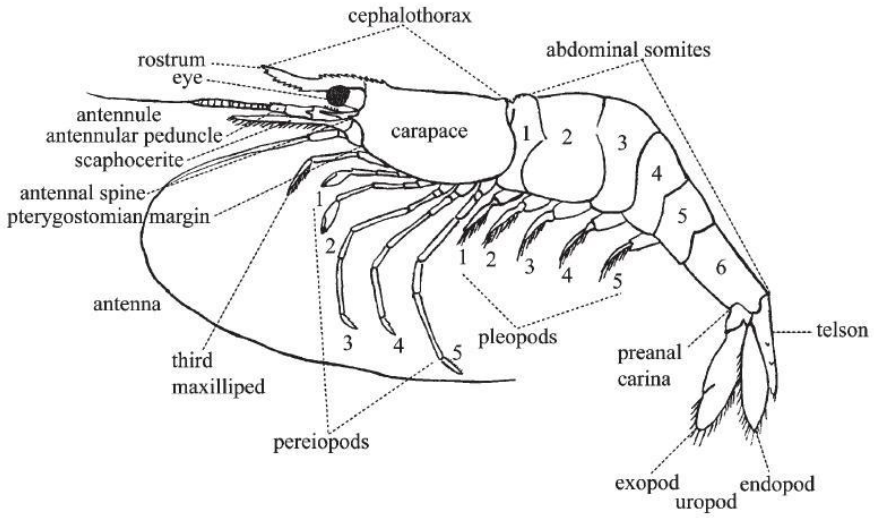

Figure 2. The external anatomy of shrimp (Ruppert et al. 2004).

2.3 Factors affecting water quality in aquaculture system

Water quality in aquaculture pond is the most important parameter affecting the health of aquatic animals and performance of aquaculture systems. The proper water quality is essential for the activities of aquatic organisms, for example, cell metabolism, growth, reproduction, respiration, movement, and excretion. The parameters affecting water quality in RAS and standard values for each parameter are as follows:

2.3.1 Ammonia; NH_3

Ammonia is an inorganic nitrogen compound which is converted from organic nitrogen through nitrogen mineralization process by the cooperation of autotrophic and heterotrophic microorganisms. Generally, the main source of nitrogen in aquaculture system is animal feed which is estimated approximately 52 to 95% (Wu, 1995). Only 25 to 30% of feed nutrients are converted for animal growth and useful energy, whereas the byproduct of protein metabolism in term of ammonia (70 to 75% of feed) is excreted as animal feces (Gutierrez-Wing and Malone 2006; Crab et al. 2007). Therefore, the biological degradation of unused feed and fecal matters by natural microorganisms

plays an important role in an ammonia generation in an aquaculture system (Achuthan et al. 2006). The small amounts of ammonia, moreover, can be diffused from the atmosphere and subsurface air bubbles during aeration.

Ammonia can be present in water in two forms, either ammonium hydroxide (un-ionized form; NH_3) or ammonium ion (ionized form; NH_4^+), mainly depending on the pH of water. The ammonium hydroxide is found in water with high pH (above 7), on the contrary, there are more ammonium ions when pH decreases to less than 7, as presented in equation 1. The pK (equilibrium constant) of the ammonia/ammonium reaction is approximately 9.5 and varies with many factors (Randall and Tsui 2002). The salt concentration and water temperature also have effects on the nitrogen forms in which high temperature raises the concentration of ammonium hydroxide, whereas ammonia in an ionized form is present in high salinity condition.

(Low pH) $NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$ (High pH) Equation 1

For aquaculture system, the toxicity of ammonia is expressed in term of total ammonia nitrogen (TAN) which is the combination of an ammonium hydroxide and an ammonium ion. An un-ionized is the principal form of toxic ammonia that is more harmful to aquatic animal health than an ionized form. According to the experiment performed by Barbieri, E. (2010), the 24 hours LC50 value of an un-ionized ammonia in white shrimp was 1.46 mg-N L^{-1} under 5‰ salinity (5 PSU) at pH 8.0 and 20°C, whereas the value of 40.72 mg-N L^{-1} was observed for TAN. Therefore, under high pH conditions, the effects of ammonia are stronger than under low pH conditions. The accumulation of ammonia in RAS may reduce the growth rate of aquatic animals by producing many physiological changes, including alterations in the metabolism. Exposure to ammonia, additionally, also increases oxygen consumption, ammonia excretion, and causes high mortality (Barbieri 2010; Cobo et al. 2014).

Ammonia toxicity divided into two levels, i.e. acute and sub-lethal. The lethal concentration of ammonia that causes organisms die for 50% of total population (LC50) can be either acute or chronic toxicity, depending on the exposure time. Acute toxicity tests are performed over a period of 2 to 7 days, while chronic tests are longer than 7 days. For acute toxicity, the TAN concentration of 30 mg-N L^{-1} caused 100% mortality in *Litopenaeus schmitti juveniles* under the 96-hour exposure period, and within 24 hours when increasing the concentration to 80 mg-N L^{-1} (Barbieri 2010). The chronic exposure to ammonia may reduce the growth rate of crustaceans, and also decrease the growth of species raised in an intensive aquaculture. Wickens (1976) reported that the slow growth rate was found during a three-week chronic test when ammonia concentrations were more than 0.10 mg-N L^{-1}. Moreover, an elevated ammonia level of 0.45 mg-N L^{-1} led to a 50% decrease in the growth of five species of penaid shrimp. The effects of ammonia on oxygen consumption and ammonia excretion were studied

by Barbieri (2010) in which the presence of TAN at 5 mg-N L^{-1} would significantly increase both oxygen consumption and ammonia excretion in white shrimp after the 2-hour exposure period. Consequently, in practical terms, the TAN should be controlled within the acceptable levels of 0.1 mg-N L^{-1} (Gutierrez-Wing and Malone 2006).

2.3.2 Nitrite; NO_2^-

Nitrite is an inorganic nitrogen compound which is oxidized from ammonia via nitritification or nitritation process by ammonia oxidizing archaea and bacteria under aerobic condition. In general, nitrite is an unstable nitrogen form which can be easily oxidized to nitrate; therefore, the accumulation of nitrite is rarely observed in natural environments. Nevertheless, the presence of an elevated nitrite concentration can occur in an intensive aquaculture with high stocking density and high feeding rate. Likewise, under some circumstances, e.g. the presence of free ammonia at higher levels and under salinity condition, nitrite is also accumulated due to the inhibition of nitrite oxidizing microorganisms (Yun and Kim 2003; Yu et al. 2004).

Nitrite toxicity in aquatic organisms is a function of the effects on the circulatory and immune systems. The presence of high nitrite concentrations can cause the hypoxia and brown blood disease which is a dark brownish color of the animal blood syndrome due to a lack of oxygen. Nitrite can enter the bloodstream and then inhibit the binding of oxygen to the iron molecule of hemoglobin. The oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) by nitrite leads to increased levels of methemoglobin instead of hemoglobin. Methemoglobin is useless as an oxygen carrier; thus, the animal blood is reduced oxygen-carrying capacity and oxygen levels, and finally loses its reddish color (Lewis and Morris 1986). For crustaceans, on the other hand, the hemocyanin is the main oxygen transporter in blood, which contains a copper-based molecule to help hold onto oxygen at the gills and presents in the blue color. The effects of nitrite on shrimp blood is not well studied; however, it is possible that nitrite effects on the copper of invertebrate's circulatory systems (Schuler 2008). Furthermore, the accumulation of nitrite in RAS may reduce the growth and survival rates of aquatic animals. Schuler, D.J. (2008) reported that the LC50 value for nitrite in the Pacific white shrimp, L. vannamei, at the post-larvae stage (approximately 25 to 45 days old) was 153.75 mg-N L^{-1} under salinity of 10 PSU at pH 7.8 and 28 °C. While Gross et al. (2004) presented that the exposure to nitrite level of 4 mg-N L^{-1} under low-salinity brackish water (less than 10 PSU) for 2 days did not affect the survival rate; nevertheless, this concentration reduced the growth of white shrimp. The safe concentration of nitrite for shrimp production in RAS should be maintained less than 0.45 mg-N L^{-1} (Gross et al. 2004) or 1 mg-N L^{-1} (Gutierrez-Wing and Malone 2006).

2.3.3 Nitrate; NO_3^-

Nitrate is the end product of nitrification, which is converted from nitrite via nitratification or nitratation process by nitrite oxidizing microorganisms under aerobic condition. Generally, nitrate is a stable nitrogen form which has a much lower toxicity compared to other nitrogenous wastes, i.e. ammonia and nitrite. However, under high dissolved oxygen concentrations, ammonia completely be oxidized to nitrate and leads to the accumulation of nitrate at higher levels.

In aquaculture system, nitrate does not directly kill or cause the disease in short-term like ammonia or nitrite; nevertheless, the long-time exposure to high levels can cause damage to aquatic organisms. The main toxic action of nitrate is due to the conversion of oxygen-carrying pigments, e.g. hemoglobin in fish and hemocyanin in crustaceans, to an incapable form of oxygen carrier (Camargo et al. 2005). Also, nitrate accumulation in RAS may cause animal stress, as well as reduces the growth, feed consumption and reproduction rates. The toxicity of nitrate depends on many factors: animal species, life stage, body size, water salinity and environmental adaptation. The freshwater organisms appear to be more sensitive to nitrate toxicity than marine species due to the presence of salt in an aquaculture system (Furtado et al. 2015). The results obtained in the research study by Furtado et al. (2015) showed that the elevated concentrations of nitrate up to 177 mg-N L^{-1} were acceptable for white shrimp (*L. vannamei*) in the BFT at a salinity of 23 PSU. Meanwhile, a nitrate concentration of 10 mg-N L^{-1} can adversely affect freshwater animals, at least during long-term exposures. The LC50 values of nitrate in freshwater species range from 5 to 2,107 mg-N L^{-1}, while the higher values of 2.2 to 5,050 mg-N L^{-1} are observed in marine organisms (Gutierrez-Wing and Malone 2006). The early life stages of marine crustaceans, e.g. larvae and broodstock stages of shrimp, may be very sensitive to nitrate toxicity, whereas an increase in life stages and body size can contribute to decrease the negative effects. In practical terms, nitrate is usually controlled below 60 mg-N L^{-1} (Van Wyk and Scarpa, 1999; Piérri et al., 2014); however, the marine white spot disease has been linked to nitrate concentration above 30 mg-N L^{-1} (Burgess, 1995). Therefore, a maximum level of 20 mg-N L^{-1} may be acceptable for marine environments, while the nitrate concentration of 2 mg-N L^{-1} would be appropriate for protecting the most sensitive freshwater species (Camargo et al. 2005).

2.3.4 Dissolved oxygen

Dissolved oxygen concentration is one of the important factors for the activities of both aquatic animals and microorganisms. According to nitrification process, an oxygen is strongly required for the conversion of ammonia to nitrate. Low oxygen levels cause an incomplete ammonia oxidation due to the shift from autotrophic to heterotrophic microorganisms. Therefore, the build-up of ammonia and nitrite at high

levels can occur in poorly oxygenated waters, and raises the negative effects on aquatic organisms.

Many previous research studies conducted on the relationships between shrimp behaviors and oxygen concentrations in aquaculture systems. Egusa (1961) reported that the stress response in P. japonicus was observed with dissolved oxygen of 1.4 ppm, while P. schmitti was the majority of shrimp began swimming at the water surface when the level of oxygen was reduced to 1.2 ppm (MacKay, 1974). Low dissolved oxygen leads to an increase in animal respiration rate (Thurston et al. 1981). The research study by Allan et al. (1990) supported that the reduced DO levels had been shown to significantly increase the acute toxicity of ammonia in P. monodon related to the increase in both respiration rates and uptake of dissolved nitrogenous compounds. However, the oxygen requirements for growth and survival are different, depending on the animal species and age/stage of aquatic organisms. For instance, the oxygen demand for white shrimp is approximately 6 ppm, which is more than the need for tiger prawn because the *P. vannamei* swim and move faster than *Penaeus monodon*. Consequently, the adequate levels of dissolved oxygen should be prepared for increasing the production rate in RAS. Many workers have suggested that the minimum level of oxygen needed for shrimp survival must be more than 2 ppm; however, the best concentration should be equal or higher than 5 ppm (Nonwachai et al. 2011).

Table 1. Effects of oxygen concentrations on shrimps (Nonwachai et al. 2011).

Dissolved oxygen (ppm)	Effects on shrimp
< 1.0	Shrimp die
< 2.0	Anoxia in shrimp, shrimp may die
< 3.0	Shrimp cannot grow up
< 4.0	Shrimp grows slowly
4.0 – 5.0	Shrimp grows normally
5.0 – 7.0	Shrimp grows healthily and rapidly

2.3.5 pH

The pH is the concentration of hydrogen ion (H^+) in the water and used to specify the acidity or basicity of aquatic environments. In aquaculture system, the pH is an indicative of the fertility or potential productivity of RAS. The aquatic organisms normally live in water with a neutral pH in the range of 6 to 8, while both acidic and basic conditions are not appropriate for animal cultivation. The growth and the survival rates of aquatic animals decrease with pH values less than 6 or greater than 9. Likewise, the outer surfaces, e.g. gills, eyes, and skin, are damaged, as well as an ability to dispose of metabolic wastes is inhibited. The effects of pH on shrimps are presented in table 2.

According to the ammonia toxicity, moreover, the pH of water is an important factor to specify the forms of ammonia. Ammonium hydroxide (toxic ammonia) is found under high pH conditions, whereas there are more ammonium ions when pH decreases to less than 7 (Randall and Tsui 2002). Earlier research by Magallon Barajas et al. (2006) showed that the LC50 values of ammonia for multiple species of shrimp were decreased when pH increases up to 9. Accordingly, in order to avoid the ammonia toxicity and increase the shrimp production, the pH in an aquaculture system should range between 7.5 and 9 (Tharavathy 2014)

Table 2. Effects of pH on shrimps.

pH	Effects on shrimp
< 5	Dangerous and make shrimp die
5 – 7	Decrease growth and feed consume rates, and shrimp may die under long contact time
7.5 – 8.5	Shrimp grows healthily and rapidly
8.5 – 10.5	Decrease growth and feed consume rates, and shrimp may die under long contact time
> 10.5	Dangerous and make shrimp die

2.3.6 Alkalinity

Alkalinity is the capacity of water to neutralize hydrogen ion which is measured and reported in terms of equivalent calcium carbonate ($CaCO_3$). The alkalinity of water always presents in three forms, i.e. hydroxide (OH^-), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), depending on pH.

Alkalinity becomes an important parameter due to the ability to resist changes in pH upon the addition of small amounts of either acid or base (Tharavathy 2014), as shown in figure 3. In addition, the bicarbonate alkalinity can also reduce the toxicity of nitrite on shrimp, but less than 1% as effective as chloride (Lewis and Morris 1986). However, according to nitrification process, the bicarbonate alkalinity is consumed by nitrifying microorganisms in the conversion of ammonia to nitrate under aerobic conditions; hence, the alkalinity of water tends to decrease continuously. The broad pH variations can occur under low alkalinity conditions, resulting in shrimp stress, reduced growth, and even mortality (Ching, 2007). Accordingly, an alkalinity in aquaculture system should be maintained in the range of 80 to 120 ppm or 120 to 150 ppm (Limsuwan, 2005).

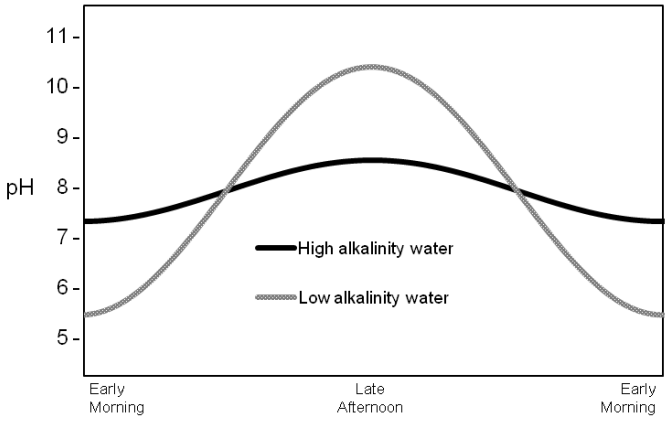

Figure 3. Changes in pH during a 24-hour period in waters of high and low total alkalinities (Wurts and Durborow, 1992).

2.3.7 Temperature

The aquatic organisms are poikilothermic animals whose internal temperature vary depending on the external environments; thus, the water temperature is one of the important factors for their growth and activities. The metabolic rates of aquatic animals always increase by 2 or 3 times when the external temperatures increase every 10°C (Tharavathy 2014), which means an oxygen requirement for aquatic species in warm water is higher than in cold water. The previous report by Regnault (1986) supported that an increased oxygen consumption in crustaceans could be linked to an increase in ammonia and nitrite uptake, as well as an increase in nitrogenous excretion, possibly due to the increased metabolic rates. Likewise, Niu et al. (2003) reported that an increase in temperature was coupled with increased respiration rates in the freshwater shrimp, *M. rosenbergii*. The temperature variation with a few degrees can delay or impede the development of the larvae stage in shrimp, while an extreme variation can cause the thermal shock and ultimately mortality (Gutierrez-Wing and Malone 2006).

Temperature also correlates with other water quality parameters, e.g. dissolved oxygen concentration and the forms of ammonia ion. The water temperature, as well as pH, plays a role in the partitioning of ammonium hydroxide and ammonium ion in aqueous environments. High temperature raises the concentration of toxic ammonia in term of unionized form, and also decreases the level of dissolved oxygen. The optimum temperature for many aquatic species is in the range of 25 to 30°C (Boyd and Tucker, 2012).

2.3.8 Hydrogen sulfide; H_2S

Hydrogen sulfide is the end product of dissimilatory sulfate reduction process by sulfate-reducing bacteria (SBR) under anaerobic conditions. The sulfate-reducing microorganisms can use oxygen in sulfate (SO_4^{2-}) as an electron acceptor to produce

sulfide (S^{2-}), as shown in equation 2. Generally, the sulfide in water has three forms as hydrogen sulfide (H_2S), hydrosulfide ion (HS^-) and bisulfide ion (S^{2-}), depending on the pH, as shown in equations 3 and 4. The hydrogen sulfide is the most dangerous form found in water with a low pH, whereas there are more hydrosulfide and bisulfide ions when pH is neutral.

$$\text{Organic matter} + SO_4^{2-} \rightarrow S^{2-} + H_2O + CO_2 \quad \text{Equation 2}$$

$$S^{2-} + H^+ \leftrightarrow HS^- \quad \text{Equation 3}$$

$$HS^- + H^+ \leftrightarrow H_2S \quad \text{Equation 4}$$

Hydrogen sulfide is usually produced at the bottom layer of mud, sludge, and bioflocs where oxygen is absent. The generation of hydrogen sulfide, even at low levels, can severely affect aquatic animals, as shown in table 3. Under the presence of hydrogen sulfide between 0.1 and 0.2 ppm, the shrimps appear to lose their equilibrium, and die suddenly when the concentration reaches 4 ppm. The effects of hydrogen sulfide on shrimp occur due to an interference of H_2S on the oxygen transfer process. However, the toxicity of hydrogen sulfide is mainly dependent on the concentration of dissolved oxygen. The prevalence of sulfate-reducing microorganisms is observed under oxygen absent conditions, whereas the levels of oxygen above 3 ppm can inhibit the sulfate reduction pathway and block hydrogen sulfide production. The pH and water temperature, moreover, also have effects on the toxicity of hydrogen sulfide in which low pH and temperature raise the H_2S toxicity. For this reason, these three parameters should be maintained within the appropriate conditions in order to avoid hydrogen sulfide production. A research study by Merican (2016) showed that the safe levels of hydrogen sulfide for tiger shrimp *(Penaeus monodon)* was 0.033 ppm, while the white shrimp *(P. vannamei*) at post-larvae and juveniles stages could tolerate up to 0.0087 and 0.0185 ppm, respectively. In practice, the concentration of hydrogen sulfide in RAS should be controlled less than 2 ppm.

Table 3. Symptoms and causes of shrimp affected by hydrogen sulfide (Panakorn, 2016).

Symptoms	Causes
Black gills	Exposure to H_2S when shrimp search for feed at the tank bottom
Abnormal color of gill and body	Stress after long exposure to H_2S
Mortality following moulting	Shrimp needs more oxygen

Table 3. Symptoms and causes of shrimp affected by hydrogen sulfide (Panakorn, 2016) (continued).

Symptoms	Causes
White feces disease (WFD)	H_2S toxicity irritates soft tissue in shrimp gut, causing the release of fat and mucous
Rotten egg smell	H_2S bubbling in the middle of pond. Discharge water color is too black with rotten egg smell
High ammonia and nitrite	Nitrifying bacteria destroyed by H_2S

2.3.9 Suspended solid; SS

Suspended solids (SS) in aquaculture system are generated from the artificial food pellets, animal excretion and the growth of microbial cells. Normally, the suspended solids can basically be calculated as equal to 25% of dry feed (Gutierrez-Wing and Malone 2006). Within the BFT, the suspended solids are required to maintain at the high concentrations to achieve nitrogen treatment process (Azim and Little 2008). Nevertheless, the elevated levels of solid have a negative impact on physical, chemical and biological properties of RAS. The excessive solids decrease dissolved oxygen concentrations in the aquaculture pond to reach the levels below the recommendation for cultivated species. In addition, suspended solids are the major physiological stressors to aquatic organisms which cause gill abrasion and behavior change (Yang et al. 2017). The shrimp at the larvae stage requires high water quality due to their sensitivity to suspended solids and bacterial infections (Gutierrez-Wing and Malone 2006). High concentrations of suspended solids increase the susceptibility to diseases, for example, white spot syndrome virus (WSSP), infectious hypodermal and hematopoietic necrosis virus (IHHNV), and *vibrio parahaemolyticus* (VP). Therefore, to achieve good water quality and high production rate, the suspended solids should be controlled within the acceptable levels below 20 mg-SS L^{-1}, or should not exceed 40 mg-SS L^{-1} (Muir, 1982).

2.4 Biological nitrogen removal process

Biological nitrogen removal (BNR) is the process driven by microorganisms to remove nitrogen compounds, mainly inorganic forms, i.e. ammonia, nitrite and nitrate, in aquatic environments. The BNR consists of three main sequential processes, i.e. nitrogen mineralization, nitrification under aerobic conditions and denitrification under anoxic conditions, as follows:

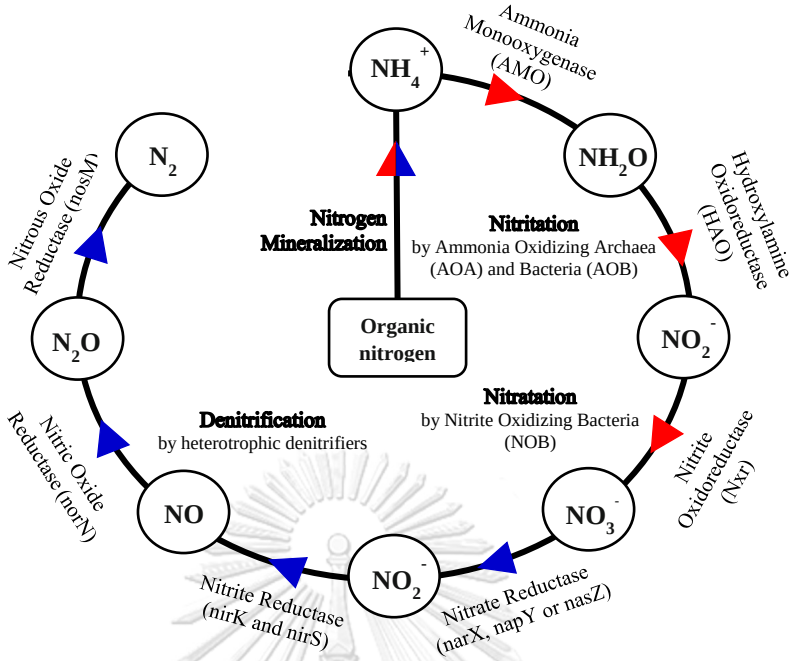

Figure 4. The overall biological nitrogen removal process.

2.4.1 Nitrogen mineralization

Mineralization is a process to convert an organic nitrogen to inorganic form as ammonia, which is driven by the cooperation between autotrophs and heterotrophs. The nitrogen mineralization consists of two-step continuous processes, i.e. aminization and *ammonificatio*n, respectively. The complex proteins are primarily broken down into the simpler substances, for example amino acids, amides, and amines, through aminization **process. H**eterotrophic microorganisms are the main drivers in the first step of mineralization, in which the organic compounds are consumed by these organisms as carbon and energy sources for cell growth. Thereafter, instead of heterotrophs, the autotrophic **organisms** *play the role in the second step, ammonification.* The amino group (-NH_2) is removed from the amino acid which is called deamination, and finally converted to inorganic form as ammonia (Schimel and Bennett 2004; Sylvia et al. 2005). The reactions of nitrogen mineralization by aminization and *ammonificatio*n are as follows:

Aminization:

$$\text{Proteins} \longrightarrow R^{+}\text{-}NH_2 + \text{R-OH} \qquad \text{Equation 5}$$

Ammonification:

$$\text{R-}NH_2 + H_2O \longrightarrow NH_3 + \text{R-OH} \qquad \text{Equation 6}$$

Ammonia production rate by nitrogen mineralization depends on many factors, for instance dissolved oxygen, pH, water temperature and especially organic nitrogen concentration. High production rate of ammonia can be achieved under either normal pH or weak base condition. The suitable temperature for converting an organic nitrogen ranges from 20°C to 35°C, while the rate of ammonia generation keeps increasing until 40°C (Kladivko and Keeney 1987). Ammonia generated from nitrogen mineralization can be adsorbed within clay micelle through ammonia fixation. Moreover, under high oxygen conditions, ammonia can be used as a nitrogen source for plant growth and converted to nitrate by nitrification process.

2.4.2 Nitrification

The biological nitrification is a key process in nitrogen cycle for converting ammonia to nitrite and nitrate, respectively. The oxidation of ammonia and nitrite can be carried out by both heterotrophic, e.g. *Aspergillus* spp. and *Arthrobacter* spp., and autotrophic microorganisms, e.g. *Nitrosomonas* spp. and *Nitrobacter* spp. However, on a relative basis, this process is mainly driven by autotrophic nitrifiers under aerobic condition using either carbon dioxide (CO_2) or bicarbonate (HCO_3^-) as carbon source. The ammonia monooxygenase (AMO) enzyme is used to convert ammonia to hydroxylamine (NH_2OH), which is further oxidized to nitrite via the hydroxylamine oxidoreductase (HAO) enzyme. Finally, nitrite is completely oxidized to nitrate by using nitrite oxidoreductase (Nxr) enzyme (Klotz and Stein 2011), as shown in equation 7.

$$NH_4^+ \xrightarrow{AMO} NH_2O \xrightarrow{HAO} NO_2^- \xrightarrow{Nxr} NO \qquad \text{Equation 7}$$

Nitrification consists of two-step processes as follows:

First step: Nitritification or nitritation process is the step to oxidize ammonia to nitrite, as shown in equations 8 and 9. Ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) are two main drivers in this process. *Nitrosomonas* spp. is the most frequently identified genus associated with this step. The other genera, including *Nitrosococcus* spp. and *Nitrosospira* spp., and some subgenera, including *Nitrosolobus* spp. and *Nitrosovibrio* spp., can also autotrophically oxidize ammonia (Watson et al. 1981). The yield coefficient of *Nitrosomonas* is between 0.04 and 0.13 g-VSS/g NH_4-N (0.29 g-VSS/g NH_3-N of theoretical yield), while the oxygen consumption for nitritation is equal to 3.43 mg-O_2/mg NH_3-N (Yu et al. 2004). (Stratton & McCarty, 1967)

$$55NH_4^+ + 76O_2 + 109HCO_3^- \longrightarrow C_5H_7O_2N + 54NO_2^- + 57H_2O + 104H_2CO_3$$

Equation 8

$$55NH_4^+ + 76O_2 + 5CO_2 \longrightarrow C_5H_7O_2N + 54NO_2^- + 52H_2O + 109H^+$$

Equation 9

Second step: Nitratification or nitratation process is the step to convert nitrite to nitrate as the final product, as shown in equations 10 and 11. Nitrite oxidizing bacteria (NOB), especially the genus *Nitrobacter* spp., are the major players in this process. Moreover, the genera *Nitrospina* spp., *Nitrococcus* spp., and *Nitrospira* spp. also contribute to oxidize nitrite (Watson et al. 1981). The yield coefficient of *Nitrobacter* is between 0.02 and 0.07 g-VSS/g NO_2-N (0.084 g-VSS/g NO_2-N of theoretical yield), while the oxygen consumption for nitratation is equal to 1.14 mg-O_2/mg NO_2-N (Yu et al. 2004). (Stratton & McCarty, 1967)

$$400NO_2^- + NH_4^+ + 4H_2CO_3 + HCO_3^- + 195O_2 \longrightarrow C_5H_7O_2N + 3H_2O + 400NO_3^-$$

Equation 10

$$400NO_2^- + NH_4^+ + 5CO_2 + 195O_2 + 2H_2O \longrightarrow C_5H_7O_2N + 400NO_3^- + H^+$$

Equation 11

The overall nitrification process can be written to include the consumption of carbonate alkalinity and cell synthesis reactions as follows:

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \longrightarrow 0.021C_5H_7O_2N + 0.98NO_3^- + 1.04\,H_2O + 1.88\,H_2CO_3$$

Equation 12

According to equation 12, the theoretical nitrogenous oxygen demand (NOD) or the dissolved oxygen required for converting ammonia to nitrate is equal to 4.57 mg-O_2/mg NH_3-N. Low oxygen concentration can cause an incomplete nitrification by promoting the growth of heterotrophic instead of autotrophic microorganisms. The bicarbonate alkalinity consumed for ammonia oxidation is equal to 7.1 mg-$CaCO_3$/mg NH_3-N (ranges from 6.0 to 7.4 mg-$CaCO_3$/mg NH_3-N), while an insufficient alkalinity can decrease the rate of ammonia oxidation by lowering the pH of the system. The addition of either sodium bicarbonate ($NaHCO_3$) or carbonate (CO_3^{2-}) may be required to maintain a favorable pH. Additionally, the formation of new microbial cell from the stoichiometry of nitrification is equal to 0.17 mg-cell produced/mg NH_3-N.

2.4.3 Denitrification

Biological denitrification process is the utilization of nitrate as the terminal electron acceptor in nitrate dissimilation. The reduction of nitrate is carried out under anoxic condition by denitrifiers. The microorganisms involved in denitrification are heterotrophs, autotrophs and usually facultative anaerobes, e.g. *Pseudomonas* spp., *Bacillus* spp., *Chromobacterium* spp., *Corynebacterium* spp., *Serratia* spp., *Achromobacter* spp. and *Paracoccus* spp. Denitrification consists of four steps: (1) the reduction of nitrate to nitrite by membrane-bound respiratory nitrate reductase (narX), periplasmic nitrate reductase (napY) or assimilatory nitrate reductase (nasZ); (2) the reduction of nitrite to nitric oxide (NO) by nitrite reductase (nirK and nirS); (3) the reduction of nitric oxide to nitrous oxide (N_2O) by nitric acid reductase (norN); and (4) the reduction of nitrous oxide to nitrogen gas by nitrous oxide reductase (nosM) (Zumft, 1997), as shown in equation 13.

$$NO \xrightarrow[\text{reductas}]{\text{Nitrate}} NO \xrightarrow[\text{reductase}]{\text{Nitrite}} NO \xrightarrow[\text{reductase}]{\text{Nitric oxide}} N_2 \xrightarrow[\text{reductase}]{\text{Nitrous oxide}} N_2$$

Equation 13

According to the characteristic of denitrification, this process is modeled as a first-order reaction that becomes pronounced under high organic loading and oxygen absent condition (Zhang et al. 2012). The microorganisms involved in denitrification process utilize organic carbon as an energy source for metabolism, growth, and cell synthesis. Likewise, an organic carbon can be used in deoxygenation to minimize the concentration of dissolved oxygen in aquatic environments. The organic carbon for denitrifiers is derived from two sources as internal and external sources. Internal carbon sources are the organic substrates obtained within the influent wastewater or from the accumulated materials stored within microbial cells, whereas the additional substrates, e.g. methanol, ethanol, acetate, glycerin and molasses, are external supplemental sources (Pungrasmi et al. 2016; van Rijn et al. 2006; Hamlin et al. 2008). The dissimilation reactions for denitrification using the several common organic substrates are as follows:

Methanol:

$$5CH_3OH + 6NO_3 \longrightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH \qquad \text{Equation 14}$$

Ethanol:

$$5CH_3CH_2OH + 12NO_3 \longrightarrow 6N_2 + 10CO_2 + 9H_2O + 12OH \qquad \text{Equation 15}$$

Acetic Acid:

$$5CH_3COOH + 8NO_3 \longrightarrow 4N_2 + 10CO_2 + 7H_2O + 8OH \qquad \text{Equation 16}$$

The overall denitrification process can be written to include the consumption of methanol used as carbon source and cell synthesis reactions, as follows:

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \longrightarrow 0.056C_5H_7O_2N + 0.47N_2 + 1.68H_2O + HCO_3^-$$

Equation 17

In addition, the concentration of methanol supplied in systems with the presence of nitrate, nitrite and dissolved oxygen can be calculated by the following equation.

$$C_m = 2.47(NO_3\text{-}N) + 1.53(NO_2\text{-}N) + 0.87\ (DO) \quad \text{Equation 18}$$

where: C_m is the concentration of methanol (mg L^{-1})

NO_3-N is the concentration of nitrate in wastewater (mg-N L^{-1})

NO_2-N is the concentration of nitrite in wastewater (mg-N L^{-1})

DO is the concentration of dissolved oxygen in system (mg-O_2 L^{-1})

According to equation 17, the methanol used as an external carbon source for nitrogen transformation to convert nitrate back to the atmosphere as an elemental nitrogen gas, is equal to 2.9 mg-CH_3OH/mg NO_3-N. The limitation of carbon source leads to nitrite accumulation during denitrification process (Rocher et al. 2015; van Rijn et al. 2006). The nitrate dissimilation gains an alkalinity of 3.6 mg-$CaCO_3$/mg NO_3-N; therefore, the cooperation of nitrification and denitrification can be able to eliminate the use of alkalinity supplements. The new cell formation from the stoichiometry is equal to 0.45 mg-cell produced/mg NO_3-N, while the yield coefficient of heterotrophic denitrifiers is equal to 0.4 g-VSS/g COD. Furthermore, the presence of oxygen inhibits denitrification by suppressing the nitrate-reducing enzyme production in the facultative anaerobes. Thus, the biological denitrification needs to be carried out in the absence of oxygen condition to maintain microbial efficiency.

2.5 Factors affecting nitrogen removal efficiency

Inorganic nitrogen removal by nitrification and denitrification co-processes are the collaboration between aerobe and anaerobe microorganisms. An adjustment of the suitable environmental conditions is necessary for the growth and the functions of both microbial cells. Water quality parameters, for example, salinity level, nitrogen loading, dissolved oxygen (DO), pH and temperature, play a key role in defining the function and distribution of microbial communities. The effects of environmental conditions on microorganisms involved in nitrogen cycle are as follows:

2.5.1 Salinity

Salinity is the measure of dissolved salts, mainly sodium chloride (NaCl), in the water, and expressed in term of parts per thousand (PPT) or practical salinity units (PSU). For marine aquaculture system, it is well known that salt concentration is considered as a common stress factor for microorganisms. The main cations in saline water, i.e. K^+, Na^+, Ca^{2+}, Mg^{2+} and SO_4^{2+}, are indispensable to cell growth, whereas an anion, chloride (Cl^-), is able to reduce microbial metabolic activities (Yu et al. 2004). The effects of salinity on microbial cells depend on an osmotic balance required for the growth of organisms. Some organism needs high concentrations of NaCl to begin the multiplication, whereas others are killed immediately in the presence of salt.

In terms of microorganisms in the nitrogen cycle, nitrifying bacteria are particularly susceptible to inhibition by salt. Aslan and Simsek (2012) reported that the abundance and community density of ammonia oxidizing bacteria (AOB) in biofilm were greater when wastewater was salt-free than after adding NaCl solution. High salinity levels have an inhibitory effect on the growth of both *Nitrosomonas* spp. and *Nitrobacter* spp., which involved in ammonia and nitrite oxidation, respectively (Cortés-Lorenzo et al. 2015). A research study conducted by Magalhães et al. (2005) showed that the decreased nitrification activity and inactivated nitrifying cells were found in an environment with a high salinity level (30 PSU). Similarly, Cortés-Lorenzo et al. (2015) indicated that the salt concentration of 15 g-NaCl L^{-1} (approximately 27 PSU) could deteriorate the nitrification efficiency. While the ammonia oxidation rate significantly decreased approximately 50%, and nitrite was accumulated when the salt concentration was more than 24.1 g-NaCl L^{-1} (approximately 43.5 PSU). The presence of nitrite accumulation during nitrification is resulted from the inhibition of nitritation or nitrite oxidation due to the higher sensitivity to high salinity levels of nitrite oxidizing bacteria (NOB) than AOB (Yu et al. 2004; Dincer and Kargi 1999). According to the characteristics of microbial growth, the *Nitrobacter* spp. are much sensitive to the external environmental changes than *nitrosomonas* spp.; hence, the growth of NOB is inhibited at the initial stages of the presence of toxic materials. Dincer and Kargi (1999) reported that the effects of salt inhibition on nitrite accumulation became more significant at concentrations above 2% (approximately 20 PSU). Nevertheless, when the addition of seawater was stopped, the concentration of nitrite declined gradually at the same time that nitrate increased (Yu et al. 2004).

On the other hand, some previous research studies promoted that lower salinity levels could encourage the growth of biological organisms. The presence of high numbers of *Nitrosospira* spp. was observed at salinity levels below 3.7 g-NaCl L^{-1} (approximately 6.7 PSU), whereas AOB disappeared when the salt concentration was more than 24.1 g-NaCl L^{-1} (approximately 43.5 PSU). Meanwhile, the *Nitrosomonas europaea* and *Nitrosococcus mobilis* lineage were the most abundant microorganisms under low salinity conditions (Cortés-Lorenzo et al. 2015). Yu et al. (2004) supported

that this was probably related to the characteristics of salt tolerance of some halophilic bacteria, which could survive in the presence of seawater by changing their endurable power. Cortés-Lorenzo et al. (2015) reported that the salinity level of 1 g-NaCl L^{-1} had a positive effect on the nitrification process. The removal efficiency of ammonia was stimulated when salinity increased from 0 to 15 PSU (Magalhães et al. 2005). Meanwhile, the maximum nitrification activity was found in intermediate salinities between 5 and 10 PSU (Jones and Hood, 1980) or between 0 and 20 PSU (MacFarlane and Herbert, 1984).

For denitrification rate, similar to nitrification, the efficiency of nitrate removal under no-salt condition was taken as 100%, while it slightly decreased when increasing the salinity levels (Yang et al. 1995). At salt concentrations above 1% (approximately 10 PSU), the significant decrease in denitrification efficiency was observed with high concentrations of nitrate in the effluent (Dincer and Kargi 1999). Likewise, Yang et al. (1995) reported that the denitrification capacity of an entrapped microbial cell immobilization (EMCI) reduced to 75% and 60% when adding sodium chloride at 20 and 30 g-NaCl L^{-1} (approximately 36 and 54 PSU), respectively. In terms of denitrifiers, *Marinobacter alkaliphilus* exhibited remarkably higher denitrification at concentrations of 0.5 to 1 M NaCl than at 2 and 3 M NaCl (Nakano et al. 2010). However, it seems like denitrification is more sensitive to salt compared to nitrification. The salt inhibition constant for nitrification (K_{TN}) and denitrification (K_{TD}) conducted by Dincer and Kargi (1999) indicated that the K_{TD} was found to be 15.2 g-NaCl L^{-1} (approximately 27.5 PSU), which was significantly lower than the K_{TN} (142 g-NaCl L^{-1} or approximately 256.5 PSU). The results demonstrated that ammonia removal could proceed under an extremely high–salt condition where denitrification could not occur.

Notwithstanding, in the natural environments, the activity and function of denitrifying microorganisms were less influenced by the presence of sea salts. It was probably due to the multiplication of halotolerant denitrifiers within biofilm layers or sediments, which could protect microbial cells from the external conditions (Magalhães et al. 2005). This reason was supported by Yang et al. (1995), which reported that the EMCI could tolerate higher levels of the inorganic salts. It might be related to the salt concentration gradient developed within the polymeric carrier for the relief of salt inhibition, which was similar to the mechanisms for protecting organisms from low pH and toxic materials. Also, the denitrification performance was slightly affected up to 2% salinity (approximately 20 PSU), because of the diffusion barrier for the entrapped cells (Dincer and Kargi 1999). Furthermore, it seems like the attached-growth system has greater salinity tolerance than suspended growth process due to the protection of a biofilm layer (Dincer and Kargi 1999).

2.5.2 Stocking density

According to aquaculture system, stocking density is the ratio of an animal population to the surface area of the tank, or an animal liveweight to the volume of water. Stocking density is usually related to the amount of input nitrogen from artificial feed. Normally, the dairy feeding rate is calculated from 3 to 5% of total animal liveweight. Only 25 to 30% of feed nutrients is converted into animal growth and useful energy, whereas 70 to 75% are excreted as animal feces. Therefore, the generation of high ammonia concentrations from ammonification of unconsumed feed and biological degradation of animal excretion is found in the intensive aquaculture system with high stocking density.

Although ammonia is an excellent nitrogen source for nitrifiers, the elevated ammonia levels are considered as a stress factor for microorganisms. The addition of ammonium approximately 20 µM could stimulate nitrification efficiency by 35%, but inhibited process at the concentration of 200 µM (Magalhães et al. 2005). A research study by Butturini et al. (2000) indicated that a strong ammonia regulatory caused the negative effects on nitrification activity in stream sediment biofilms. Likewise, the elevated levels of free ammonia nitrogen (FAN) might cause the inhibition of *Nitrobacter* spp. (Yu et al. 2004). Similar to the effects of salinity, it seems like NOB are more sensitive to ammonia than AOB. The activity and function of *Nitrobacter* spp. were inhibited when FAN was more than 1 mg-N L^{-1} (Anthonisen, 1976; Chaarls, 1998). The inhibition of free ammonia on nitrite oxidizers is the major factor for nitrite accumulation. Yun and Kim (2003) reported that the number of NOB in the nitrite accumulating system was less than that in the normal nitrification system due to the long-term inhibition of ammonia. Nevertheless, the activity of NOB could recover quickly as the FAN decreased below the threshold inhibition concentration (Yun and Kim 2003). High free ammonia inhibits not only NOB, but also AOA. Similar to NOB, the AOA appear to be more sensitive to ammonia than AOB. Prosser and Nicol (2012) mentioned that the AOA were being inhibited in the range of ammonia between 0.04 and 0.36 µM, while AOB inhibition was observed at 39 to 4500 µM NH_3-N. For denitrification efficiency, Della Rocca et al. (2006) reported that the denitrifiers were inhibited by ammonium at the concentration of 14.62 mg L^{-1} as NH_4-N. At the beginning of incubation, the elevated ammonia levels significantly affected the activities of nitrate and nitrite reductase, which involved in nitrate and nitrite reduction, respectively. However, both reductase enzymes had adapted themselves to high concentration of 21.22 mg L^{-1} as NH_4-N after 1 week of immobilization (Liu et al., 2014).

2.6 Biological filtration system

Among the available technologies, the biological filtration has been widely deployed in aquaculture system due to the abilities of both physical and biological treatment by using an immersed filter material (Rocher et al. 2015). During the biofiltration treatment, the wastewater is simply passed through a media, which acts as a filter and as a support for the growth of nutrient consuming bacteria. To achieve nitrogen removal, the basic knowledges of biofilm formation, types of biofilters, and acclimation methods are necessary.

2.6.1 Biofilm formation

Biofilm formation is the fundamental process of microorganisms to attach and grow on the surface area of material. The extracellular polymeric substance (EPS) is a slime matrix secreted by these organisms, and involves in the cell adhesion, microbial aggregation and biofilm formation. The EPS is a polymeric conglomeration generally composed of polysaccharides, proteins, nucleic acid, lipids and humic substances. Moreover, the lipopolysaccharide (LPS) is also found in the outer membrane of some bacteria (Uhrig 2017; Dogsa et al. 2005). The slime layer contributes to enhance the efficiency of microbial attachment, and protect bacterial cell from the effects of an adverse environment. Biofilm maturation is the complex developmental process involving five stages as follows:

Stage 1: Initial attachment

Biofilm formation begins with the accumulation of both organic nutrients and inorganic molecules on the material surface, which is referred to as a conditioning layer. The surface conditioning is essential for the biofilm establishment in which the aggregation of these substrates can create the suitable condition for the growth and multiplication of microorganisms. Initially, the conditioning layer starts as a thin sheet before turning into a thick layer within few seconds due to the adhesion of suspended cells or natural free-living organisms in the aquatic environments (Sauer et al. 2002). The reversible attachment is mostly driven by a physical process, with the combination of electrostatic, van der Waals forces, and hydrophobic interactions (Flint et al. 1997). Thus, the initial bonds between microorganisms and conditioning layer are rather weak, and can be easily destroyed by shear strength caused by the water flow. Nevertheless, with the prolonged period, the microbial adhesion becomes stronger and achieves the irreversible attachment due to the presence of dipole-dipole interactions, and covalent and hydrogen bonds.

Stage 2: Irreversible attachment

Irreversible attachment refers to the permanent adhesion of biofilm which can weather shear forces and maintain a steadfast grip on the media surface. The development of permanent biofilm is achieved within few hours after the initial

attachment of microbial cells (Sauer et al. 2002). Once attached to the surface and each other, microorganisms are able to colonize, replicate, and form the complex multispecies communities known as a biofilm. The extracellular substance is secreted by these organisms to generate the single slime layer which acts as a bridge between microbial cells and conditioning layer. This extracellular matrix can increase the efficiency of microbial adhesion, prevent organisms from the external environments, and enhance the adsorption of nutrients. Hence, during this stage, the biofilm is capable of supporting rapid growth in thickness related to the efficient nutrient entrapment (Das and Dash 2015).

Stage 3: Maturation I

After completing an irreversible attachment, the first stage of maturation is observed to occur when microbial cells became progressively layered (Sauer et al. 2002). Once microbial colonization has begun, the biofilm grows through a combination of cell division and recruitment. The development of mature biofilm is achieved within few days after the permanent film formation. Within the mature biofilm, microorganisms are able to send the signals and communicate with each other by using quorum sensing (QS) which is the small diffusible signal molecules. The QS can control a variety of physiological functions, for example, motility, conjugation, competence, sporulation, and virulence (Hammer and Bassler 2003). The cellular communication enables microorganisms to restrict the expression of specific genes to higher cell densities. Consequently, by increasing the thickness of film layer, the growth and multiplication of microbial cells contribute to developing the vertical or three-dimensional structure of mature biofilm.

Stage 4: Maturation II

Biofilm grows continuously until reaching to the maximum dimensions in the second stage of maturation which lasts for a few days (Sauer et al. 2002). At this stage, the mature film begins to develop the multiple layers with the linking channels for substrate and nutrient exchange (Coughlan et al. 2016). Microbial cells in the forming colony work together in the coordinated cooperative system for transporting nutrients to biofilm and removing waste products. This framework contributes to make the elaborate structure of biofilm, which suits to thriving in the particular environments. For this reason, the mature biofilm reaches an equilibrium that delivers oxygen, food, and nutrients while carrying away fermentation products and sloughed cells.

Stage 5: Dispersion

The final stage of biofilm formation is known as dispersion, in which microbial cells are released from the biofilm and transported to the new location for recolonization (Uhrig 2017). In addition, microbial dispersion also refers to the change in shape and size of the mature biofilm. Dispersal encourages the spreading of microorganisms by allowing the biofilm to act as a reservoir releasing cells back into the environment to

carry out the new cycle elsewhere (Coughlan et al. 2016). This stage occurs within few weeks after biofilm reaches a maximum dimension (Sauer et al. 2002). Within the mature biofilm, the presence of nutrients in close proximity to microbes leads to reduce the motility requirements and energy demands. Hence, the microbial dispersion is strongly required to remove excess biofilm for lifespan extension. Normally, the pieces of film layer may periodically slough off due to many factors, for example, flow rate dynamics, water shear strength, chemical contaminants, and changing properties of biofilm (Flint et al. 1997). The released cells may remain in the fluid as a contaminant or transport to a new surface where biofilm formation can start again.

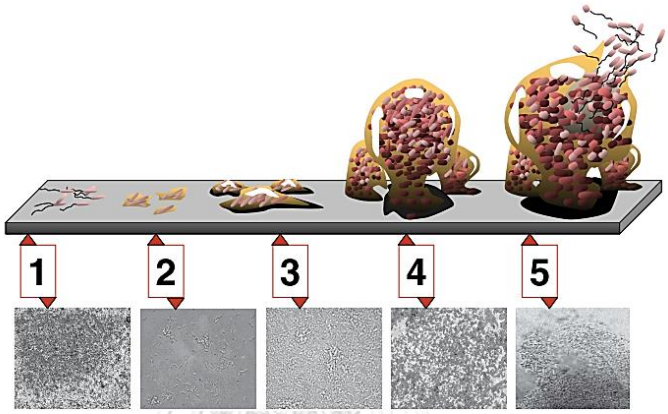

Figure 5. Five stages of biofilm formation (Monroe 2007).

2.6.2 Types of biofilter media

Biological filters are the devices to culture microorganisms that provide a space for microbial adsorption and multiplication. The acclimated biofilter is required for maintaining the water quality conditions needed to sustain healthy animal populations in aquaculture systems. The primary concern for selecting an appropriate biofilter is a space for the attachment of microorganisms. The unit of media space is usually referred to as the specific surface area (SSA). The material with a large surface area per unit volume can provide more surface available for cell growth and give the effective nitrogen removal. Furthermore, the other characteristics, for example, porosity, durability, and non-toxicity are also considered for choosing biofilter in RAS. Material with high porosity can reduce the chances of particle clogging, which causes the oxygen limitation inside biomedia. The high endurance and long lifetime capacities can reduce the maintenance cost of the biological filtration system. And importantly, biofilter must be made from the non-toxic material, which does not present potential harm to both aquatic life and microorganisms. The common biofilters applied in recirculating aquaculture systems are as follows:

2.6.2.1 BCN

BCN is made from a high-density polyethylene (HDPE) material in the cylinder shape. The outer hub ring has added thickness for superior crush resistance, while the interior striations provide additional surface area for the attachment of microorganisms. The BCN-009 (figure 6 (a)) and BCN-012 (Figure 6 (b)) are the famous series of plastic media in an aquaculture system in Thailand, which have the large SSA of 836 and 859 m^2 m^{-3}, respectively. In addition, BCN also has the large protected surfaces with small external dimensions that contribute to protect organisms from water shear strength and cell washout. The BCN-012 has more ability to provide the shelters for protecting the biomass, related to the protected surface area of 704 m^2 m^{-3}, which is higher than the protected area of BCN-009 (494 m^2 m^{-3}). In terms of the applications, the BCN can be used variable as a media in wastewater treatment and aquaculture systems by applying in the static bed filter or the moving bed filter. According to the low density of 0.95 g cm^{-3} and the light weights of 165 kg m^{-3} for BCN-009 and 150 kg m^{-3} for BCN-012, the BCN can be suspended and circulated with water masses in the moving bed biofilm reactor (MBBR) for both aerobic and anoxic applications. The movement direction of biomedia is adjusted by either aeration or mechanical stirring, depending on the design of reactor and effluent requirements. The advantages of the floating biomedia are related to the abilities to optimize microbial growth in parallel with the self-cleaning. The movement of materials with water masses can increase a change of attached organisms to contact the nutrients and oxygen. At the same time, the water shear strength contributes to maintain the appropriate thickness of biofilm layers, which can decrease the clogging problems as well as minimize the dead spots. Nevertheless, related to the low biomass carrying capacity, the nitrification and denitrification under aerobic and anoxic conditions cannot be simultaneously performed on BCN biomedia.

(a) BCN-009 (b) BCN-012

Figure 6. BCN biomedia

There are some research studies applied BCN as a media for the attachment of microorganisms in aquaculture systems. Boonpuak et al. (2011) used BCN-009 as a pre-material for the colonization of nitrite oxidizing bacteria in an indoor recirculating tank for shrimp cultivation under the salinity condition of 30 PSU, before transferring

the microbial cell into chitosan flake for studying the effects of immobilization period and pH adjustment. While the BCN-012 was used as the moving bed biofilters of marine aquaculture system in the research study conducted by Keuter et al. (2017). The bioreactor was filled with 26.5% of carriers, which were moved continuously by aerator arranged circularly at the tank bottom. The nitrification efficiency of BCN acclimated with the starter aquatic animals was 17.6 mg-TAN m^{-2} day^{-1}. Furthermore, BCN-012 was also applied in an electro moving bed membrane bioreactor (eMB-MBR) with the 30% filling ratio for wastewater treatment system. The integration of electrochemical processes into the biological treatment system could improve the performance of nutrient removal in which the enhancement of orthophosphate (PO_4-P) and ammonia nitrogen (NH_4-N) removal efficiencies were achieved up to 55.0% and 98.7%, respectively (Borea et al. 2017).

2.6.2.2 Biocord biofilter

Fibrous Biocord™ is produced from a hydrophobic polypropylene (PP) core strand covered with rings of thread, with the rope core and overall diameters of 5 and 45 mm, respectively. The SSA is equal to 2.8 m^2 m^{-1} length of biofilter or around 1,760 m^2 m^{-3}, which is sufficient for the attachment of microorganisms. In the water, the thread rings of biofilter have a positive charge, whereas microbe cells are negatively charged; therefore, microorganisms can be easily attached to material surface caused by the opposite charges. According to the polypropylene fabric in the helical structure, as shown in figure 7, the complex structure of this biofilter gives microorganisms the best conditions to multiply at both surface and interior of the cord. Thus, it is possible for the living together of aerobic and anaerobic microbial cells. Aerobes can multiply on the biofiler surface with high oxygen availability while the intricate fibers can prevent oxygen from reaching the cored center where anaerobes prevail. The outer layer of microorganisms and their biofilm can help to protect the inter layer from the toxic shock load as well as cell washout; hence, the sufficient amount of biomass is maintained on biofilter throughout the operation period. Besides, anaerobes can consume the excreta of aerobes, which makes the system becomes a balanced food chain and produces less excess sludge. The effective porosity of this biofilter is more than 99%, which eliminates the possibility of the cord clogging and provides the mass transfer. In terms of operating cost, Biocord™ has property to treat large quantities of water with the extremely low head loss and energy consumption. The aeration requirement for attached growth systems is lower when compared to the activated sludge process. Moreover, due to the living together of two organisms, nitrogen removal used biofilter reduces the chemical supply for maintaining alkalinity by simultaneous nitrification and denitrification process (Li et al. 2008). For the maintenance, the fibrous biofilter can be cleaned simply by washing in tap water for an elimination of excess solid deposit. The average life expectancy of Biocord™ is

approximately 5 years due to an endurance of synthetic fiber; however, the polypropylene fiber can be deteriorated by ultraviolet radiation, which makes this biofilter is not suitable for the outdoor aquaculture system. Biocord^{TM} can be installed easily within the aquaculture pond or used as a media in many types of bioreactors, for example, submerged membrane bioreactor (SMBR) and trickling filter (TF), related to its weight of 34 g m^{-1} length of the biofilter.

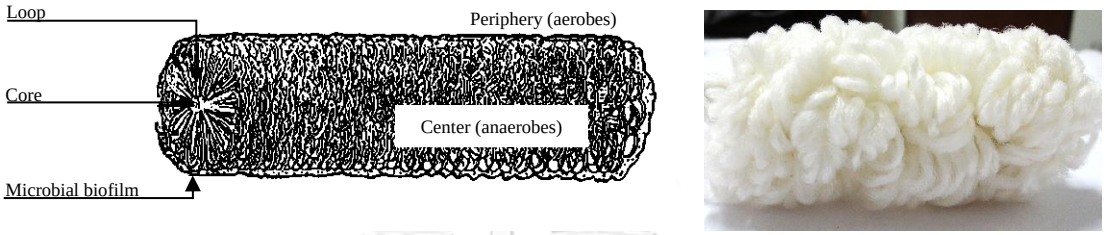

Figure 7. Fibrous Biocord^{TM} biofilter structure

There are some research studies applied Biocord^{TM} as a biomedia in water treatment process. Zhang et al. (2012) located fibrous biofilter vertically in the bio-contact oxidation system upstream, followed by an ecological floating bed, and a vertical moveable eco-bed downstream to improve water quality in Taihu Lake Basin, China. Fibrous biofilter had the highest BOD oxidation rate to convert the main pollutant, organic nitrogen, to inorganic compounds which would be more easily adsorbed by following systems. While the continued study by Yuan et al. (2012) applied Biocord^{TM} as solid capture device to eliminate suspended solid and turbidity in wastewater. The results showed that the total efficiencies of solid and turbidity removal were 87.2% and 84.9%, respectively, at the flow rate of 4 L min^{-1}. Additionally, the fibrous biofilter was acclimated in the river for 50 days in the range of 18 to 23°C to allow a self-formed biofilm, and then used for nitrogen and phosphorus removal. In terms of aquaculture systems, the study performed by Sesuk et al. (2009) functioned Biocord^{TM} as a nitrifying biofilter in RAS with pre-immobilization in synthetic wastewater for 78 days. The completed nitrification was occurred within 3 weeks during acclimation period, and nitrifying biofilter had high efficiency to keep ammonia and nitrite lower than 1 mg-N L^{-1} throughout the 44 days of experiment, while inorganic loading increased from 1.24 to 2.78 mg-N L^{-1}. Besides, the nitrifying biofilm process and single solid separating were operated in a Tilapia aquaculture system by Khammi et al. (2015). The fibrous biofilter length of 6.0 m was able to accommodate the stocking density as high as 5.0 kg m^{-3}, which corresponded to nitrogen loading rates of 8.4 mg-N L^{-1} day^{-1}.

2.6.2.3 Japanese filter mat

Japanese filter mat is the square media made of the curled polyester (PE) fibers with a small diameter of 0.25 mm, as shown in figure 8. According to the characteristics of synthetic polymer, the polyester fibers are noted for being biologically inert, water resistant, and environmentally friendly. The specific surface area of Japanese filter mat is equal to 300 m^2 m^{-3}, which is lower than the values of BCN and Biocord™. However, according to the complicated structure, the curled polyester fibers make the ideal environment and dwelling for many different kinds of microorganisms. The filter mat is especially designed to provide a suitable place for colonization of both aerobes and anaerobes. Similar to Biocord™ biofilter, the aerobic microorganisms can multiply on material surface, while the intricate polyester fibers can prevent the inter anaerobes from oxygen diffusion. Therefore, the Japanese filter mat can perform the simultaneous nitrification and denitrification under both aerobic and anoxic conditions within the structure. For the application, this biofilter is widely used in aquaculture, aquarium, and aquaponics. The filter mat can be utilized for biological nitrogen removal in fixed-bed reactors, as well as applied in the filtration unit for removing solid particles. According to the previous experiment conducted by Khammi et al. (2015), Japanese mat acted as a filter media in filtration unit of RAS. The excess solids were kept within the curled fibers which contributed to control the concentration of suspended solids in the range of 20 and 35 mg-SS L^{-1}. In addition, the solid debris preserved within biomedia can be used as an internal carbon source for heterotrophic denitrifiers, which can reduce the addition of extra carbon substrates. Likewise, the occurrence of anoxic process contributes to pH balance and decrease the chemical supply for maintaining alkalinity in the systems.

Figure 8. Japanese filter mat

2.6.3 Biofilter acclimation

The primary essential process to develop the stable and viable microbial biofilm on media used in aquaculture system is the biofilter acclimation. The short period of time and complete nitrogen removal through nitrification and denitrification co-

processes are expected for this step (Zhu et al. 2016). Normally, an immobilization process for nitrifying biofilm always takes a long time (range, 28 to 60 days) at the temperatures between 21 and 26°C, and more than 40 days in the system without seeding (Achuthan et al. 2006). Moreover, the changes in environmental conditions, for example, salinity level, nitrogen loading, and temperature, have a negative effect on the growth and functions of microorganisms. Incomplete nitrification with nitrite accumulation is the main issue in marine and brackish aquaculture systems. The salt concentration is considered as a common stress factor, which is able to reduce the microbial metabolic activities, especially for nitrite oxidizing bacteria. Therefore, this problem appears to justify the development of new immobilization procedures (Gutierrez-Wing and Malone 2006).

Many previous research studies involved in improving method for biofilm establishment in aquaculture systems, as shown in table 4. The desirable method for biofilm formation is the seeding, which is to develop microorganisms within biofilter media before starting an aquaculture system. This method can improve water quality by controlling the nitrogen concentration since the first day of culture period, even in high stocking density or high feeding rate. The natural colonization in synthetic wastewater is the popular seeding method to develop the mature biofilm for fresh and marine aquaculture (Kuhn et al. 2010). The ammonium solution, ammonium chloride (NH_4Cl), is normally used as a nitrogen source for nitrifers. The supplemental nutrients, moreover, are added to stimulate the growth of microorganisms. This method is not complicated and easy to operate without the outbreak disease. However, the natural colonization in synthetic wastewater always takes a long time (approximately 4 to 8 weeks) to develop the stable nitrification ability and nitrifier populations in marine biofilter, which possibly due to the influence of environmental stresses (Kumar et al., 2013).

Using sludge from the full-scale treatment plant can solve the problem of long-time acclimation by applying the ready sludge in aquaculture systems. Nevertheless, the sludge from other systems can be easily washed out from biofilter due to the weak attaching ability on carriers. Besides, it may carry a potential pathogen, which increases the risk of disease outbreak in RAS. Using of the exogenous extracellular polymeric substance (EPS) and the inoculated carries from stable system can also give the rapid biofilm establishment (Tsuneda et al., 2001; Zhu et al., 2016). The microbial cells and their biofilm are released from the inoculated carries which have the homogenous surface characteristics for colonization on new carriers. Thus, the strong attachment of microbial cells on the cleaned biofilters can prevent biomass from wash-out. Zhu et al. (2016) reported that the 15% inoculated carries were enough to establish complete nitrification on new carries, whereas the reactor without mature biofilm was found nitrite accumulation at 60 mg-N L^{-1}. In reactors with higher seeding ratios, however, the new biofilm could be inhibited by mature biofilm through the substrate competition.

Although these methods have an outstanding performance, they are not suitable for aquaculture system in Thailand because of high operating cost and complicated operation. A report conducted by Dennis and Thomas (2012) showed that the natural microorganisms can also introduce by the small numbers of starter animals from an already operating aquaculture system. The growth of aquatic animals can proceed at the same time of the bacterial colonization on the media surface without having an activated biofilter. Hence, this start method is suitable mainly for operating by aquaculturists. Additionally, the advantage of using organisms entered with starter animals is the attribute that allows an appropriate microorganism for each system.

The natural colonization with starter aquatic animals has a higher nitrification efficiency than the immobilization in synthetic wastewater (Keuter 2011). Rapid acclimation of virgin plastic media in aquaculture system is desirable to minimize biofilter activation time and to reduce the accumulation of toxic ammonia and nitrite. Keuter et al., (2017) reported that the nitrifying potentials of AOB on material acclimated in bioreactor which feed on ammonia and nitrite was equal to 1.14 mg-TAN m^{-2} day^{-1}, whereas the potential ammonia oxidizing activity of 17.6 mg-TAN m^{-2} day^{-1} was observed when introducing the 21.3 kg of Turbot fish.

Notwithstanding, some diseases caused by pathogen infection can be found in the system started with this method related to the pathogen adhesion on animal skin. The disinfection by low concentrations of either chlorine or formalin is required before beginning RAS. Furthermore, the rapid increase in nitrogen concentration at the first culture period due to an inactive biofilter is one of the most concerned problems, especially in intensive aquaculture system. Consequently, exchanging water is strongly recommended to reduce the effect on aquatic organisms.

Table 4. Methods for biofilm establishment in aquaculture system.

Method	System	Advantages	Disadvantages	Reference
Freeze-dried inoculates of nitrifiers	Freshwater aquarium	- Rapid establishment of nitrification	- Inconsistent performance	Spotte (1992)
Exogenous extracellular polymeric substance (EPS)	Aquaculture	- Without outbreak disease - Rapid establishment	- Complicated and expensive - Not suitable for large systems	Tsuneda et al. (2001)
Availability of ammonia binding inoculum liquid (ABIL)	Freshwater aquarium	- Useful in freshwater - Complete nitrification	- Sensitive to salinity variations - Not useful for shrimp hatcheries	Grommen et al. (2002)

Table 4. Methods for biofilm establishment in aquaculture system (continued).

Method	System	Advantages	Disadvantages	Reference
Natural colonization in synthetic wastewater	Freshwater and marine aquaculture	- Without outbreak disease - Easy to operate	- Take a long time (4-8 weeks) for establish only nitrification	Kuhn et al. (2010); Sesuk et al. (2009)
Natural colonization with starter aquatic animal	Freshwater and marine aquaculture	- Rapid establishment of nitrification - Complete nitrification - Well-suited microorganisms	- Risk of outbreak disease - Stressful for aquatic animal	Dennis and Thomas (2012); Keuter et al. (2017)
Inoculated carries from stable system	Marine aquaculture	- Complete nitrification - Rapid establishment (32 days in 15% of seeding ratio)	- New biofilms could be inhibited by inoculated carries due to substrate competition - Risk of outbreak disease	Zhu et al. (2016)

2.7 Microbial community involved in nitrogen removal

Microorganisms play important roles in the biological nitrogen removal. The understanding of microbial characteristics and their functions leads to the development of improved nitrogen removal efficiency. The microorganisms involved in nitrification and denitrification are as follows:

2.7.1 Ammonia oxidizing archaea (AOA)

According to the discovery in 2005, aside from bacteria, ammonia oxidizing archaea (AOA) are considered as the additional group of microorganisms involved in ammonia removal through nitritation (Konneke et al. 2005). The AOA are classified as *Crenarchaeota*, and placed into the archaeal phylum *Thaumarchaeota* (Pester et al. 2011). The ammonia monooxygenases subunit A (*amo*A) genes that are members of the copper-containing membrane-bound monooxygenase (CuMMOs) enzyme family, are carried in AOA genomes (Pester et al. 2012). In previous studies, the pure cultures of *Nitrosopumilus maritimus* (Konneke et al. 2005) and *Nitrososphaera gargensis* (Hatzenpichler et al., 2008) were confirmed to carry archaeal *amo*A. Furthermore, the *amo*A gene was also observed in the genome of uncultured *Cenarchaeum symbiosum* (Hallam et al., 2006).

Archaea have been detected in ubiquitous environments, including fresh waters, marine waters, coral reefs, estuaries, sediments, and soils. According to the previous study, the archaea were identified as the dominant ammonia oxidizers in freshwater aquaculture related to the low ammonia concentrations in aquaculture systems (Sauder et al. 2011). The AOA can survive under nutrient limitation conditions with the ammonia levels lower than 0.2 µM (Martens-Habbena et al., 2009). While the substrate threshold of TAN for the growth of Thaumarchaeote *Nitrosopumilus maritimus* is as low as 10 nM (Pester et al. 2011). Pester et al. (2012) indicated that the preference of archaea for low substrate concentrations was associated with their physiological characterization. However, the AOA were being inhibited in the range of ammonia between 0.04 and 0.36 µM (Prosser and Nicol 2012).

Archaea can be found in the extreme environments, for example, low pH, poor oxygen availability, high temperature, and sulfide contamination. The *Candidatus* Nitrosotalea devanaterra can grow in the pH range 4.0 to 5.5 (Gubry-Rangin, 2011) due to the development of new mechanisms for ammonia removal under acidic conditions (Lehtovirta-Morley et al., 2011). The AOA are numerically predominant in aquaculture sediments where feeding debris are accumulated (Lu et al., 2015). The abundance of archaea in under this organic-rich conditions are correlated with the resistance of low dissolved oxygen levels (Francis et al. 2005). Moreover, the archaea are able to grow at high temperature due to the thermotolerant or heat-resistant of their membrane lipids (Koga 2012). Notwithstanding, under higher light intensities at water surface, the archaeal growth is prohibited due to the characteristic of photoinhibition (Merbt et al. 2012).

2.7.2. Ammonia oxidizing bacteria (AOB)

For more than hundred years, the first step of nitrification, nitritation process, has long been considered to be carried out solely by ammonia-oxidizing bacteria (AOB) (Winogradsky, 1890). The chemolithoautotrophic AOB belong to *Beta-* and *Gamma-proteobacteria*, which use oxygen as an electron acceptor to oxidize ammonia via two steps by using ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) enzymes (Teske et al., 1994; Purkhold et al., 2000). The β-proteobacterial AOB consist of the genera *Nitrosomonas* and *Nitrosospira*, while the *Nitrosococcus spp.* belongs to γ-*proteobacteria* (Prosser, 2007). In addition, some subgenera, including *Nitrosolobus* spp. and *Nitrosovibrio* spp., can also autotrophically involve in ammonia oxidation (Watson et al. 1981). The *Nitrosomonas* is frequently identified as the dominant genus of AOB (Sharma and Ahlert 1977). The biological characteristics of *Nitrosomonas* spp. are shown in table 6.

The chemolithoautotrophic nitrifying bacteria have been found in fresh waters, marine waters, sewage systems, sediments, and soils. The AOB can multiply under various nutrient conditions. In ammonium-rich systems, the *Nitrosomonas* spp. is

dominant in the range of ammonia between 5 and 70 μM (Itoi et al. 2006) or between 20 and 100 μM (Foesel et al., 2008). While *Nitrosospira* spp. is abundant in terrestrial habitats where ammonia concentrations are lower than 1 μM (Bruns et al., 1999; Kowalchuk and Stephen, 2001). Although the optimum pH for cell growth is between 7.6 and 7.8, the AOB can survive under both acidic and basic conditions. The growth of AOB under inappropriate conditions might be possible by the formation of cell aggregates within the biofilm layer (Schmidt et al. 2002). In terms of dissolved oxygen, the AOB are considered to have a strictly chemolithoautotrophic aerobic metabolism, in which the oxygen is necessary for their growth and functions. Theoretically, the oxygen consumption for driving nitritation by nitrifying bacteria is equal to 3.43 mg-O_2/mg NH_3-N (Yu et al. 2004). Nevertheless, it is interesting to note that the *Nitrosomonas* spp. is aerobic nitrifiers that can survive in anoxic environments (Abeliovich and Vonhak, 1992). A research study by Wu et al. (2010) reported that the AOB were predominant in the sediments of Eastern Taihu Bay where oxygen was absent, which was likely due to the presence of rich organic substances. The AOB could denitrify with either hydrogen or organic compounds as electron donors due to the metabolic versatility of various substrates (Bock et al. 1995). It is well known that the temperature is a factor to select the different lineages, to change the diversity, and to control the biogeographic distribution of AOB (Wu et al. 2013). The optimal temperature for the bacterial growth is around 30°C, while the AOB activity begins to decline at 35°C (Gabarró et al. 2012).

Table 5. Optimum growth conditions for AOB genera (Watson et al., 1989; Holt et al., 2000).

Conditions	*Nitrosomonas*	*Nitrosococcus*	*Nitrosospira*	*Nitrosolobus*	*Nitrosovibrio*
pH	7.5 – 8.0	7.5 – 8.0	7.5 – 8.0	7.5	7.5 – 7.8
Temp	25 – 30°C	25 – 30°C	20 – 35°C	25 – 30°C	25 – 30°C
Media	Chemo-lithotroph	Chemo-lithotroph	Obligate chemo-lithotroph	Chemo-lithotroph	Chemo-lithotroph

2.7.3 Nitrite oxidizing bacteria (NOB)

Nitrite oxidizing bacteria (NOB) are the main players in the second step of nitrification. Similar to AOB, the NOB are known as the chemolithoautotrophs, which obtain energy from the oxidation of inorganic compounds. During nitratation process, nitrite is used as a nitrogen source for NOB, and completely oxidized to nitrate using nitrite oxidoreductase (Nxr) enzyme (Klotz and Stein, 2011). Normally, the NOB consist of four validly described genera, i.e. *Nitrobacter*, *Nitrospina*, *Nitrococcus* and *Nitrospira* (Bartosch et al., 1999). The *Nitrobacter* is frequently identified as the

dominant genus in nitratation (Sharma and Ahlert 1977). The biological characteristics of *Nitrobacter* spp. are shown in table 6. Notwithstanding, some research study claimed that the *Nitrospira* spp. had been found to be the main nitrite oxidizers in wastewater treatment plants and marine aquaculture systems due to the better scavengers of nitrite and oxygen than *Nitrobacter* spp. (Brown et al. 2013).

The NOB are known to be especially sensitive to the external environment (Abeliovich 2006). There are many factors that affect the growth and functions of NOB, for example, ammonia concentration, pH, dissolved oxygen, and temperature. The elevated levels of organic nitrogen and ammonia cause the inhibition of NOB. The activity and function of *Nitrobacter* spp. are disabled when the FAN levels are over than 1 mg L^{-1} (Anthonisen, 1976; Chaarls, 1998). A research by Yu et al. (2004) reported that the occurrence of nitrite accumulation at high concentrations was observed when the ammonia concentrations were more than 40 mg L^{-1}, and the pH values were between 7.5 and 8.0. Theoretically, the oxygen consumption for driving nitratation by NOB is equal to 1.14 mg-O_2/mg NO_2-N. Nevertheless, in practical terms, the growth of *Nitrobacter* spp. is inhibited under the conditions of dissolved oxygen lower than 3 mg L^{-1} (Yu et al. 2004). In terms of temperature, the growth of NOB is inhibited at the temperature between 25 and 28°C. Meanwhile, in the range of 30 to 35°C, the difference between the growth rates of *Nitrobacter* spp. and *Nitrosomonas* spp. give rise selection pressure, resulting in nitrite accumulation due to the inhibition of NOB growth (Yu et al. 2004).

Table 6. Biological characteristics of *Nitrosomonas* spp. and *Nitrobacter* spp. (Yu et al. 2004)

Parameter	***Nitrosomonas* spp.**	***Nitrobacter* spp.**
Bacteria size, µm	1 × 1.5	1 × 1.5
Generation cycle, h	8 – 36	12 – 59
Trophic type	Autotrophic	
Type of bacteria	Strictly aerobic	
Shape	Ellipse or bar shape	
The most ratio growth rate, µm/ h	0.04 – 0.08	0.02 – 0.06
Yield coefficient, Y	0.04 – 0.13	0.02 – 0.07
Saturation constant K, mg L^{-1}	0.6 – 3.6	0.3 – 1.7

Table 7. Optimum growth conditions for NOB genera (Watson et al., 1989; Holt et al., 2000).

Conditions	*Nitrobacter*	*Nitrospina*	*Nitrococcus*	*Nitrospira*
pH	6.5 – 8.5	7.0 – 8.0	7.5 – 8.0	7.6 – 8.0
Temp	5 – 37°C	20 – 30°C	25 – 30°C	20 – 30°C
Media	Facultative Chemo-lithotroph	Obligate chemo-lithotroph	Obligate chemo-lithotroph	Chemo-lithotroph

2.7.4 Denitrifying microorganisms

Denitrifiers are heterotrophic microorganisms that involve in the reduction of nitrate to an elemental nitrogen gas by consuming either inorganic or organic substrates as sources of carbon and energy. Most denitrifiers are the facultative anaerobic bacteria which belong to a wide range of various subclasses of *Alpha-*, *Beta-*, *Gamma-*, *Epsilon-proteobacteria*, high-, and low-GC Gram-positive bacteria (Ambus and Zechmeister-Boltenstern 2007). Moreover, denitrification is also found among the *Firmicutes, Actinomycetes, Bacteroidetes,* and *Aquificaceae* as well as among the archaea (Braker and Conrad, 2011). There are more than 50 genera of denitrifiers that function in nitrate removal. The genera *Pseudomonas, Ralstonia, Alcaligenes, Paracoccus, Rhodobacter, Rubrivivax, Thauera, Burkholderia, Bacillus,* and *Streptomyces* have been pointed out as the dominant denitrifiers in various environments, while the marine denitrifiers are dominated by *Shewanella baltica* and *Marinobacter* spp. (Mrkonjic Fuka et al. 2007).

Denitrifiers are commonly found in many natural surroundings, for example, soils, marine and freshwater sediments (Mrkonjic Fuka et al. 2007). Normally, the denitrifying bacteria represent around 10 to 15% of the total bacterial population in environments (Ambus and Zechmeister-Boltenstern 2007). Due to their characteristics, the major factors affecting denitrifiers are oxygen and carbon source. Hernandez and Rowe (1987) reported that the oxygen had effect on nitrate respiration. The inhibitory effect of oxygen was maximum at around 0.2% oxygen saturation. Nevertheless, in the presence of oxygen, the denitrification activities have also been observed due to the multiplication of denitrifiers within biofilm layers or sediments, which can protect microbial cells from the external conditions (Sirivedhin and Gray 2006). It is well known that the denitrifiers utilize either inorganic or organic carbon as an energy source for metabolism, growth, and cell synthesis. Rocher et al. (2015) indicated that the nature of the carbon source could affect nitrite accumulation by inhibiting nitrite reduction. Methanol seemed to be the best source for denitrifiers, indicating by the lowest concentration of nitrite accumulation of 0.05 g NO_2-N per g NO_3-N. Besides, the optimal COD: Nitrate-N ratio of 5:1 can allow a complete denitrification without any nitrite accumulation (Pungrasmi et al. 2013).

Chapter 3
Methodology

3.1 Experimental framework

This research was divided into two main studies, including:

Study 1: Effects of salinity, stocking density, and acclimation period on nitrogen removal efficiency and microbial community

Biofilter acclimation process was accomplished in the indoor aquaculture tank in order to allow the attachment and growth of natural microorganisms. The Pacific white shrimps were used as the experimental animals in the RAS. The effects of salinity on nitrification and denitrification efficiencies, and microbial diversity were studied by varying the salt concentrations at 5, 15 and 25 PSU. For the effects of nitrogen loading, the stocking densities of shrimps in an aquaculture tank were adjusted at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) levels. The experiment was continuously operated under aerobic condition approximately 60 days without water exchange. The operating conditions in RAS were maintained as the following: DO > 4 mg-O_2 L^{-1}, pH = 7.5 – 9, temperature = 28 – 31ºC and alkalinity 100 – 150 mg-$CaCO_3$ L^{-1}. Thereafter, the shrimps were harvested before switching the aquaculture tank to anoxic condition for denitrification process approximately 20 days. Methanol was supplied as an external organic carbon source for denitrifiers at COD: Nitrate-N ratio of 5:1. The biofilter samples were collected at week 2, 4, 6, and 8 during biofilm formation mode, and on the last day of the experiment (day 80) for the estimation of nitrification and denitrification rates. Nitrogen removal efficiency was calculated and analyzed using one-way analysis of variance (ANOVA) to determine the optimal conditions of salinity level, stocking density, and acclimation period for RAS. The surface morphology of biofilms on week 8 at the different salinity levels were observed by scanning electron microscopy (SEM). Furthermore, the microbial communities in biofilter were monitored by applying the next-generation DNA sequencing method (MiSeq) using universal primers for bacteria.

Study 2: Application of biofilter in marine RAS for long-term operation

The Biocord^{TM} biofilter was installed in the 2000 L aquaculture tanks, which was adjusted the salinity level of 25 PSU and shrimp stocking density of 1 kg m^{-3}. The biofilter acclimation was performed during the first 60 days, and thereafter the aerobic shrimp cultivation with complete nitrification was continuously operated for 60 days without water exchange, except for adding water to compensate for lost water due to an evaporation. During the experimental period, the biofilters were monthly cleaned for elimination of excess solid deposit as well as for prevention of H_2S production. Moreover, the shrimp was randomly recorded the body weight for food quantity adjustment by maintaining the feeding rate in the range of 3 to 5% of total body weight per day. After 120 days, the experiment was switched to anoxic condition for nitrate removal through denitrification process. The shrimp was harvested as well as locating the submersible water pump in aquaculture tank instead of air stone diffusers. The methanol at COD: Nitrate-N ratio of 5:1 was supplied for denitrifiers, and then adjusting periodically when finding that the nitrate concentration tends to remain constant. The anoxic condition was performed approximately 10 days or until nitrate concentrations are lower than 10 mg-N L^{-1}. After finishing the first crop, the air pump was turned on again to re-oxygenate approximately 1 to 2 days before beginning the next crop. To evaluate the performance of long-term operation, the experiment was operated through a second round of replication following the 60 days of cultivation and 10 days of anoxic condition. The biofilter samples were collected biweekly and after finishing denitrification process for the estimation of nitrogen removal rates. The extracted DNA obtained from biofilters were applied for microbial community analysis by the Illumina MiSeq system to study the changes of microbial diversity during long-term operation.

The overall experiment framework of this research is shown in figure 9.

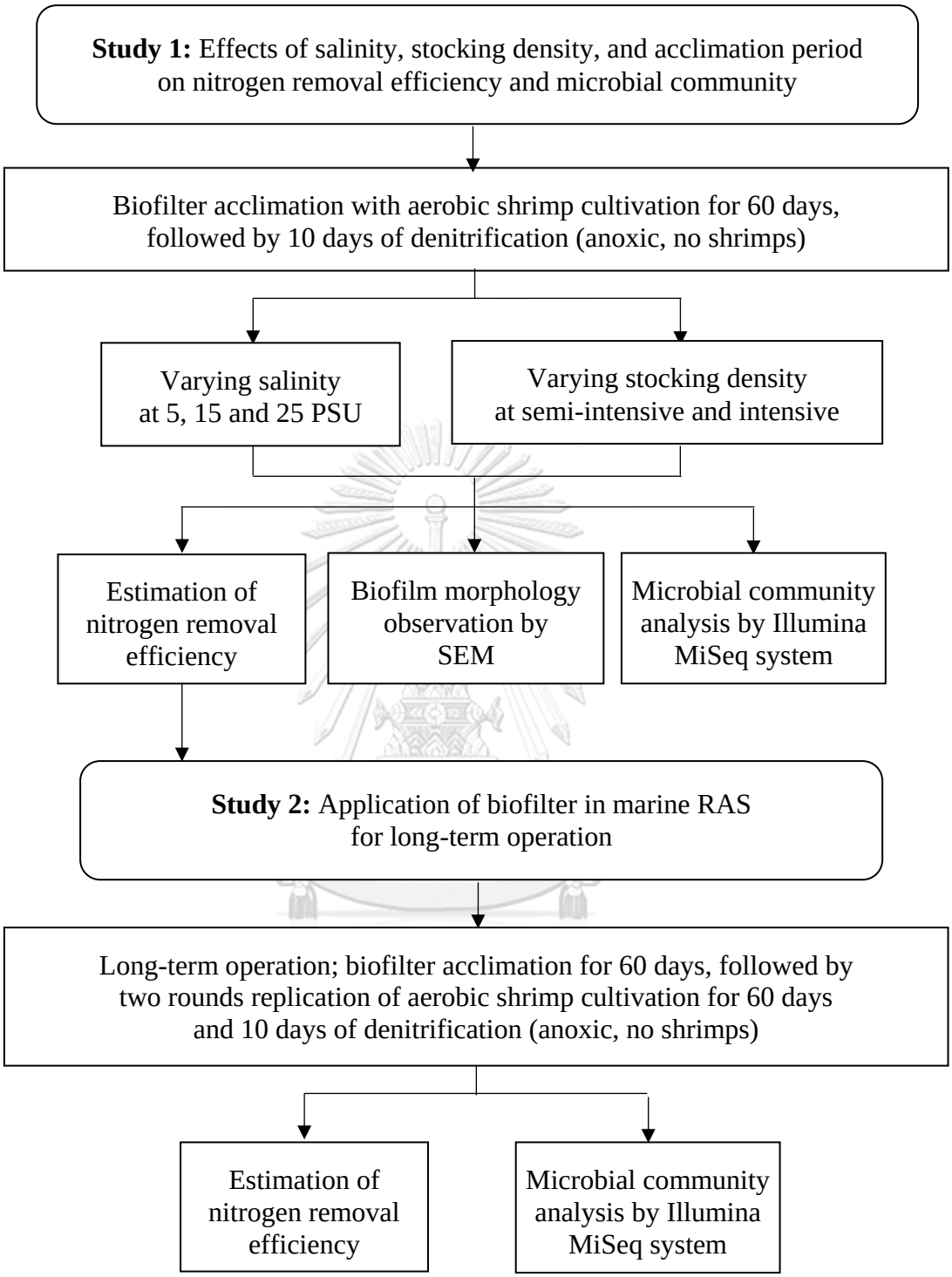

Figure 9. Experiment framework of this study.

3.2 Study 1: Effects of salinity, stocking density, and acclimation period on nitrogen removal efficiency and microbial community

The objectives of this study were to evaluate the effects of salinity, stocking density, and acclimation period on nitrification and denitrification efficiencies, and microbial diversity in biofilter during acclimation period. The experiment was divided into 4 parts including: 1) acclimation of biofilter in aquaculture tank, 2) estimation of nitrification and denitrification efficiencies and 3) analysis of microbial community as follows:

3.2.1 Acclimation of biofilter in aquaculture tank

The objective of this part was to determine the optimal conditions of salinity level, stocking density, and acclimation period for nitrification and denitrification co-processes in RAS. The 10 cm length of fibrous Biocord^{TM} biofilter (25 pieces) and the 10×23×4 cm^3 of Japanese filter mat (25 pieces) with the same specific surface area of 0.28 m^2 were washed in tap water, dried in the hot air oven, and marked with numbers prior to use. Biofilter acclimation was performed in the 240 L indoor aquaculture tank in order to allow the attachment and growth of natural microorganisms on the material surface. The installation diagram of biofilters in aquaculture system is shown in figure 10(a). The 45-day old Pacific white shrimp, *Litopenaeus vannamei*, was used as the experimental animals in the RAS. The effects of salinity on nitrification and denitrification efficiencies, and microbial diversity were studied by varying the salt concentrations in an aquaculture tank at 5, 15 and 25 PSU. For the effects of nitrogen loading, the stocking densities of shrimps in an aquaculture tank were adjusted at two levels, i.e. semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}), with the initial densities of approximately 0.5 and 1.0 kg m^{-3}, respectively. Shrimps were fed daily at 3% feeding rate of the total weight, with an artificial feed contained more than 36% of protein by rationing at two times (10:00 h and 16:00 h). The suitable conditions in an aquaculture tank were prepared for the existence of white shrimp and aerobic microorganisms. The optimal alkalinity was adjusted on the first day of the experiment in the range of 100 to 150 mg-$CaCO_3$ L^{-1}, and sodium bicarbonate ($NaHCO_3$) was added periodically in order to maintain the pH between 7.5 and 9 (Tharavathy 2014). The major minerals, for example, calcium (Ca) and magnesium (Mg), were prepared for shrimp molting and new shell formation at the concentrations of 58 and 196 mg L^{-1} by adding calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) (Boyd et al., 2003). Air stone diffusers were installed at the bottom of the tank to keep high oxygen available (>4 mg-O_2 L^{-1}), to ensure a completely-mixed state, as well as to prevent anaerobic condition and the production of toxic metabolites. The experiment was continuously operated approximately 60 days without water exchange, except for adding water to compensate for lost water due to an evaporation.

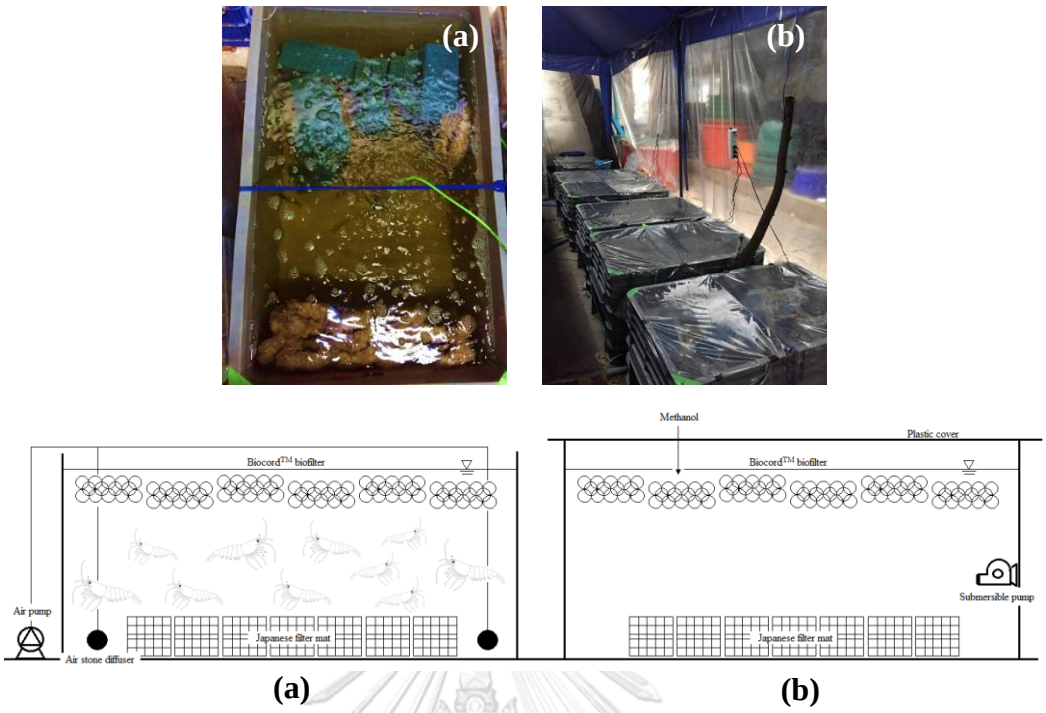

Figure 10. Installation diagram of fibrous Biocord^{TM} biofilter and Japanese filter mat in the shrimp culture tank for a) aerobic and b) anoxic conditions.

After 60 days, the experiment was switched to anoxic condition for nitrate removal through denitrification process. Shrimp was harvested and recorded the total number, length, and body weight for analysis of the growth performance and survival rate. Air stone diffusers were taken out of aquaculture tank and locating the submersible water pump instead (figure 10(b)), to circulate water mass and to avoid hydrogen sulfide (H_2S) production from anaerobic zone. Methanol (CH_3OH) was supplied as an external organic carbon source for denitrifiers at COD: Nitrate-N ratio of 5:1 (Pungrasmi et al. 2013) on day 61 of the experiment, and adjusted periodically when finding that the nitrate concentration tends to remain constant. Besides, the aquaculture tank was covered by the plastic sheet for preventing oxygen diffusion from the atmosphere. This anoxic condition was operated continuously approximately 20 days or until nitrate concentrations are lower than 10 mg-N L^{-1}. Water samples from aquaculture tank were collected daily for ammonia, nitrite, and nitrate analysis to monitor changes in the nitrogen profiles throughout 80 days of the experiment. Biofilter samples, Biocord^{TM} and Japanese mat, were collected at week 2, 4, 6 and 8, and on the last day of the experiment for the estimation of nitrification and denitrification efficiencies in section 3.2.2. Additionally, the extracted DNA obtained from biofilters were applied for microbial community analysis by the next-generation DNA sequencing method (MiSeq) in section 3.2.3.

3.2.2 Estimation of nitrification and denitrification efficiencies

The objectives of this part were to evaluate the effects of salinity, stocking density, and acclimation period on nitrification and denitrification efficiencies, and to compare the nitrogen removal rates between fibrous Biocord^{TM} biofilter and Japanese filter mat. The acclimated biofilter samples from aquaculture tanks (in section 3.2.1) were placed in 3 L test chamber for the estimation of nitrification rate, as shown in figure 11(a) and 12(a). The decrease of ammonia concentration was monitored after adding 2.5 L synthetic wastewater containing 1.5 mg-N L^{-1} of ammonium chloride. The continuous aeration was provided directly through diffuser stones to keep DO concentrations higher than 4 mg-O_2 L^{-1}; therefore it was assumed that oxygen is not a limiting factor. An optimal alkalinity for nitrifiers was maintained between 100 to 150 mg-$CaCO_3$ L^{-1} by sodium bicarbonate. All batch experiments were operated in triplicate for approximately 5 days or until ammonia concentrations are undetectable.

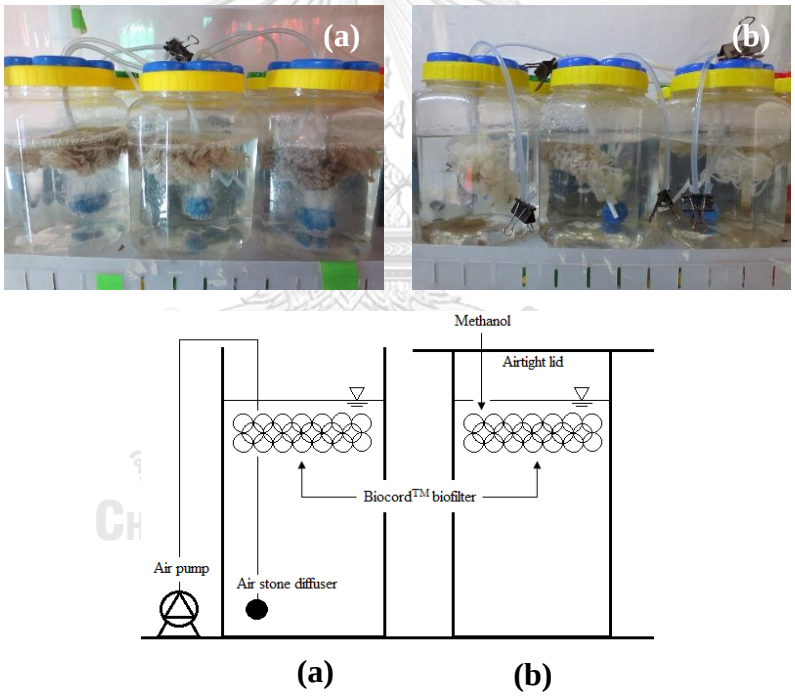

Figure 11. The test chambers for determination of a) nitrification and b) denitrification efficiencies of acclimated fibrous Biocord^{TM} biofilter.

Afterward, the wastewater in reactor was discharged carefully by avoiding biomass losses. Denitrification efficiency, on the other hand, was performed by adding 2.5 L synthetic wastewater containing 10 mg-N L^{-1} of sodium nitrate solution instead. The anoxic condition for nitrate removal was provided by turning off an air pump and covering the test chamber with the airtight lid (figure 11(b) and 12(b)). Furthermore, the COD: Nitrate-N ratio was adjusted on the first day of the experiment at 5:1

(Pungrasmi et al. 2013) by injecting the 0.105 ml of methanol as an external organic carbon source for denitrifiers. The experiment was operated for 5 days or until nitrate concentrations are undetectable.

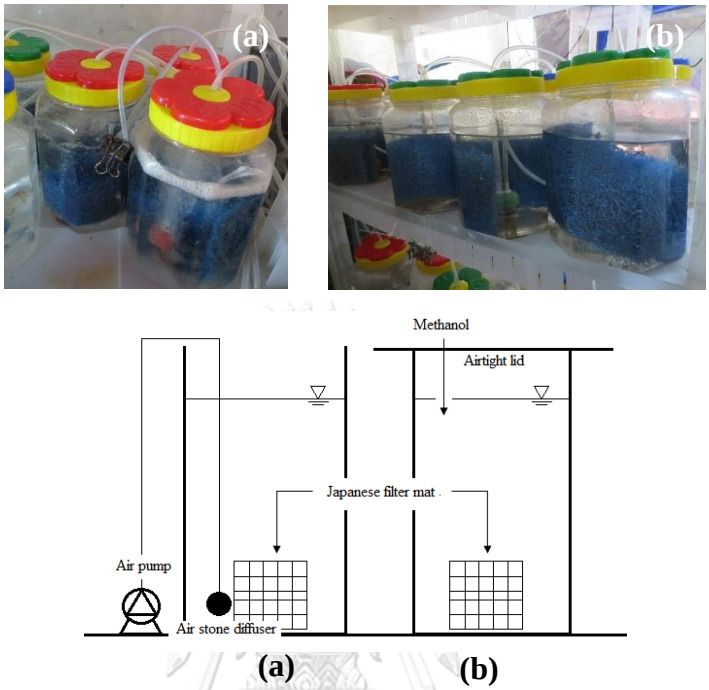

Figure 12. The test chambers for determination of a) nitrification and b) denitrification efficiencies of acclimated Japanese filter mat.

- **Analytical methods for water quality**

Water samples from aquaculture tanks (in section 3.2.1) and aerobic-anoxic test chambers (in section 3.2.2) were collected and filtered with filter papers (Whatman® glass microfiber filters; 25 mm). The filtered water was kept frozen at -20 ºC prior to analysis. Total ammonia nitrogen (TAN), nitrite-nitrogen (NO_2^--N), and nitrate-nitrogen (NO_3^--N) were analyzed by using microplate spectrophotometer (BioTek PowerWave XS2, Winooski, USA) according to Salicylate-Hypochlorite Method (Bower and Holm-Hansen 1980), Colorimetric and Spectrophotometric Method (Strickland and Parsons 1972), and Ultraviolet Spectrophotometric Method (APHA et al. 2005), respectively. Alkalinity was tested for pH change to a certain end point (pH 4.5) by titration with sulfuric acid (H_2SO_4) according to Titration Method (APHA et al. 1998). For denitrification experiments, the chemical oxygen demand (COD) was conducted periodically after adding methanol. The COD analysis was examined for color change by titration with standard ferrous ammonium sulfate titrant (FAS) according to the Closed Reflux, Titrimetric Method (ASTM 1995). Water quality in term of physical and chemical characteristics, i.e. dissolved oxygen (DO), pH,

oxidation-reduction potential (ORP), and temperature was daily measured using portable instruments (DO meter; HANNA HI 9147 and pH/ORP/temperature meter; HANNA HI 9125, Woonsocket, USA). In aerobic-anoxic test chambers (in section 3.2.2), moreover, the ORP monitoring was conducted not only in the water layer, but also in biomass within biofilter core. Finally, the nitrogen mass balance was performed on the first and last day of the experiment. Nitrogen contents in an artificial feed, aquatic animals, water, and sediments in an aquaculture tank were evaluated by CHNS Elemental Analyzer (Thermo ScientificTM Flash 2000, USA) at Scientific and Technological Research Equipment Centre, Chulalongkorn University.

Table 8. Analytical methods for water quality.

Parameter	Method/Equipment	References
Total ammonia nitrogen (TAN)	Salicylate-Hypochlorite	(Bower and Holm-Hansen 1980)
Nitrite (NO_2^--N)	Colorimetric and Spectrophotometric	(Strickland and Parsons 1972)
Nitrate (NO_3^--N)	Ultraviolet Spectrophotometric	(APHA et al. 2005)
Nitrogen content	CHN S/O Elemental Analyzer	-
Dissolved oxygen (DO)	DO meter; HANNA HI 9147	-
pH	pH meter; HANNA HI 9125	-
Oxidation-reduction potential (ORP)	ORP meter; HANNA HI 9125	-
Temp	Thermometer; HANNA HI 9125	-
Alkalinity	Titration Method	(APHA et al. 1998)
Chemical oxygen demand (COD)	Closed Reflux, Titrimetric Method	(ASTM 1995)

- **Evaluation of nitrogen removal rates**

The decrease in ammonia and nitrate concentrations were plotted versus reaction time. Nitrification and denitrification rates of biofilters at the initial ammonia and nitrate concentrations of 1.5 and 10 mg-N L^{-1}, respectively, were calculated based on the equations as follows:

$$\text{Nitrogen removal rate per specific area of biofilter (mg-N } m^{-2} \text{ day}^{-1}) = \frac{(\text{Initial conc.} - \text{Final conc.}) \times \text{Volume of solution (L)}}{\text{SSA of biofilter } (m^2) \times \text{Reaction time (day)}} \quad \text{Equation 19}$$

Or calculate based on the slope of the curve of reactant concentration versus time at t = 0 as follows:

$$= \frac{\text{Graph slope (mg-N L}^{-1}\text{ day}^{-1}) \times \text{Volume of solution (L)}}{\text{SSA of biofilter (m}^2\text{)}}$$

Equation 20

- **Statistical analysis**

The collected data was expressed as the average ± standard deviation (SD) and analyzed for significant differences between groups by using one-way analyses of variance (ANOVA) following the Duncan's multiple range tests; significance level at $P<0.05$. All analyses were run under SPSS Statistics version 21.0 for Windows (IBM, New York, USA).

- **Determination of solid deposited in biofilters**

The remarkable cleaned Biocord^{TM} biofilters (10 cm each) and Japanese filter mats (10×23×4 cm each) were dried in the hot air oven at 103 to 105ºC until constant weight and then preserved in the desiccator for 30 minutes to balance temperature. Afterward, the dried biofilters were weighted to 0.1 mg lab analytical balance (Sartorius BP210s, Goettingen, Germany) and collected data before applying in immobilization process. During the acclimation period in aquaculture tank, biofilter samples were collected at week 2, 4, 6 and 8 as well as on the last day of the experiment for weight estimation again. The amount of microorganisms and small particles contained in material structures were calculated by comparing the difference of g-dry weight (DW) between before and after acclimation process.

- **Evaluation of growth and survival of white shrimp post-larvae**

The length and weight data of white shrimp were collected before starting the experiment. In case shrimp die during the cultivation period, the dead shrimp was removed and replaced a new shrimp with the same size to maintain the stocking level in aquaculture tank. While the number of dead shrimp was used to calculate the survival rate at the end of the experiment. And after 60 days, the shrimp was harvested as well as collected the data of the length and body weight again for the estimation of growth performance. The equations are as follows:

Daily weight gain (DWG, g-shrimp day^{-1})

$$= \frac{\text{Final average weight} - \text{Initial average weight}}{\text{Experimental period}}$$

Equation 21

Average shrimp weight (g shrimp^{-1})

$$= \frac{\text{Total shrimp weight}}{\text{Total number of shrimp}} \quad \text{Equation 22}$$

Average shrimp length (cm shrimp^{-1})

$$= \frac{\text{Total shrimp length}}{\text{Total number of shrimp}} \quad \text{Equation 23}$$

Survival rate (%)

$$= \frac{\text{Number of survival shrimp}}{\text{Total number of shrimp}} \times 100 \quad \text{Equation 24}$$

Feed conversion ratio (FCR, ratio by weight)

$$= \frac{\text{Total feed given (g)}}{\text{Animal weight gain (g)}} \quad \text{Equation 25}$$

Density (kg-shrimp m^{-3})

$$= \frac{\text{Total shrimp weight}}{\text{Volume of aquaculture tank}} \quad \text{Equation 26}$$

3.2.3 Microbial community analysis

The objectives of this part were to observe the biofilm morphology on biofilter material and to study the effects of salinity, stocking density, and acclimation period on microbial community during the acclimation period.

- **Observation of biofilm morphology**

The small pieces of biofilters (Biocord^{TM} and Japanese mat) from an intensive aquaculture tank at the different salinity levels of 5, 15, and 25 PSU were cut and collected on day 60 of the experiment. The samples were preserved in either acetone or ethanol prior to analysis. The surface morphology of biofilms were observed by scanning electron microscopy; SEM (JSM-6610LV SEM, JEOL, Peabody, USA) at Scientific and Technological Research Equipment Centre (STREC), Chulalongkorn University.

- **DNA extraction and determination of nucleic acid concentration**

The pieces of fibrous Biocord™ biofilter were collected at week 1 and 8 as well as on the last day of the experiment for microbial community analysis by Illumina MiSeq system. Samples were extracted the DNA by using the *Fast DNA® SPIN Kit* (MP Biomedicals, Santa Ana, USA) following the manufacturer's instruction manual. The 0.5 g-wet biofilter sample was added to a Lysing Matrix E tube, and mixed with Sodium Phosphate Buffer (978 µl) and MT buffer (122 µl). The sample was homogenized in the FastPrep-24 (MP Biomedicals, Santa Ana, USA) instrument at the speed of 6.0 for 40 seconds, and centrifuged at 14,000 ×g for 5-10 mins to separate the mixtures. A supernatant (approximately 800 to 1000 µl) was transferred to the new 2 ml micro-centrifuge tube and mixed with PPS (250 µl) by hand 10 times to remove protein. The tube was centrifuged again at 14,000 ×g for 5 mins to pellet precipitate and transferred the supernatant (approximately 800 µl) to the clean 15 ml tube. Blinding Matrix suspension (1 ml) was added into the tube and converted by hand for 2 mins to allow binding DNA. After settling of silica matrix by placing tubes in a rack for 3 mins, the supernatant (approximately 500 µl) was carefully removed and discarded. The Blinding Matrix in the amount of supernatant was re-suspended and transferred to the SPIN™ Filter. The tube was centrifuged at 14,000 ×g for 1 min and the catch tube was emptied. The SEWS-M wash solution mixed with 100% ethanol (500 µl) was added into the tube and used to re-suspend the pellet. The tube was centrifuged at 14,000 ×g for 1 min and the catch tube was emptied. For impurity sample, the pellet was re-suspended and washed by SEWS-M (500 µl) twice. Afterwards, without any addition of the solution, the tube was centrifuged again at 14,000 ×g for 2 mins to dry the matrix of residual wash solution. The SPIN™ Filter was put into a new catch tube and dried by air at the room temperature for 5 mins. The 50 or 100 µl of DES (DNase/Pyrogen-Free water) was added into the tube and centrifuged at 14,000 ×g for 1 min to bring eluted DNA into the clean catch tube. The nucleic acid concentration was measured with NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, USA) by using DES as a blank. Finally, the extracted DNA was stored in the refrigerator at -20 ºC for extended periods.

- **Agarose Gel Electrophoresis**

Extract DNA was checked by agarose gel electrophoresis. The mixing of sample (1 µl) and 6×loading dye (2 µl) was loaded into the well of 1% agarose gel. The 100 bp DNA ladder (5 µl) which contained a set of known DNA fragments with the different sizes was also mixed with loading dye and used as molecular size standard. The electrophoresis was conducted at 100 V for 30 min. Thereafter, the gel was soaked in SYBR green solution for 30 min before observing by Chemiluminescence and Fluorescence Imaging System (Syngene, Cambridge, U.K.).

- **Microbial community analysis by Illumina MiSeq system**

The 16S rRNA gene in extracted DNA samples was amplified by polymerase chain reaction (PCR) technique using the Thermal cycler (BIO-RAD, Hercules, USA) following the conditions in table 11 (first stage). The universal primer sequences for bacterial gene targeting V3-V4 variable regions used for PCR amplification are listed in table 9 (Herlemann et al. 2011), and the chemical mixtures for each PCR reaction are in table 10. After completing the first stage PCR amplification, the free primers and primer dimers contaminated in 16S amplicon were removed by AMPure XP beads (Beckman Coulter, Brea, USA), and then the Illumina sequencing adapter was labeled to the PCR products using Nextera XT Index primer (Illumina Inc., San Diego, USA). The second stage PCR was performed following the conditions in table 11 (second stage). Thereafter, the final products were purified again by AMPure XP beads (Beckman Coulter, Brea, USA) before preparing the suitable concentration. The DNA concentration was measured to provide the basis for library pooling by using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA), and then adjusted to the concentration of 4 ng μl^{-1} with 10 mM Tris-HCl, pH 8.5. Cluster generation and 250-bp paired-end read sequencing were performed on Illumina MiSeq Sequencer (Illumina Inc., San Diego, USA) at the Faculty of Medicine, Chulalongkorn University.

Table 9. Sequence of universal primers used for PCR amplification.

Target genes	**Universal primers**		**Reference**
	Name	**Oligonucleotide sequence + overhang adapter (5' to 3')**	
Bacterial gene	Bakt_341F	**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CCT ACG GGN GGC WGC AG	(Herlemann et al. 2011)
	Bakt_805R	**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GA CTA CHV GGG TAT CTA ATC C	

Table 10. Chemical mixtures for PCR amplification.

Component	Volume (µl)
ddH2O	14.375
10 x PCR Buffer	2.5
dNTP mix	2
2 pmol/µl forward primer	2.5
2 pmol/µl reverse primer	2.5
Taq polymerase	0.125
10 ng/µL DNA template	1.0
Total volume	25

Table 11. PCR condition for Miseq.

Program	First stage		Program	Second stage	
	Temp (ºC)	Duration (sec)		Temp (ºC)	Duration (sec)
Pre-incubation	94	180	Pre-incubation	94	180
Amplification (25 cycles)	94	30	Amplification (8-10 cycles)	98	20
	55	30		55	30
	72	30		72	30
Final extension	72	300	Final extension	72	300
Cooling	4	-	Cooling	4	-

- **Bioinformatics analysis**

Sequencing reads quality was examined using FASTQC software. Overlapping paired end reads were assembled using PEAR. FASTX-Toolkit was applied to filter out assembled reads that do not have a quality score of 30 at least 90% of bases, and then remove reads that are less than 400bp long. Chimeras were removed by the UCHIME method (Edgar et al., 2011) as implemented in vsearch1.1.1 (Rognes et al., 2016) using –uchime_ref option against chimera-free Gold RDP database. OTU picking was performed with the pick_open_reference_otus.py command in QIIME 1.9.0, specifying that SortMeRNA was used for reference picking, and taxonomic assignments were conducted against Greengenes 97% database. Subsequently, the subsampled failure reads were clustered de novo using SUMACLUST. After the OTU picking, the OTUs that supported by less than 0.1% reads were filtered out. To ensure even sequencing depth across samples, the reasonable minimum number of sequences

per sample were randomly subsampled for analysis of bacterial communities. Then, alpha diversity estimates were computed for phylogenetic diversity (PD), chao1, observed otus, and a rarefaction curve was generated. In addition, beta diversity was estimated by computing weighted UniFrac distances between samples to create principal coordinate analyses (PCoA).

3.3 Study 2: Application of biofilter in marine RAS for long-term operation

The objectives of this study were to evaluate the performance of biofilter for long-term operation in marine RAS and to monitor the changes in microbial community during long-term operation. The experiment was divided into 4 parts including: 1) long-term operation of aquaculture system, 2) estimation of nitrification and denitrification efficiencies and 3) analysis of microbial community as follows:

3.3.1 Long-term operation of aquaculture system

The objective of this part was to apply biofilter in marine RAS for long-term operation. This experiment was operated in the 2000 L indoor aquaculture tank (outer dimension 1.95 m; inner dimension 1.85 m; tank depth 0.85 m; water depth 0.75 m), which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3}. The 50 m length of fibrous Biocord^{TM} biofilter was installed in aquaculture tanks, as showed in figure 13(a), where the 45-day old Pacific white shrimp were cultured. The length of fibrous biofilter was calculated based on the nitrogen removal rate obtained from section 3.2.2, and the nitrogen waste generation rate at the optimal stocking density obtained from section 3.2.1 (the calculations are shown in appendix D). To fix the biofilter under submerged condition throughout the experiment, the 12.5 m length of fibrous biofilter was rolled up on the PVC pipe with the dimension of ½ inch (18 mm) which was built as rectangle structure (figure 14) and 4 portions of PVC pipe structure with biofilter were installed in aquaculture tank. Moreover, the solid collection devices (figure 15) which was made from the wave pump (WP-400M, Sobo, Zhongshan, China) with the flow rate of 10,000 L hr^{-1} combined with the 150 micron stainless mesh hollow tube (dimension 0.20 m; length 0.60 m) and closed with PVC cap were also installed in the tank.

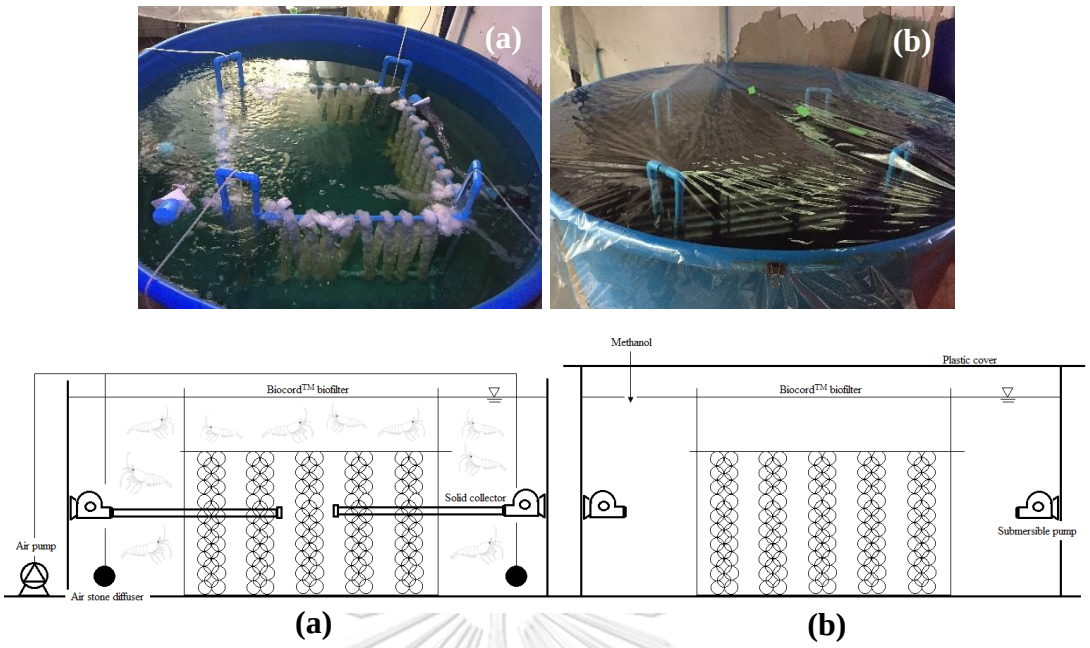

Figure 13. Installation diagram of fibrous Biocord™ biofilter and solid collection device in the shrimp culture tank for a) aerobic and b) anoxic conditions.

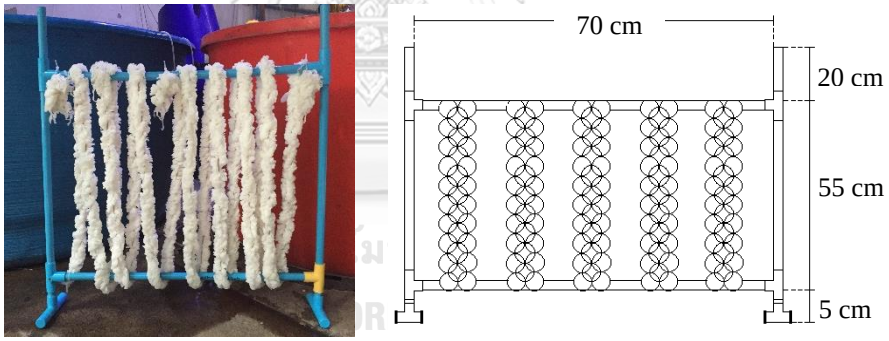

Figure 14. Diagram of Biocord™ biofilter rolled up on PVC pipe, applied in RAS.

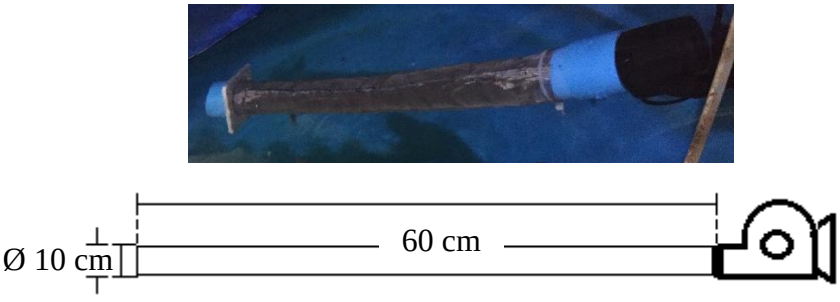

Figure 15. Diagram of solid collection device, applied in RAS.

The biofilter acclimation was performed during the first 60 days, and thereafter the aerobic shrimp cultivation with complete nitrification was continuously operated for 60 days without water exchange, except for adding water to compensate for lost water due to an evaporation. The operating conditions in RAS were maintained as the following: DO > 4 mg-O_2 L^{-1}, pH = 7.5 – 9, temperature = 28 – 31ºC and alkalinity 100 – 150 mg-$CaCO_3$ L^{-1}. During the experimental period, the biofilters were monthly cleaned for elimination of excess solid deposit as well as for prevention of H_2S production. Furthermore, the shrimp was randomly recorded the body weight for food quantity adjustment by maintaining the feeding rate in the range of 3 to 5% of total body weight per day. After 60 days, similar to section 3.2.1, the experiment was switched to anoxic condition for nitrate removal through denitrification process. The shrimp was harvested as well as locating the submersible water pump in aquaculture tank instead of air stone diffusers (figure 13(b)). The methanol at COD: Nitrate-N ratio of 5:1 was supplied for denitrifiers, and then adjusting periodically when finding that the nitrate concentration tends to remain constant. The anoxic condition was performed approximately 10 days or until nitrate concentrations are lower than 10 mg-N L^{-1}.

After finishing the first crop, the air pump was turned on again to re-oxygenate approximately 1 to 2 days before beginning the next crop. To evaluate the performance of long-term operation, this experiment was operated through a second round of replication following the 60 days of cultivation and 10 days of anoxic condition. The water samples from each tank were collected daily for ammonia, nitrite and nitrate analysis to monitor changes in the nitrogen profiles throughout 7 months of the experiment. The biofilter samples were collected biweekly and after finishing denitrification process for the estimation of nitrogen removal rates in section 3.3.2. Furthermore, the extracted DNA obtained from biofilters were applied for microbial community analysis by the Illumina MiSeq system in section 3.3.3.

3.3.2 Estimation of nitrification and denitrification efficiencies

The objective of this part was to check the nitrogen removal efficiency of biofilter during long-term operation. The biofilter samples from long-term aquaculture system (in section 3.3.1) were placed in aerobic test chamber for the estimation of nitrification rate, as shown in figure 11(a). The decrease of ammonia concentration was monitored after adding 2.5 L synthetic wastewater containing 1 mg-N L^{-1} of ammonium chloride. The operating conditions were maintained as the following: DO > 4 mg-O_2 L^{-1} and alkalinity 100 – 150 mg-$CaCO_3$ L^{-1}.

Denitrification efficiency, on the other hand, was performed in aerobic test chamber, as shown in figure 11(b), by adding 2.5 L synthetic wastewater containing 10 mg-N L^{-1} of sodium nitrate solution. The COD: Nitrate-N ratio was adjusted on the first day of the experiment at 5:1 (Pungrasmi et al. 2013) by injecting methanol as an external organic carbon source for denitrifiers. All batch experiments were operated in

triplicate for approximately 5 days or until ammonia or nitrate concentrations are undetectable. The analytical methods for water quality are described in section 3.2.2.

3.3.3 Microbial community analysis

The objective of this part was to study the microbial diversity and community dynamics during long-term operation of aquaculture system. The pieces of biofilters were collected monthly during shrimp cultivation as well as on the last day of anoxic period for microbial community analysis by the Illumina MiSeq system. The microbial DNA contained on biofilters was extracted by using the *Fast DNA® SPIN Kit* (MP Biomedicals, Santa Ana, USA) following the manufacturer's instruction manual. The 16S rRNA gene in extracted DNA samples was amplified through PCR by using the universal primer for archaeal and bacterial gene in table 12 with the chemical mixtures for each PCR reaction in table 13, and following the conditions in table 14. Thereafter, the PCR products were purified by QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) before preparing the suitable concentration. The DNA concentration was measured to provide the basis for library pooling by using Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA), and then adjusted to the concentration of 2 nmol/L. The sequencing of 16S rRNA gene were performed on Illumina MiSeq Sequencer (Illumina Inc., San Diego, USA) at Department of Civil and Environmental Engineering, Nagaoka University of Technology.

Table 12. Sequence of universal primers used for PCR amplification.

Target genes	Universal primers		Reference
	Name	Oligonucleotide sequence (5' to 3')	
Archaeal and bacterial gene	Univ515F	GTG CCA GCM GCC GCG GTA A	(Caporaso et al. 2012)
	Univ806R	GGA CTA CHV GGG TWT CTA AT	

Table 13. Chemical mixtures for PCR amplification.

Component	Volume (µl)
MQ water	6
10 µM forward primer	1
10 µM reverse primer	1
Premix Ex Taq Hot start	10
10 ng/µL DNA template	2
Total volume	20

Table 14. PCR condition for Miseq.

Program	Temp(°C)	Duration (sec)
Pre-incubation	94	180
Amplification (25 cycles)	94	45
	50	60
	72	90
Final extension	72	600
Cooling	4	-

Chapter 4

Results and discussion

4.1 Effects of salinity, stocking density, and acclimation period on nitrogen removal efficiency and microbial community

This study was focused on the evaluation of the effects of salinity, stocking density, and acclimation period on nitrification and denitrification efficiencies, and microbial diversity in biofilter during acclimation period. The experimental results were divided into 3 parts including: 1) biofilter acclimation in aquaculture systems, 2) nitrogen removal rates of fibrous biofilter and filter mat, and 3) microbial diversity at different salinity and nitrogen loading as follows:

4.1.1 Biofilter acclimation in aquaculture systems

The inorganic nitrogen profiles in aquaculture tanks which varied the salinity levels at low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU), and the stocking densities at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) are shown in figure 16.

The presence of ammonia was found in all systems during the first 10 days of grow-out period. Ammonia was naturally originated from the breakdown and conversion of nitrogenous organic matter via ammonification (van Rijn 2013; Stewart et al. 2006). For this reason, ammonia increased continuously and reached to the peak on day 6 of the experiment. Ammonia concentrations were affected by the stocking densities of white shrimp in aquaculture systems. In an intensive stocking density, the highest ammonia levels were 3.54, 4.39 and 4.92 mg-N L^{-1} for 5, 15 and 25 PSU, respectively, while the lower levels of ammonia (approximately 2 mg-N L^{-1}) were observed in the semi-intensive tanks. Moreover, it seemed like the peak of ammonia concentration under low-salinity (5 PSU) condition was lower than in the medium- (15 PSU) and high-salinity (25 PSU) systems. Thereafter, the ammonia oxidizing microorganisms were the important drivers in nitritration process to convert ammonia to nitrite, resulting in the decreased levels of ammonia. An intermediate nitrite accumulation during nitrification was result from the growth rates difference between AOB (0.29 g-VSS g NH_4^+-N^{-1}) and NOB (0.084 g-VSS g NO_2^--N^{-1}), in which AOB grow approximately 3.5 times faster than NOBs (Sharma and Ahlert 1977). Also, the NOB are much sensitive to environmental changes, especially for increasing in salinity. In consequence, nitrite accumulation is related to the inhibition of nitrite oxidation process (Lewis and Morris 1986; Yu et al. 2004). The accumulation of nitrite was presented in all experimental systems; however, related to the effects of osmotic stress, the longest accumulation time (from day 5 to day 40) was detected in both semi-intensive and intensive aquaculture tanks at the salinity level of 25 PSU. Nevertheless,

after 1 month of acclimation, the nitrification without nitrite accumulation occurred completely due to the development of sufficient nitrifying biofilm on biofilters. Report conducted by Dennis and Thomas (2012) showed that the natural microorganisms can also be introduced with the small numbers of starter animals from the already operated RAS. In this study, the growth of aquatic animals could proceed at the same time of the bacterial colonization on media surface without having the activated biofilters. According to complete nitrification process, the levels of nitrate increased continuously to the highest of approximately 50 mg-N L^{-1} (for semi-intensive), and 60 mg-N L^{-1} (for intensive) on day 59 of the experiment.

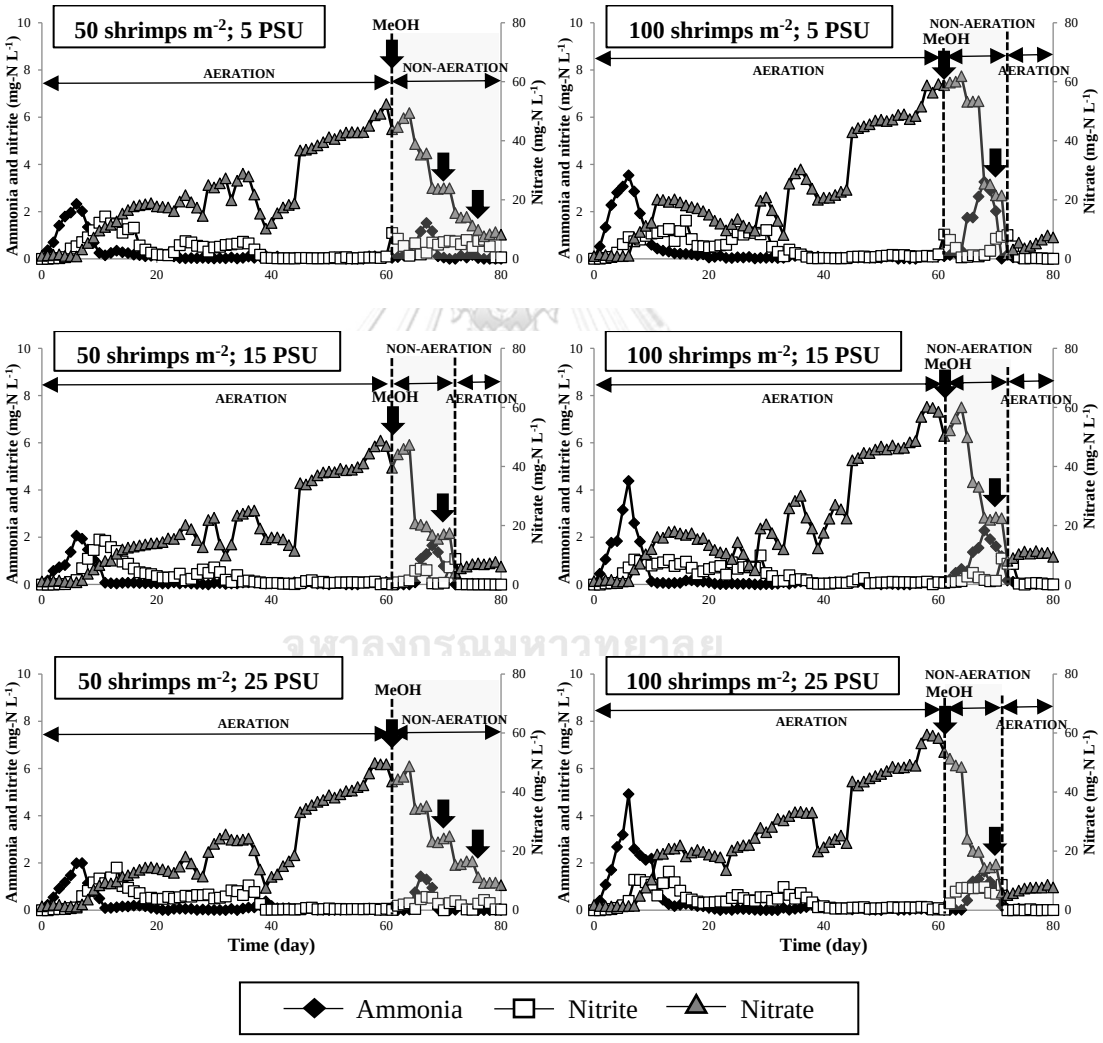

Figure 16. Inorganic nitrogen profiles in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange. Black arrows (⬇) indicate the addition of methanol.

For denitrification, after the systems were switched to oxygen absent condition, nitrate reduction was conducted by prevalent microorganisms, facultative anaerobes, with an electron donor derived from external source (methanol) at COD:Nitrate-N of 5:1 (Pungrasmi et al. 2016; van Rijn et al. 2006). The semi-intensive RAS at 15 PSU and intensive densities (at 5, 15 and 25 PSU) could remove nitrate to lower than 10 mg-N L^{-1} on day 72. Meanwhile, the nitrate concentrations of 15.50 and 15.27 mg-N L^{-1} were found in the semi-intensive tanks at 5 and 25 PSU. Therefore, the addition of methanol was more required on day 76 for reducing the remaining nitrate in these systems. Finally, when the aeration was switched back on, there were no changes in ammonia and nitrite concentrations while nitrate tended to fluctuate.

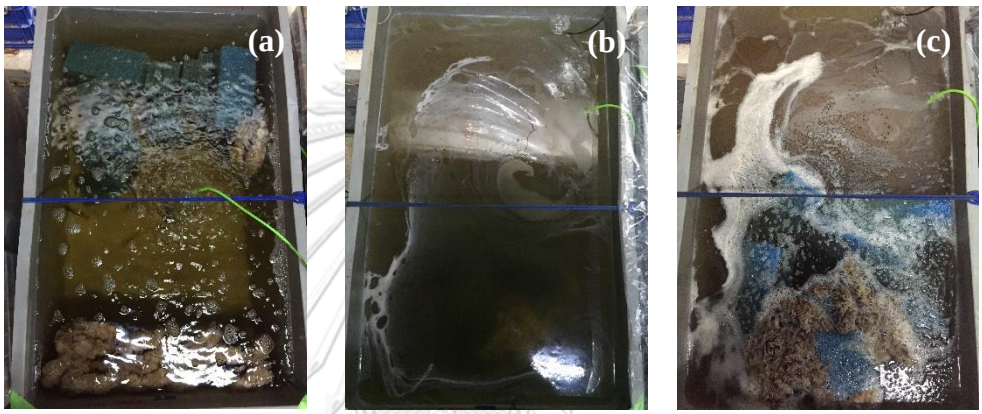

Figure 17. Experimental aquaculture system during (a) 60 days of biofilter acclimation with aerobic shrimp cultivation, (b) on day 61 with methanol supplement (anoxic, no shrimps) and (c) after 10 days of denitrification, without water exchange.

- **Water quality in aquaculture systems**

The water quality parameters in shrimp culture tanks which varied salinity levels at low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU), and the stocking densities at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) are shown in table 15.

Table 15. Water quality parameters measured in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange. Data are shown as the mean ± 1SD.

Parameters	Salinity 5 PSU				Salinity 15 PSU				Salinity 25 PSU			
	50 shrimps m^{-2}		100 shrimps m^{-2}		50 shrimps m^{-2}		100 shrimps m^{-2}		50 shrimps m^{-2}		100 shrimps m^{-2}	
	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic
DO ($mg\text{-}O_2\ L^{-1}$)	6.96± 0.92	1.60± 1.28	6.70± 0.98	0.51± 0.87	6.84± 0.92	0.39± 0.46	6.42± 0.86	0.38± 0.33	6.56± 0.84	1.23± 1.21	6.60± 0.91	0.36± 0.30
Temperature (ºC)	28.70± 0.79	30.30± 0.87	28.89± 0.89	30.76± 0.61	28.82± 0.89	30.88± 0.61	28.96± 0.87	30.78± 0.58	28.80± 0.82	30.42± 0.77	28.99± 0.91	30.70± 0.71
pH	7.93± 0.19	7.45± 0.34	7.99± 0.26	7.14± 0.32	7.88± 0.22	7.29± 0.39	7.87± 0.21	7.35± 0.33	7.77± 0.12	7.33± 0.18	7.79± 0.23	7.45± 0.15
Alkalinity ($mg\text{-}CaCO_3\ L^{-1}$)	128.69± 19.47	190.74± 20.94	136.52± 27.96	153.69± 33.41	143.93± 32.88	211.79± 80.33	143.13± 38.00	198.37± 81.27	131.31± 25.18	189.89± 39.91	138.34± 35.42	235.31± 63.06
ORP (mV)	139.10± 10.77	−14.10± 73.39	136.44± 17.91	−55.69± 88.98	142.21± 12.52	−30.87± 68.82	139.14± 13.59	−40.80± 85.31	134.31± 14.31	−2.66± 74.45	144.68± 16.24	−49.90± 77.27
COD ($mg\text{-}COD\ L^{-1}$)	Nm	75.36± 55.88	Nm	122.90± 85.02	Nm	106.65± 64.86	Nm	154.46± 98.31	Nm	107.98± 55.84	Nm	144.32± 70.48

Nm = not measure

During 60 days of biofilter acclimation with aerobic shrimp cultivation, all parameters were within the suitable levels for the normal growth of white shrimp and aerobic microorganisms. The DO concentrations (figure 18) were more than 4 mg-O_2 L^{-1} (Nonwachai et al. 2011) which were appropriated for shrimp, whereas the low oxygen levels cause an incomplete ammonia oxidation due to the shift from autotrophic to heterotrophic microorganisms. The temperature values were within the range of 25 to 30°C (Boyd and Tucker, 2012), and the pH values (figure 19) were between 7.5 and 9. Normally, the shrimp can grow healthily and rapidly at the pH values between 7.5 and 8.5 (Tharavathy 2014); however, in order to avoid the ammonia toxicity and increase the shrimp production, the pH in an aquaculture system should range between 7.5 and 9 (Tharavathy 2014). The gradual decline of alkalinity during biofilter acclimation with aerobic shrimp cultivation was the major indicator of nitrification process (figure 20). The theoretical alkalinity consumption for ammonia oxidation is equal to 7.1 mg-$CaCO_3$/mg NH_3-N, while an insufficient alkalinity causes a decreased nitrification rate (Sharma and Ahlert 1977; Hagopian and Riley 1998). Therefore, the sodium bicarbonate ($NaHCO_3$) was added on days 0, 40 and 49 to maintain the alkalinity within the range of 100–150 mg-$CaCO_3$ L^{-1}. The ORP values (figure 21) in aquaculture systems were between 50 and 150 mV, which was consistent with the recommendation for efficient nitrification (Chapentier et al. 1998; Li and Irvin 2007).

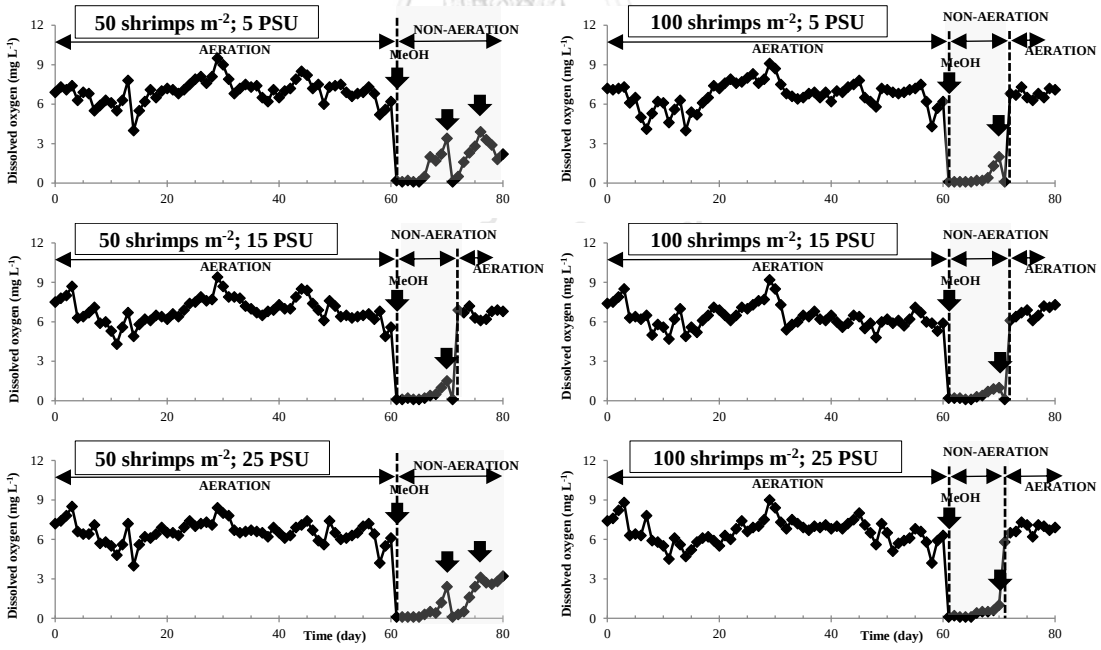

Figure 18. Dissolved oxygen in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange. Black arrows (⬇) indicate the addition of methanol.

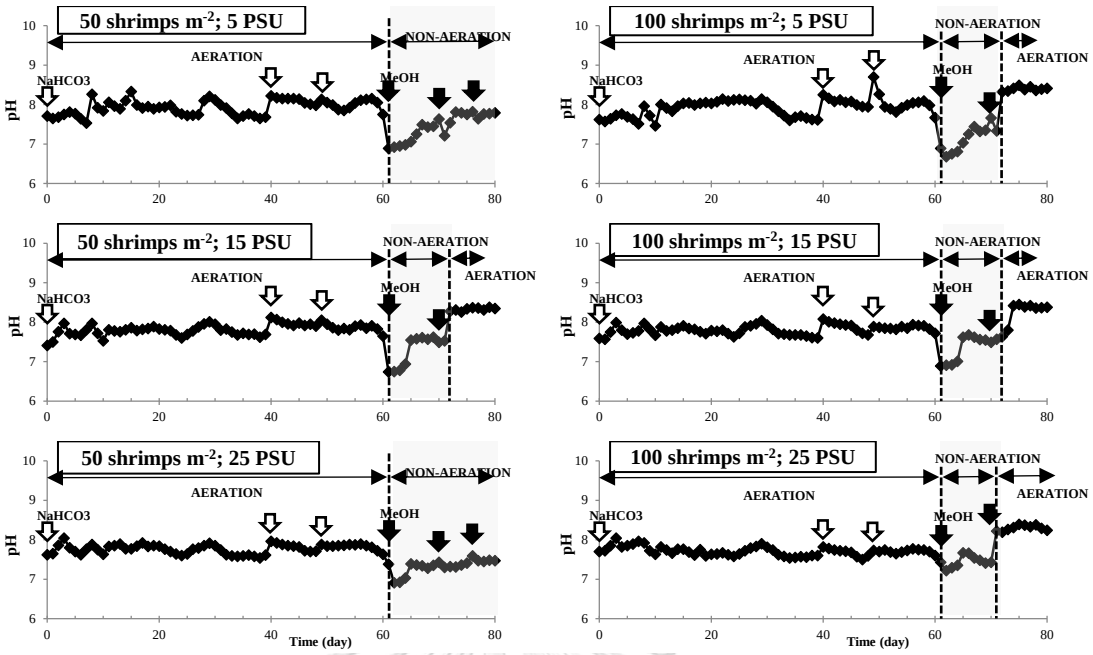

Figure 19. The pH in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange. White arrows (⇩) indicate the addition of sodium bicarbonate and black arrows (⬇) indicate the addition of methanol.

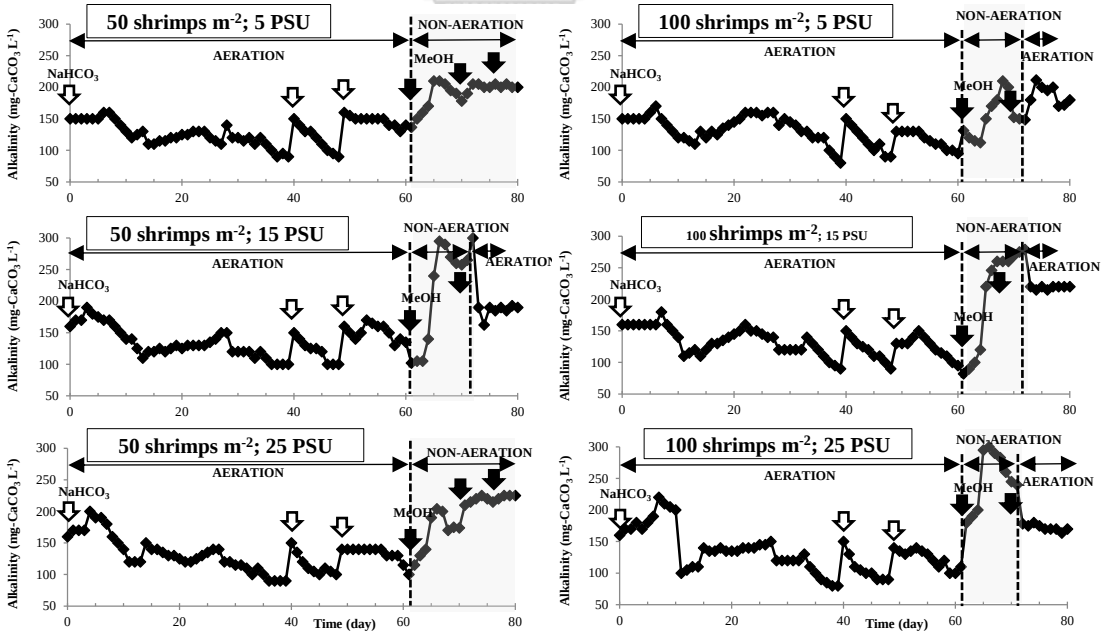

Figure 20. Alkalinity in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange. White arrows (⇩) indicate the addition of sodium bicarbonate and black arrows (⬇) indicate the addition of methanol.

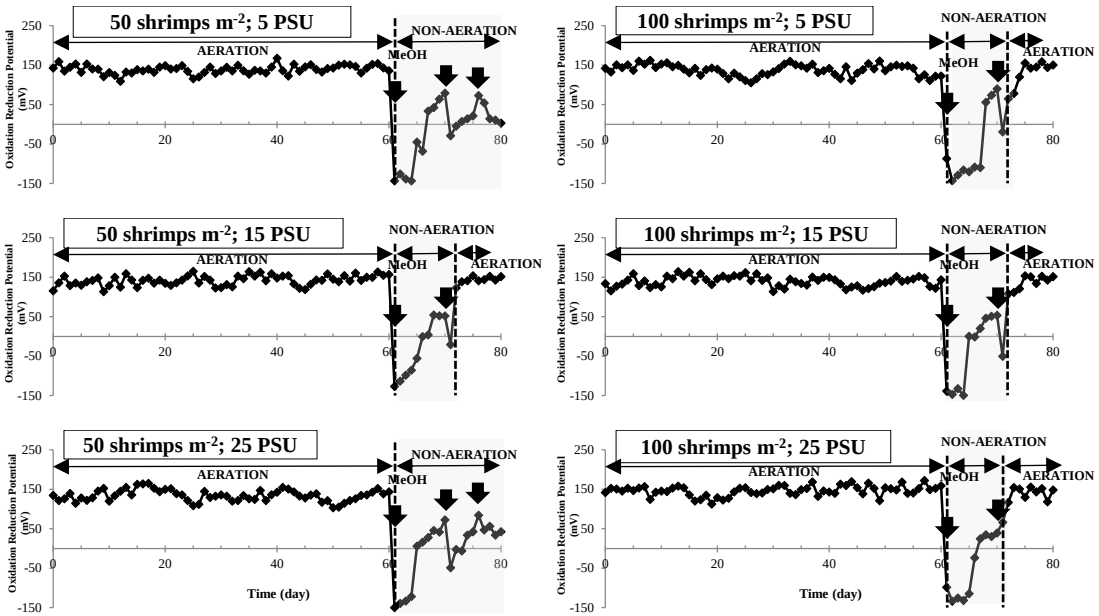

Figure 21. Oxidation-reduction potential in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange. Black arrows (⬇) indicate the addition of methanol.

For the denitrification, the average DO concentration in all systems was sharply decreased from 6.05±0.26 mg-O_2 L^{-1} (on day 59) to 0.13±0.05 mg-O_2 L^{-1} (on day 60) within 1 day after methanol addition. During 10 days of anoxic period, the DO concentration were between 0.1 and 2 mg-O_2 L^{-1}, indicating that the nitrate removal was successfully performed. In terms of alkalinity, theoretically, nitrate dissimilation can gain an alkalinity of 3.57 mg-$CaCO_3$/mg NO_3-N (van Rijn et al. 2006). The increase of alkalinity was found in all experimental systems during the denitrification treatment, especially under medium- (15 PSU) and high-salinity (25 PSU) conditions. The required ORP values for complete nitrate removal are in the range of −200 and −400 mV (Lee et al. 2000). In this study, the average values observed in anoxic systems were between −40 and −55 mV which were below zero, indicating the oxygen absent condition that allowed the denitrification process to occur. The COD concentration was conducted after methanol addition, as shown in figure 22. The decrease of COD level was related to the decrease of nitrate in which the COD concentrations in all tanks decreased from 300 mg-COD/L on day 61 to 123–154 mg-COD/L on day 70 and to less than 50 mg-COD/L on day 72.

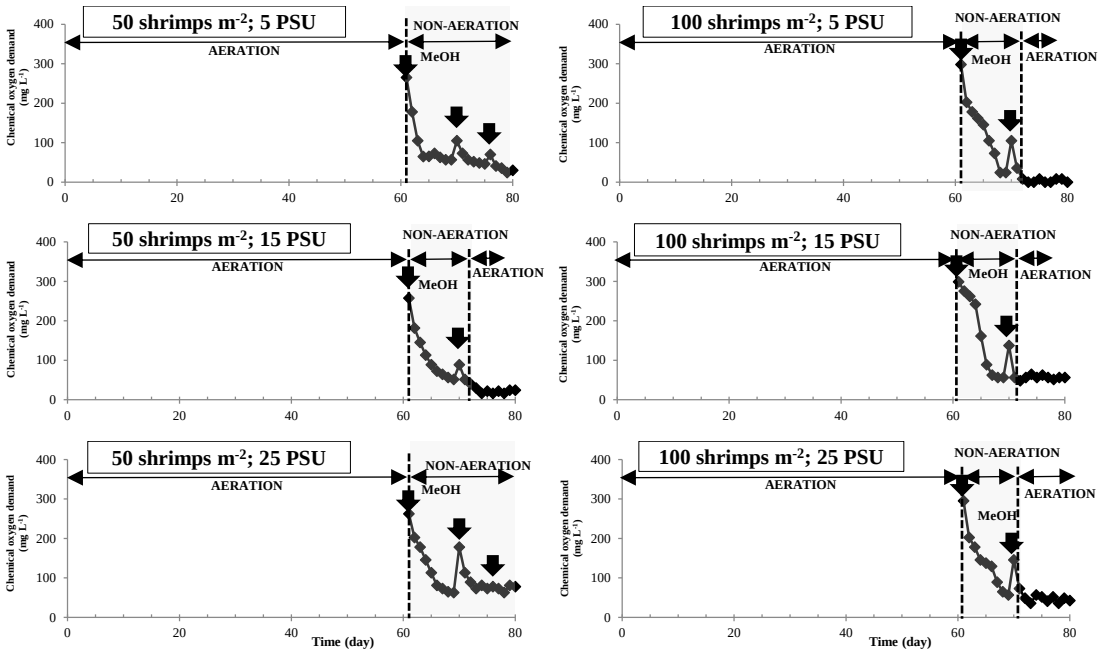

Figure 22. Chemical oxygen demand in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange. Black arrows (⬇) indicate the addition of methanol.

- **Growth of white shrimp in aquaculture systems**

Growth of white shrimp cultured in aquaculture systems varied salinity levels at low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU), and the stocking densities at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) are shown in table 16.

At the beginning of the experiment, shrimp were started to culture at the average weight and length of 3.62±0.83 g and 7.51±0.58 cm, respectively, as shown in figure 23. The growth of shrimp increased significantly throughout two months of culture period. The maximum growth was observed in intensive RAS at 5 PSU with the final average weight and length of 7.67±2.23 g and 10.20±2.14 cm, respectively, and with the daily weight gain (DWG) of 0.08 and 0.06 g-shrimp day^{-1} for the culture period of 1 and 2 months, respectively. The shrimp cultured in high-salinity systems took the second place on growth rate and there were no significant difference in average weight and length between semi- and intensive aquaculture tanks under the salinity of 25 PSU. Related to previous study, the white shrimp normally grow best in high-salinity systems at 20 and 30 PSU with the higher final weight, weight gain and specific growth ratio than other treatments (at 2 and 10 PSU) (Gao et al. 2016). With the survival rate, the results clearly indicated that the shrimp could survive better in low stocking density tanks with the survival rate of 55.56±16.44% whereas there were only 33.33±8.66%

observed in the intensive RASs. Comparison among different salinity levels, the medium- and high-salt concentration systems could gain the shrimp survival rate (56.67%) over than in low-salinity RAS (36.67%). For the feed conversion ratio (FCR), the shrimp cultured in intensive RAS at 5 PSU had the minimum FCR of 1.33 and 2.67 for the culture period of 1 and 2 months, respectively. Also, the intensive RAS at 25 PSU had the low FCR of 1.45 and 2.78 for the culture period of 1 and 2 months, respectively. Finally, in term of shrimp density, the initial and final density of shrimp cultured in semi-intensive RASs were 0.56±0.05 and 1.01±0.07 kg m^{-3}, respectively, while there were two times higher of 1.06±0.05 and 2.15±0.18 kg m^{-3} in intensive aquaculture tanks.

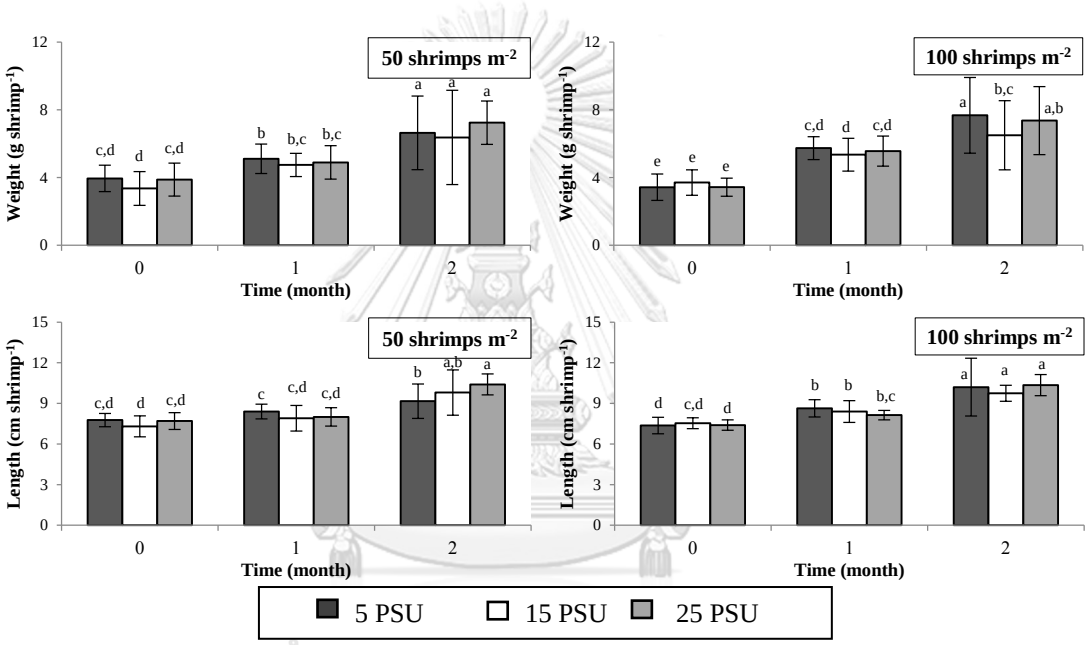

Figure 23. Comparison of shrimp average weight and length which cultured in different salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) at the variations of culture periods.
Data are shown as the mean ± 1SD. Means with a different letter are significantly different ($P < 0.05$ ANOVA and Duncan's multiple range test).

Table 16. Growth of shrimp cultured in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation. Data are shown as the mean ± 1SD. Means with a different letter are significantly different ($P < 0.05$ ANOVA and Duncan's multiple range test).

Condition	Time (month)	DWG (g day^{-1})	Avg. weight (g $shrimp^{-1}$)	Avg. length (cm $shrimp^{-1}$)	Survival rate (%)	FCR	Density (kg m^{-3})
50 shrimps m^{-2}; 5 PSU	0	-	3.94±0.78	7.77±0.50	100.00	-	0.59
	1	0.04	5.10±0.87	8.40±0.54	56.67	3.07	0.77
	2	0.05	6.64±2.18	9.16±1.27	36.67	3.00	1.00
100 shrimps m^{-2}; 5 PSU	0	-	3.42±0.78	7.37±0.61	100.00	-	1.03
	1	0.08	5.74±0.68	8.60±0.64	41.67	1.33	1.72
	2	0.06	7.67±2.23	10.20±2.14	23.33	2.67	2.30
50 shrimps m^{-2}; 15 PSU	0	-	3.35±1.00	7.30±0.77	100.00	-	0.50
	1	0.05	4.74±0.69	7.90±0.95	56.67	2.17	0.71
	2	0.05	6.37±2.78	9.80±1.67	56.67	2.63	0.95
100 shrimps m^{-2}; 15 PSU	0	-	3.70±0.75	7.53±0.40	100.00	-	1.11
	1	0.05	5.35±0.97	8.40±0.8	41.67	2.02	1.61
	2	0.03	6.50±2.04	9.75±0.59	38.33	4.19	1.95
50 shrimps m^{-2}; 25 PSU	0	-	3.88±0.97	7.70±0.62	100.00	-	0.58
	1	0.03	4.89±0.99	8.00±0.68	56.67	3.46	0.73
	2	0.07	8.63±2.54	11.05±1.17	56.67	1.88	1.09
100 shrimps m^{-2}; 25 PSU	0	-	3.43±0.53	7.40±0.39	100.00	-	1.03
	1	0.07	5.56±0.89	8.13±0.35	41.67	1.45	1.67
	2	0.06	7.36±2.01	10.35±0.78	38.33	2.78	2.21

4.1.2 Nitrogen removal rates of fibrous biofilter and filter mat

This part was focused on the evaluation of nitrification and denitrification efficiencies and the comparison of nitrogen removal rates between fibrous Biocord^{TM} biofilter and Japanese filter mat. The experimental results are as follows:

- **Nitrification rates of biofilters**

The comparison of nitrification rates between fibrous Biocord^{TM} biofilter and Japanese filter mat acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) stocking density aquaculture systems are shown in figure 24.

For fibrous Biocord^{TM} biofilter, the acclimation of biofilter in an intensive aquaculture tank under low-salinity level had the highest nitrification rate since week 2, whereas other conditions were found in week 4. The maximum ammonia removal rate occurred in an intensive aquaculture tank under 5 PSU as 100.42±5.97 mg-N m^{-2} day^{-1} on week 2, followed by under 15 PSU as 95.16±5.52 mg-N m^{-2} day^{-1} on week 4. Meanwhile, the Japanese filter mat acclimated in an intensive aquaculture tank under low-salinity level for 6 weeks had the highest rate of 145.43±1.17 mg-N m^{-2} day^{-1}, followed by in a semi-intensive tank under same salinity level as 144.40±4.37 mg-N m^{-2} day^{-1}. These results indicated that the immobilization of biofilter under low-salinity level (5 PSU) had higher nitrification efficiency than other salinity (15 and 25 PSU) levels, which was probably due to the inhibition of nitrifying microbial growth by dissolved salts (Cortés-Lorenzo et al. 2015). Moreover, the biofilter acclimated in an intensive aquaculture tank (100 shrimp m^{-2}) had higher ammonia removal rate than in semi-intensive system (50 shrimp m^{-2}), in which high stocking density could provide higher organic residue and microbial population than in low shrimp density. Related to the comparison of nitrogen removal performance between two types of biofilter, the nitrification rate of filter mat was 44.8% higher than observed in fibrous biofilter. According to the features of Japanese filter mat, the complicated structure of curled polyester fibers and the characteristic of submersibility might gain more attached microbes and preserve more solid particles than the fibrous Biocord^{TM} biofilter which is floating biofilter. The increase of nitrification efficiency during the first period of acclimation (from week 2 to week 6) was resulted from the attachment and growth of natural microorganisms on material surface. Nevertheless, the ammonia removal rate tended to decrease when the immobilization time was extended. The significant decrease of process performance occurred after switching the systems to anoxic condition. The lowest nitrification rate was observed in an intensive aquaculture tank under 15 PSU as 17.91±5.61 and 33.25±8.05 mg-N m^{-2} day^{-1}, for fibrous biofilter and filter mat, respectively. The results clearly indicated that although the system was operated under anoxic condition for 10 days, nitrifying microorganisms could still play role in nitrification. This was probably due to the resistance of some ammonia oxidizing

microorganisms to low dissolved oxygen concentrations (Francis et al. 2005). However, the decreased rate was resulted from dissolved oxygen absence (van Niel et al. 1993) and methanol addition (Munz et al. 2011) which stimulate the growth of heterotrophs that has a higher competitive of nutrient consumption and biofilm formation than autotroph.

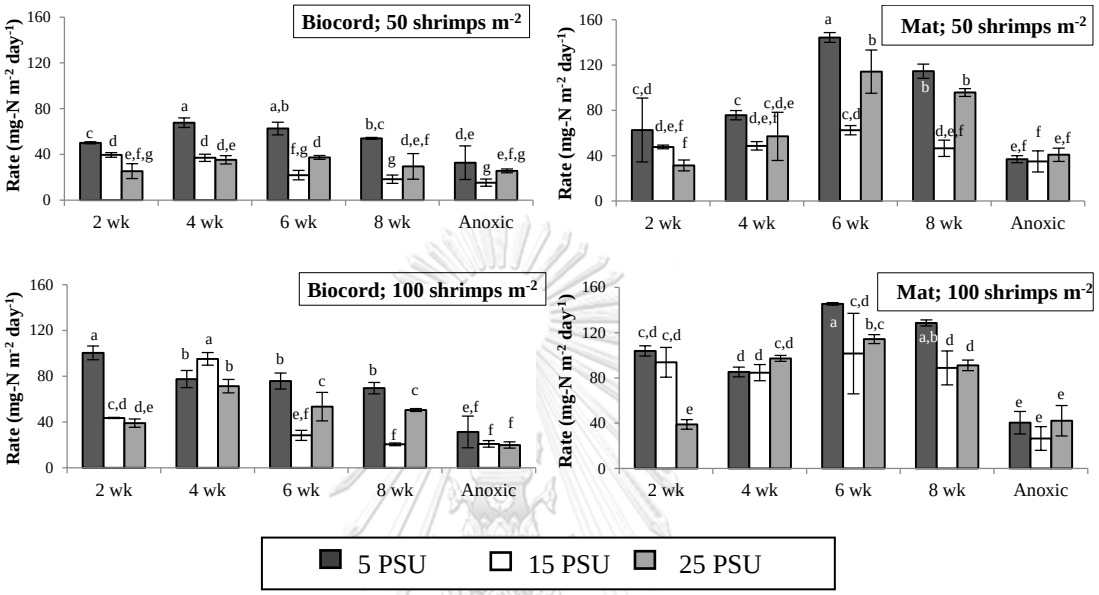

Figure 24. Comparison of nitrification rates between Biocord^{TM} biofilter and Japanese filter mat acclimated in different salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) at the variations of acclimation times. Data are shown as the mean ± 1SD, derived from three repeats. Means with a different letter are significantly different ($P < 0.05$ ANOVA and Duncan's multiple range test).

- **Denitrification rates of biofilters**

The comparison of denitrification rates between fibrous Biocord^{TM} biofilter and Japanese filter mat acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) stocking density aquaculture systems are shown in figure 25.

The estimation of denitrification rate was performed in test chamber under anoxic condition; therefore, the efficiencies during 8 weeks of biofilter acclimation were represented the ability of biofilter to reduce nitrate via anoxic denitrification process. The results showed that the nitrate removal could be occurred during 8 weeks of biofilter acclimation in shrimp cultivation tank even though the system was operated under aerobic condition. Previous study reported that this was related to the multiplication of anaerobes within biofilter structure where oxygen was absent (van Rijn et al. 2006). Denitrification rate increased with acclimation time, which was resulted from the gradual accumulation of solid debris on biofilter media. The

efficiency of nitrate removal in each experimental systems was not significant different during first 6 weeks of immobilization period. However, the denitrification rates were significantly increased on week 8, and all experimental systems had the highest rates after switching the condition from aerobic to anoxic. For fibrous biofilter, the maximum denitrification efficiency was found in an intensive aquaculture at 25 PSU as 81.86±4.40 mg-N m^{-2} day^{-1}, followed by a semi-intensive tank at 25 PSU as 78.02±10.92 mg-N m^{-2} day^{-1}. Meanwhile, the filter mat acclimated in an intensive aquaculture tank at 5 PSU as 165.80±50.17 mg-N m^{-2} day^{-1}, followed by at 25 PSU as 124.37±25.63 mg-N m^{-2} day^{-1}. Unlike nitrification, the results showed that the activity and function of denitrifiers were less influenced by the presence of sea salts. It was due to the multiplication of halotolerant denitrifiers within biofilm layers, which can protect microbial cells from the external conditions (Magalhães et al. 2005). Research study by Yu et al. (2004) supported that related to the characteristics of salt tolerance of some halophilic bacteria, these microorganisms can survive in the presence of seawater by changing their endurable power. In term of the effect of shrimp stocking density, same as nitrification, the nitrate removal rate of biofilter acclimated in an intensive aquaculture tank (100 shrimp m^{-2}) was greater than in a semi-intensive system (50 shrimp m^{-2}). And the denitrification performance of filter mat was 102.5% higher than observed in fibrous biofilter due to the characteristics of submersible filter mat.

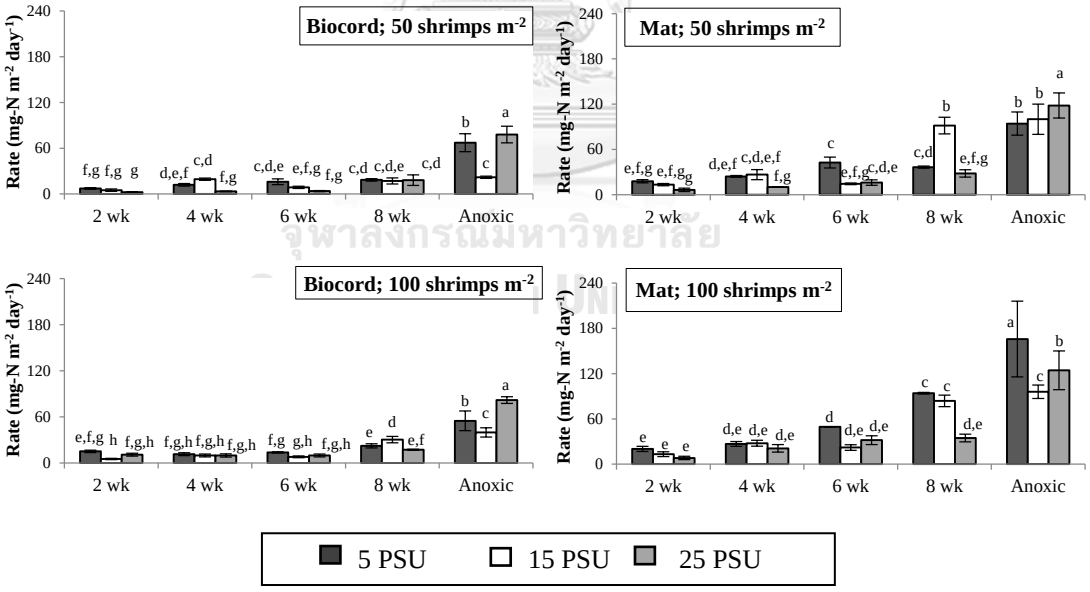

Figure 25. Comparison of denitrification rates between Biocord™ biofilter and Japanese filter mat acclimated in different salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) at the variations of acclimation times. Data are shown as the mean ± 1SD, derived from three repeats. Means with a different letter are significantly different ($P < 0.05$ ANOVA and Duncan's multiple range test).

4.1.3 Microbial diversity at different salinity and nitrogen loading

This part was focused on the microbial diversity on biofilter during acclimation period with aerobic shrimp cultivation, followed denitrification (anoxic, no shrimps) at different salinity and nitrogen loading. The experimental results are as follows:

- **Biofilm morphology on biofilters**

The SEM images of biofilters (fibrous Biocord^{TM} biofilter and Japanese filter mat) acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels in an intensive aquaculture system (100 shrimp m^{-2}) after 60 days of aerobic shrimp cultivation are shown in figure 26 and 27, respectively. The images showed that the biofilters were covered with filamentous microorganisms, especially under high-salinity of 25 PSU (figure 26(e), and 27(e) and (f)).

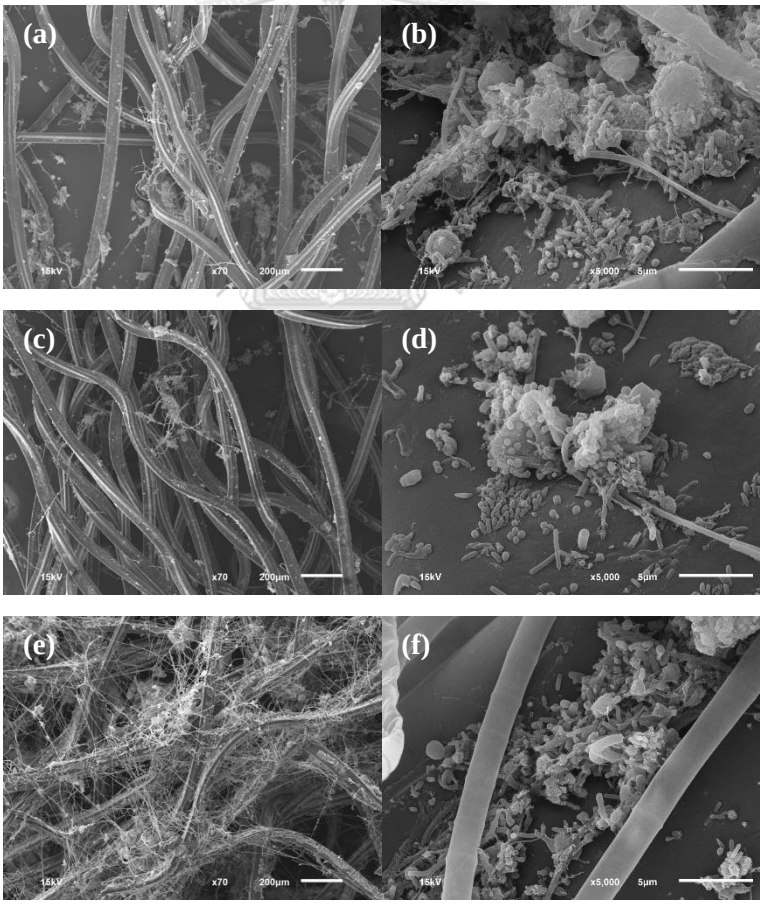

Figure 26. SEM images of the fibrous Biocord^{TM} biofilter after use for 60 days in aerobic shrimp cultivation at high stocking density (100 shrimp m^{-2}) in (a, b) low- (5 PSU), (c, d) medium- (15 PSU) and (e, f) high-salinity (25 PSU) systems. Images are shown at (a, c, e) 70 x and (b, d, f) 5,000 x magnification.

On biofilters, both spherical and rod-shaped bacteria with the average length of 0.5 to 1 μm were among the dominant species observed in this study. Nevertheless, the bacterial communities on biofilters were no difference among the three samples which operated under different salinity level. To clarify the microbial community on the filters, their DNA was extracted and analyzed by Illumina MiSeq DNA sequencing.

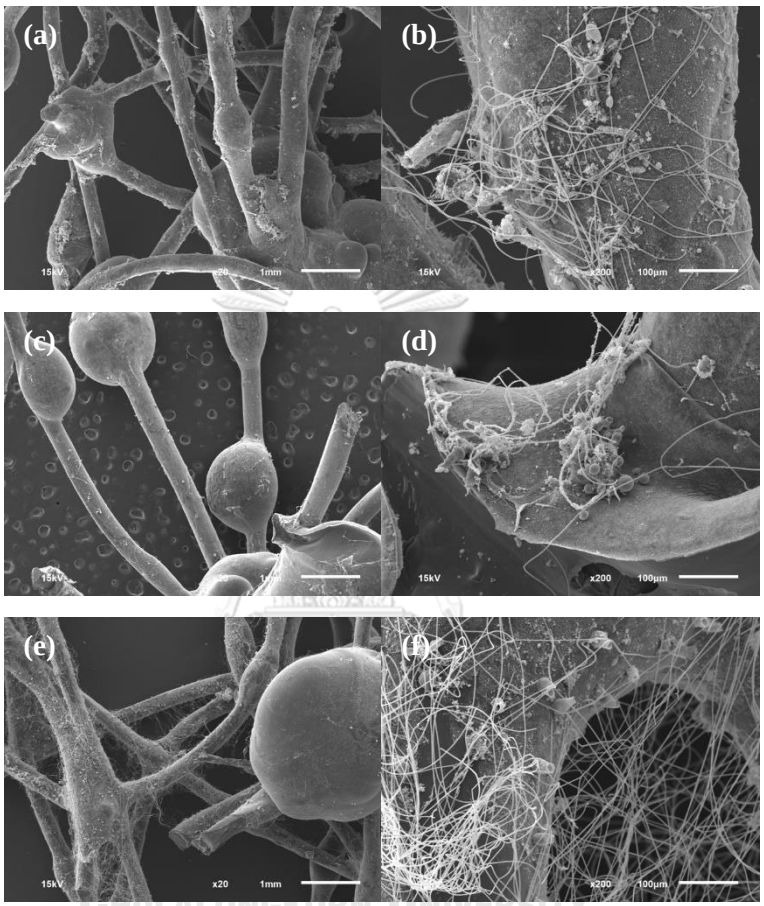

Figure 27. SEM images of the Japanese filter mat after use for 60 days in aerobic shrimp cultivation at high stocking density (100 shrimp m^{-2}) in (a, b) low- (5 PSU), (c, d) medium- (15 PSU) and (e, f) high-salinity (25 PSU) systems. Images are shown at (a, c, e) 20 x and (b, d, f) 200 x magnification.

- **Bacterial community structure at the taxonomic levels**

The relative abundances of bacterial phyla and class observed on the fibrous Biocord^{TM} biofilter acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels in semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (on week 1 and 8), followed by anoxic denitrification are shown in figure 28 and 29, respectively.

The fourteen different bacterial phylum with the relative abundance >1% in at least one sample were found in this study. Almost all analyzed sequences belonged to eight phyla of *Proteobacteria* (65.51%), *Bacteroidetes* (21.74%), *Actinobacteria* (3.07%), *Planctomycetes* (0.97%), *Firmicutes* (0.95%), *Chloroflexi* (0.86%), *Gemmatimonadetes* (0.80%) and *Caldithrix (*0.56%), while unassigned phyla and phylum with low relative abundance were included in others. At class level, there were twenty-four bacterial class (>1% in at least one sample*)* and almost sequences belonged to *Gammaproteobacteria* (25.22%), *Alphaproteobacteria* (16.50%), *Flavobacteriia* (14.21%), *Deltaproteobacteria* (13.42%), *Betaproteobacteria* (10.08%), *Saprospirae* (5.42%), *Actinobacteria* (1.80%), *Cytophagia* (1.59%) and *Acidimicrobiia* (1.24%).

Proteobacteria (Alpha-, Beta-, Delta- and Gamma-) were the outstanding group, as from 23.50 to 51.20% (aerobic) and from 95.72 to 96.14% (anoxic) in low-, from 36.06 to 71.98% (aerobic) and from 90.17 to 96.60% (anoxic) in medium- and from 34.44 to 83.47% (aerobic) and from 75.09 to 97.45% (anoxic) in high-salinity systems. The another important phylum was *Bacteroidetes* (mainly dominated by the class *Cytophagia, Flavobacteriia* and *Saprospirae*) with the relative abundances from 21.10 to 72.56% (aerobic) and from 3.23 to 3.62% (anoxic) in low-, from 25.23 to 31.66% (aerobic) and from 3.33 to 8.33% (anoxic) in medium- and from 14.12 to 23.74% (aerobic) and from 2.05 to 16.82% (anoxic) in high-salinity systems. These results were related to many studies that *Proteobacteria* and *Bacteroidetes* are the most abundant bacterial phylum in marine RASs (Martins et al. 2013; Wietz et al. 2009). Interestingly, class *Gammaproteobacteria* belonged to phylum *Proteobacteria* were observed in all samples, especially on biofilter acclimated under medium- and high-salinity levels under anoxic condition, whereas class *Betaproteobacteria* were predominant under low-salinity system. As consistent with previous studies (Wang et al. 2012), class *Alpha-* and *Beta-proteobacteria* have been enriched in the freshwater sediment while class *Gammaproteobacteria* have been isolated from marine sediment. *Actinobacteria* (represented by the class *Acidimicrobiia* and *Actinobacteria*) are normally widely distributed in marine environments, e.g. seawater and sediments (Puttaswamygowda et al. 2019). In this experiment, during aerobic shrimp cultivation, the abundance of *Actinobacteria* was higher in high-salinity system as 0.30 to 7.56% at 5 PSU, 0.23 to 10.56% at 15 PSU and 0.18 to 13.94% at 25 PSU whereas the low relative levels were detected under anoxic condition. *Planctomycetes* related to the anaerobic ammonia-oxidizing (anammox) bacteria were more dominant under aerobic (between 0.15 and 3.29%) than anoxic (between 0.00 and 0.03%) condition. Likewise, the results from marine RAS bioreactors also indicated that Planctomycetes were found only on the nitrifying biofilter with high oxygen availability whereas there were no detection in anoxic bioreactor (Brailo et al. 2019).

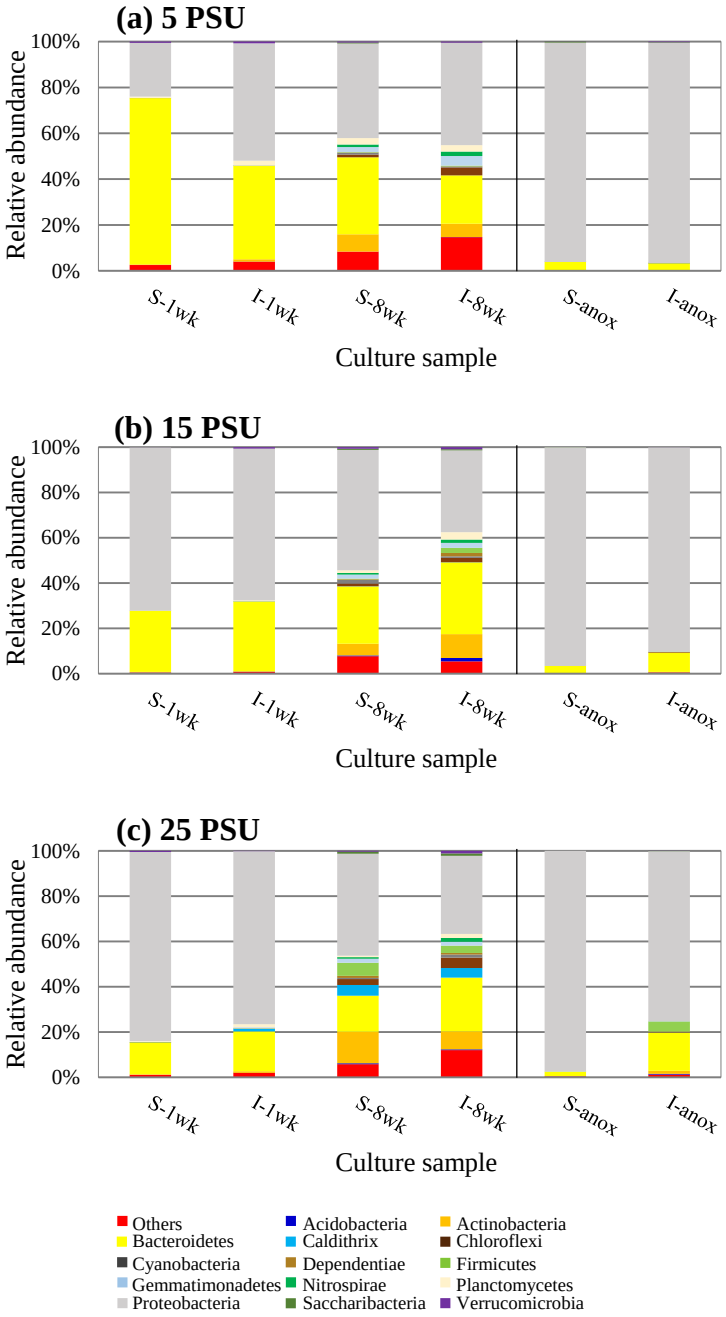

Figure 28. Relative abundances (%) of bacterial phyla, as determined by Miseq pyrosequencing, observed on fibrous Biocord^{TM} biofilter acclimated under (a) low- (5 PSU), (b) medium- (15 PSU) and (c) high-salinity (25 PSU) levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

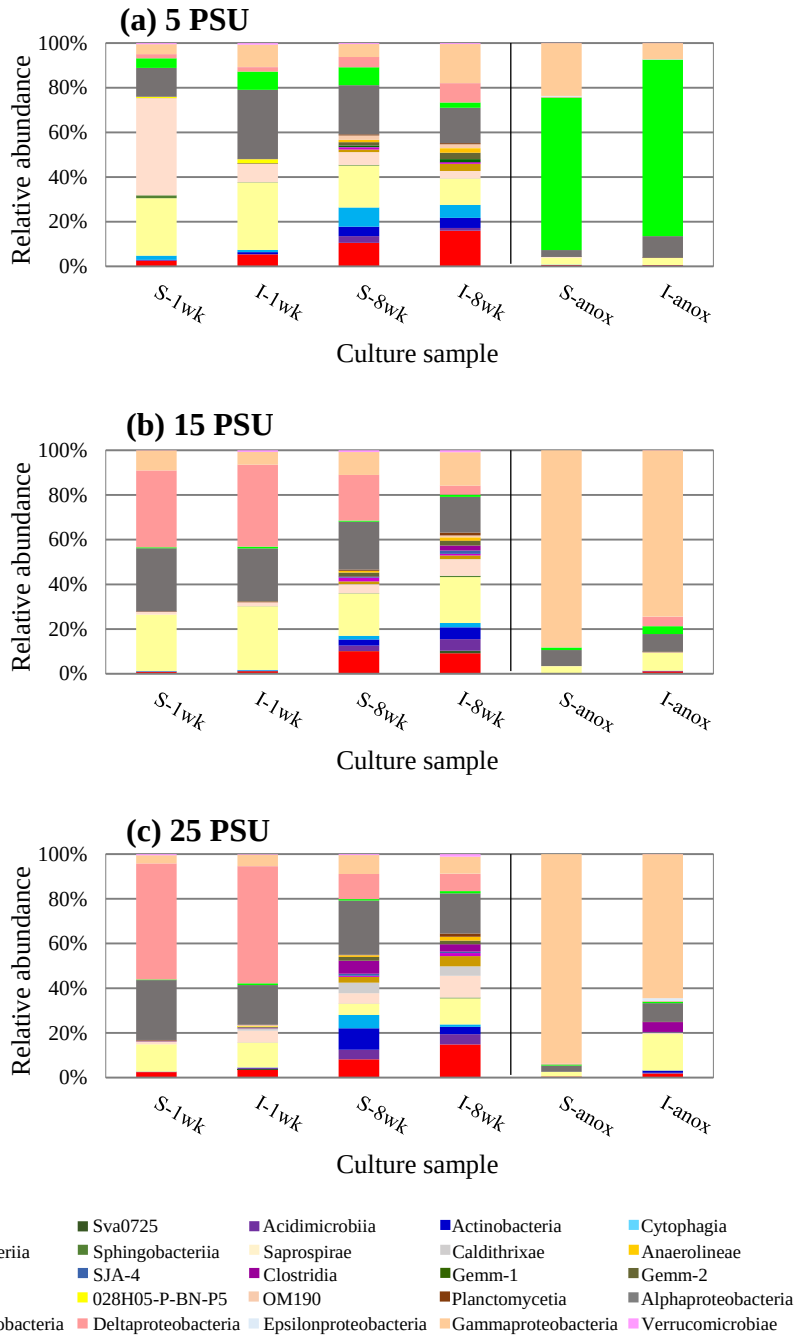

Figure 29. Relative abundances (%) of bacterial class, as determined by Miseq pyrosequencing, observed on fibrous Biocord^{TM} biofilter acclimated under (a) low- (5 PSU), (b) medium- (15 PSU) and (c) high-salinity (25 PSU) levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

Beta diversity analysis of phyla diversity among biofilters acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels in semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (on week 1 and 8), followed by anoxic denitrification are shown in figure 30.

The results indicated that the diversity patterns of bacterial phylum were more influenced by acclimation time than salinity level, and were less influenced by shrimp stocking density. The phylum diversity increased with operational period, and then decreased when aerobic was switched to anoxic condition. The relative abundance of *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes* and *Nitrospirae* observed on fibrous biofilter acclimated for 8 weeks were *obviously higher* than other periods. For the effect of salinity, some important phylum in nitrogen cycle was promoted under low-salinity condition. Many previous studies have already verified that *Nitrospirae*, related to the chemolithoautotrophic nitrifiers, are sensitive to high salt concentrations (Yu et al. 2004; Dincer and Kargi 1999; Abeliovich 2006; Cortés-Lorenzo et al. 2015). In this study, *Nitrospirae* could be found in all experiments at various salinity levels, but were more dominant in low-salinity system. Contrarily, the relative abundance of *Acidobacteria* and *Caldithrix* were higher when salt concentration was increased. Finally, in term of the initial shrimp stocking density, there were no difference between bacterial phyla observed on the fibrous biofilter acclimated in semi-intensive and intensive aquaculture systems under low- and medium-salinity levels. At the salinity of 25 PSU, nevertheless, the phylum diversity was greater in high-density shrimp culture tank.

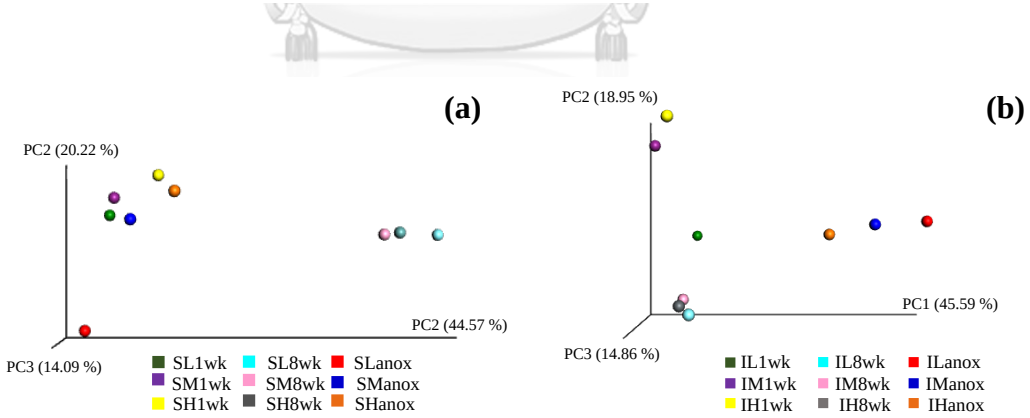

Figure 30. Dissimilarity (beta diversity) among bacterial communities observed on fibrous Biocord^{TM} biofilter acclimated under L = low- (5 PSU); M = medium- (15 PSU); H = high-salinity (25 PSU) levels in (a) semi-intensive (S; 50 shrimp m^{-2}) and (b) intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1^{st} and 8^{th} weeks), followed by anoxic denitrification, calculated as Principal Coordinates Analysis (PCoA) 3D plot of the weighted UniFrac distance matrix.

Bacterial community structure at the genus level

The richness heat-map of the bacterial genera observed on the fibrous Biocord^{TM} biofilter acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels in semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (on week 1 and 8), followed by anoxic denitrification are shown in figure 31.

Results demonstrated that the predominant genus on filter acclimated at salinity of 5 PSU was different from that in medium- and high-salinity systems. During the first period (week 1) of experiment, *Lewinella* (OTU579046, 3.80 to 40.69% abundance), *Arenibacter* (OTU809275, 11.95 to 12.23% abundance) and *Flavobacteriaceae bacterium* (OTU69822, 6.82 to 7.69% abundance) were the most abundant genus in low-salinity system. According to the NCBI blast, the OTU579046 is related to *Lewinella cohaerens* strain ATCC 2312 (NCBI Reference Sequence: NR_115012.1) from the beach sediments while the OTU809275 is related to *Arenibacter sp.* TBL_45 (GenBank: JX854294.1) from the North Sea on solid media and finally the OTU69822 is related to *Maribacter flavus* strain KCTC 42508 (NCBI Reference Sequence: NR_144593.1), respectively. *Arenibacter* are member of family *Flavobacteriaceae* which have been isolated from various marine environments (Bakunina et al. 2013). Meanwhile, the *PB19* bacterium were dominant at other salinity (15 and 25 PSU) levels in which the OTU4915 with 45.98% maximum abundance was promoted in semi-intensive tanks and the OTU220 with 38.58% maximum abundance was promoted in intensive aquaculture systems. Both OTUs are related to the uncultured bacterium clone IZ1RPV404EDW2P (GenBank: KP947345.1) which have been detected in intestines of the black tiger shrimp and the Pacific white shrimp. In correlation with another RAS, the *PB19* bacterium had also been found in the Turbot, *Scophthalmus maximus*, culture system at the salinity of 24 PSU (Martins et al. 2013). Under anoxic denitrification, furthermore, the growth of *Methylotenera* (OTU571984, 42.43 to 49.24% abundance and OTU549523, 24.08 to 27.26% abundance) was promoted in low-salinity system while *Methylophaga* were the outstanding group under other salinity conditions. Within *Methylotenera*, both OTU571984 and OTU549523 are related to the *Methylomonas clara* strain D22 (GenBank: CP033953.1) which grow in high methanol concentration medium. For *Methylophaga*, the semi-intensive systems contributed to promote the growths of both OTU226125 (*Methylophaga sp.* DG1507; GenBank: KC295387.1) and OTU4366292 (*Methylophaga sp.* M1; GenBank: KU524454.1) with 44.17 and 63.88% abundance at 15 and 25 PSU, respectively, while the intensive systems contributed to promote the growth of OTU278985 (uncultured *Methylophaga sp.*, clone SARG_54; GenBank: AM238581.1), as ranging from 37.51 to 47.51%. Both genus, *Methylotenera* and *Methylophaga*, were linked with the methanol cycle in association with denitrification. Previous research studies reported that *Methylotenera* have been isolated from freshwater sediments (Kalyuzhnaya et al. 2012; Kalyuhznaya et al. 2009;

Kalyuzhnaya et al. 2009) and low-salinity RAS (Satanwat et al. 2019) while *Methylophaga* are typically isolated form brackish and marine environments (Rissanen et al. 2016; Auclair et al. 2012).

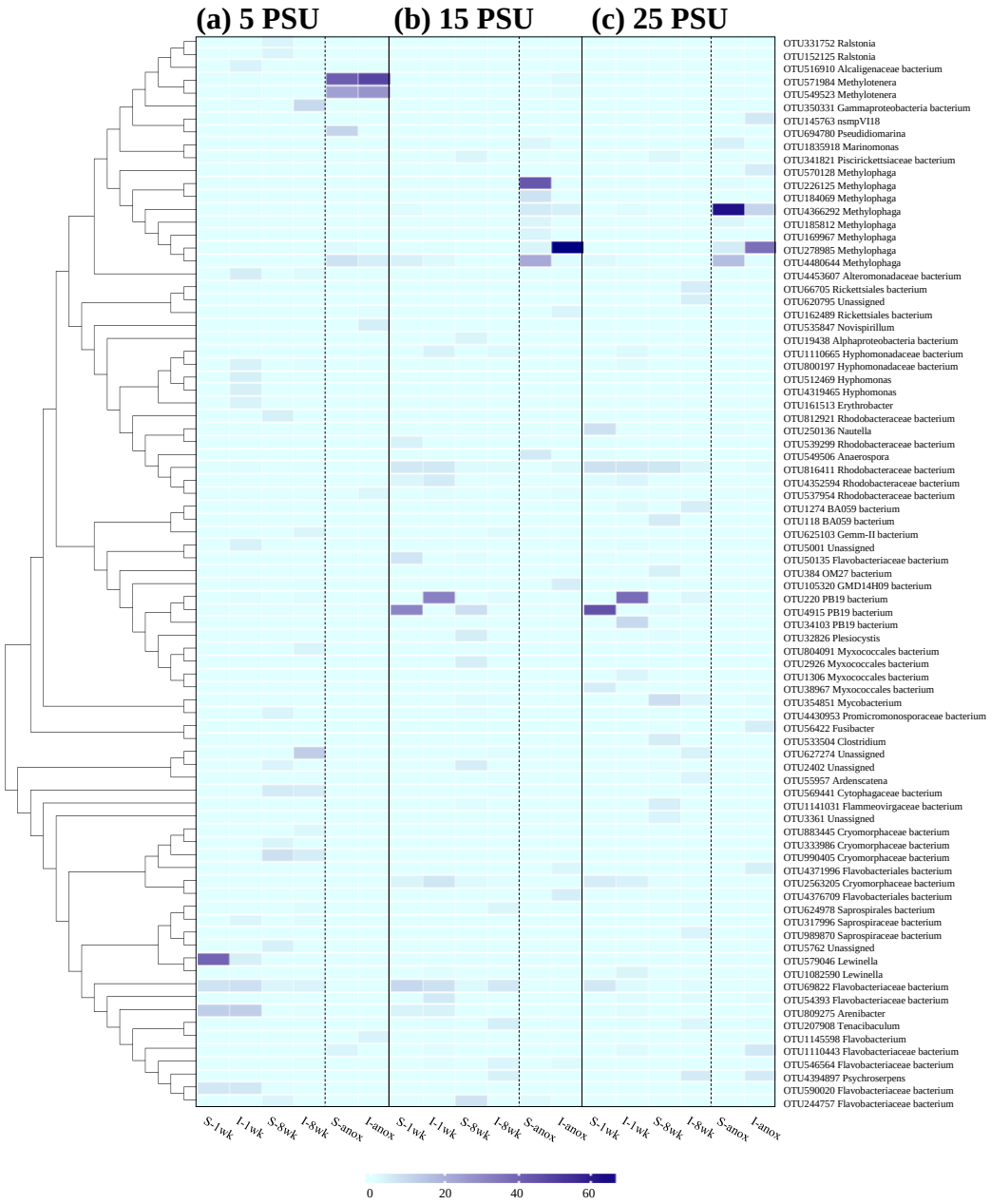

Figure 31. Richness heat-map of the bacterial genera observed on fibrous Biocord^{TM} biofilter acclimated under (a) low- (5 PSU), (b) medium- (15 PSU) and (c) high-salinity (25 PSU) levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

- **Nitrifying community structure**

Nitrosomonadaceae and *Nitrospiraceae* were the families of nitrifying bacteria observed on the fibrous Biocord^{TM} biofilter acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels in semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) aquaculture systems, as shown in figure 32.

Family *Nitrosomonadaceae* belong to phylum *Proteobacteria* and consist of two genera, i.e. *Nitrosomonas* and *Nitrosospira*. The phylogeny of this family bases on ammonia monooxygenase (*amoA*) gene that catalyzes the first step of nitrification (Prosser et al. 2014). For chemolithoautotrophic aerobic NOB, family *Nitrospiraceae* are in phylum *Nitrospirae* and contain genera *Nitrospira* (Daims 2014). In this study, there were 6 OTUs of *Nitrosomonadaceae* and 5 OTUs of *Nitrospiraceae*. Almost OTUs in the family *Nitrosomonadaceae* could be found on fibrous filter since first week of acclimation while only one OTU of *Nitrospiraceae*, the uncultured *Nitrospira sp.* clone Bb_17_10_ HH_clone55 (GenBank: JQ900201.1), was observed. The absence of NOB was probably due to the short period of acclimation in which some species could be identified after 3–6 months (Abeliovich 2006). Similar to the results in richness heat-map of the bacterial genera, the predominant AOB in low-salinity system was different from that under medium- and high-salinity conditions. The uncultured *Nitrosomonadaceae* bacterium clone 2d_95589 (GenBank: MG802020.1) was dominant under 5 PSU while the relative abundance was lower under 15 PSU and it was absolutely absent under 25 PSU. Meanwhile, other OTUs, were preferred to live in high-salinity system. Notwithstanding, the AOB could not survive during denitrification with methanol supplement (Munz et al. 2011), indicating by the disappearance of *Nitrosomonadaceae* in the last period (anoxic) of experiment. For NOB, the growth of uncultured *Nitrospira sp.* clone Bb_17_10_HH_clone55 (GenBank: JQ900201.1) was promoted under low-salinity condition while the relative abundance of uncultured *Nitrospira sp.* clone Bb_28_10_HH_clone81 (GenBank: JQ900198.1) increased in high-salinity system. Interestingly, the uncultured Nitrospira sp. clone Bb_17_10_HH_clone55 (GenBank: JQ900201.1) and the uncultured *Nitrospira sp.* clone Bb_28_10_HH_clone81 which belong to the family *Nitrospiraceae* could still be found, but with the low abundance, under anoxic condition after methanol addition in both medium- and high-salinity systems. Similar to the research conducted by Schramm et al. (2000) that the genus Nitrospira were also found at the oxic-anoxic interface of biofilter in reactor (Schramm et al. 2000).

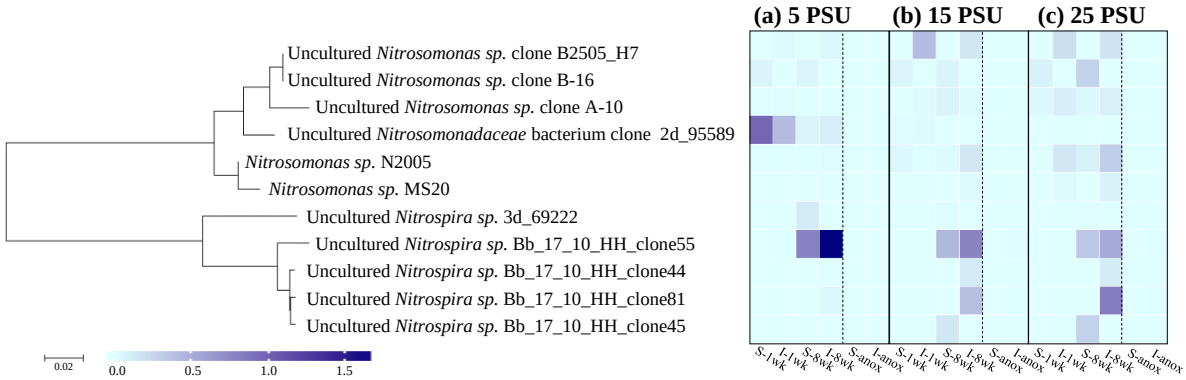

Figure 32. Richness heat-map of the nitrifying bacteria observed on fibrous Biocord^{TM} biofilter acclimated under (a) low- (5 PSU), (b) medium- (15 PSU) and (c) high-salinity (25 PSU) levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

- **Anaerobic ammonia-oxidizing (anammox) bacterial group**

Planctomyces were the genus of anammox bacteria observed on the fibrous Biocord^{TM} biofilter acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels in semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) aquaculture systems, as shown in figure 33.

Taxonomically, the genera *Planctomyces* belong to phylum *Planctomycetes*, class *Planctomycetia*, order *Planctomycetales* and family *Planctomycetaceae*. In this study, there were 3 OTUs of *Planctomyces* related to bacteria that can anaerobically oxidize ammonium, with nitrate or nitrite, to nitrogen gas. Results indicated that almost anammox bacteria were found in intensive shrimp culture systems since first week of acclimation. During the prolonged operation (week 8), the uncultured *Planctomycete* clone BO592 (GenBank: DQ368077.1) was promoted in high-salinity system at maximum abundance of 0.60%. Related to the previous study, the uncultured *Planctomycete* clone BO592 and clone BO821 (GenBank: DQ368113.1) have also been isolated from the seawater in Black Sea's suboxic zone where oxygen is limited (Kirkpatrick et al. 2006). The uncultured *Planctomycete* clone CIS36 (GenBank: MH630164.1) that has been detected in another RAS water, was abundance in medium- (0.15% abundance) and low-salinity (0.08% abundance) systems, respectively. Strangely, even though Kirkpatrick et al. (2006) reported the prevalent of some *Planctomycete* under the permanent anoxic condition, all OTUs in this experiment were absent under anoxic denitrification with methanol addition. Meanwhile, the results from marine RAS also indicated the absence of phylum *Planctomycetes* in anoxic bioreactor (Brailo et al. 2019).

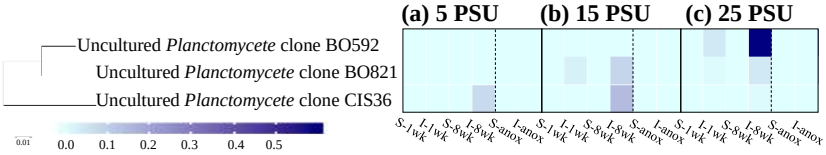

Figure 33. Richness heat-map of the anammox bacteria observed on fibrous Biocord^{TM} biofilter acclimated under (a) low- (5 PSU), (b) medium- (15 PSU) and (c) high-salinity (25 PSU) levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

- **Vibrio bacterial group**

The richness heat-map of the *Vibrio* bacteria observed on the fibrous Biocord^{TM} biofilter acclimated under low- (5 PSU), medium- (15 PSU) and high-salinity (25 PSU) levels in semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (on week 1 and 8), followed by anoxic denitrification are shown in figure 34.

Vibrio is the gram-negative bacterial genus belonged to phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Vibrionales* and family *Vibrionaceae*. Generally, *Vibrio* cause the anorexia, behavioural changes and mortality, ranging from insignificant to 100%, particularly in post-larvae (PL) and young juvenile shrimp (Karunasagar et al., 1994). In this study, 8 OTUs of *Vibrio* were observed. Some OTU, e.g. OTU106027, could be presented since first week of experiment. The OTU106027 related to *Vibrio vulnificus* strain CMCP6 (GenBank: CP037932.1) was accounted for 0.08 and 0.38% in semi- and intensive systems, respectively, at the salinity of 5 PSU. Thereafter, the numbers of *Vibrio* obviously increased when the experiments were operated for 8 weeks. The OTU559632 related to *Vibrio sp.* T12 (GenBank: LC184187.1) was the predominant in semi-intensive RAS at high-salinity level with the maximum abundance of 0.84% at 25 PSU, followed by 0.48% at 15 PSU. While the OTU578606 related to *Vibrio sp.* K22-41 (GenBank: EU333880.1) was abundance in semi-intensive RAS at low-salinity level with 0.46% at 5 PSU, followed by 0.25% at 15 PSU. These results were consistent with previous study that the *Vibrio* can survive better in seawater while the salt concentration varies for the different species (Percival and Williams 2014). Under anoxic denitrification, the low level of expression was clearly found; however, the growth of some sequence, e.g. *Vibrio sp.* K22-41, which has also been isolated from a cold desert of the Indian Himalayas could still be presented.

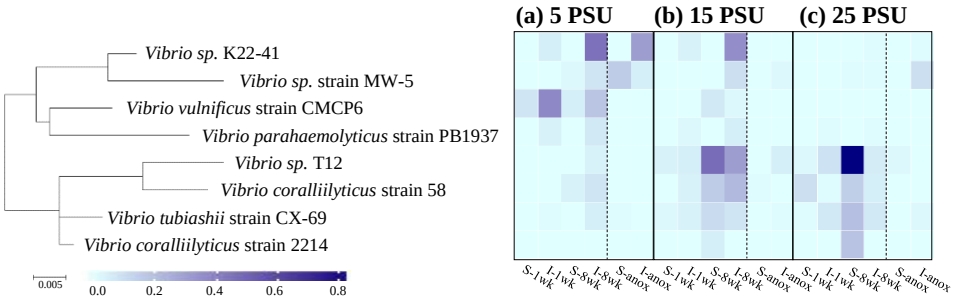

Figure 34. Richness heat-map of the Vibrio bacteria observed on fibrous Biocord^{TM} biofilter acclimated under (a) low- (5 PSU), (b) medium- (15 PSU) and (c) high-salinity (25 PSU) levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

4.2 Application of biofilter in marine RAS for long-term operation

This study was focused on the evaluation of the performance of biofilter for long-term operation in marine RAS and to monitor the changes in microbial community during long-term operation. The experimental results were divided into 3 parts including: 1) long-term operation of aquaculture system, 2) nitrogen removal rates of fibrous biofilter and 3) microbial diversity in long-term operation of aquaculture system as follows:

4.2.1 Long-term operation of marine RAS

The inorganic nitrogen profiles in long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 35.

During the first 60 days of biofilter acclimation, the accumulation of ammonia and nitrite at high concentrations were observed. Ammonia was naturally converted from nitrogenous organic matter through ammonification (van Rijn 2013; Stewart et al. 2006), and increased rapidly before reaching the level of 2.60±1.70 mg-N L^{-1} on day 6. To prevent the toxicity of ammonia at elevated concentrations on shrimp (over than 1 mg-N L^{-1}), the water was periodically applied to exchange. The increase in ammonia, however, was still found with the maximum concentration of 2.92±0.04 mg-N L^{-1} on day 10. Afterward, the level of ammonia began to decrease continuously at the same time as nitrite increased, indicating the occurrence of first step nitrification, nitritation process, driven by ammonia-oxidizing microorganisms (Ward 2008). Results was supported by Keuter et al. (2017) that the activity of ammonia oxidizing microorganisms increased quickly and ammonia concentrations remained acceptable

after the systems were stocked with aquatic animals. For nitrite, similar to study 1, the long-term accumulation (more than 1 month) was presented thereafter in the experimental system with the maximum level of 6.96±0.39 mg-N L^{-1} on day 37. Nitrate started to increase after 1 month of biofilter acclimation which probably related to the function of NOB. Complete nitrification without nitrite accumulation occurred after 54 days of acclimation due to the development of sufficient nitrite-oxidizing biofilm on biofilters. Nevertheless, the initial shrimp density of 1 kg-shrimp m^{-3} (intensive stocking density) was higher than the nitrogen removal capacity of RAS using non-acclimated biofilter.

For the first crop of aerobic shrimp cultivation (after biofiter acclimation), the acclimated fibrous biofilter could control ammonia and nitrite within the acceptable levels with the average concentrations of 0.16±0.09 and 0.12±0.11 mg-N L^{-1}, respectively. According to the complete nitrification process, the levels of nitrate increased continuously to the highest of 54.72±0.61 mg-N L^{-1} on day 120. Denitrification was performed after shrimp cultivation by switching the system to anoxic condition with methanol addition at COD:Nitrate-N ratio of 5:1 (Pungrasmi et al. 2016; van Rijn et al. 2006). The acclimated biofilter was effective to remove nitrate to 5.91±3.80 mg-N L^{-1} within one week (day 127). Therefore, the system was re-oxygenated (for 3 days) and then started a second round of replication on day 130. Similar results were obtained from second crop of aerobic shrimp cultivation in which ammonia and nitrite were controlled with the average concentrations of 0.17±0.13 and 0.17±0.18 mg-N L^{-1}, respectively, while the concentration of nitrate raised continuously to 71.38±2.38 mg-N L^{-1} on day 183. It seemed like the amount of nitrate accumulated in the second crop was higher than in previous crop. This was probably related to the accumulation of organic nitrogen in RAS during long-term operation. With the methanol supplement, denitrification was also operate completely within one week to remove nitrate to the concentration of 8.39±1.83 mg-N L^{-1} on day 189. To evaluate the probability of third crop cultivation, finally, the shrimp was cultured for more two weeks and the results presented the similar trend of inorganic nitrogen compounds. The average levels of ammonia and nitrite were 0.11±0.09 and 0.07±0.14 mg-N L^{-1} while the nitrate concentration stimulated to 14.69±0.82 mg-N L^{-1} on the last day of the experiment (day 210).

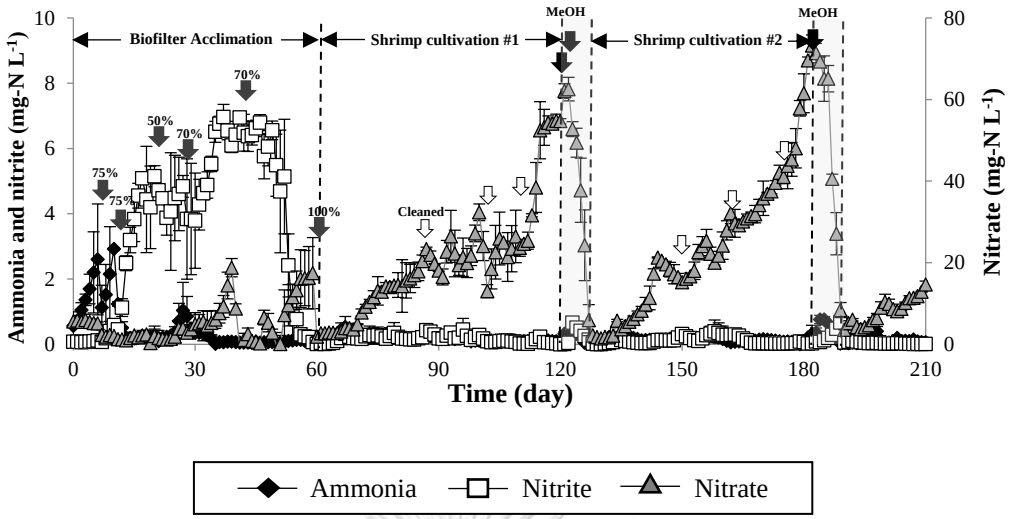

Figure 35. Inorganic nitrogen profiles in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps). Grey arrows (⬇) indicate the percentage of water exchange, white arrows (⇩) indicate the cleaning of biofilter and black arrows (⬇) indicate the addition of methanol.

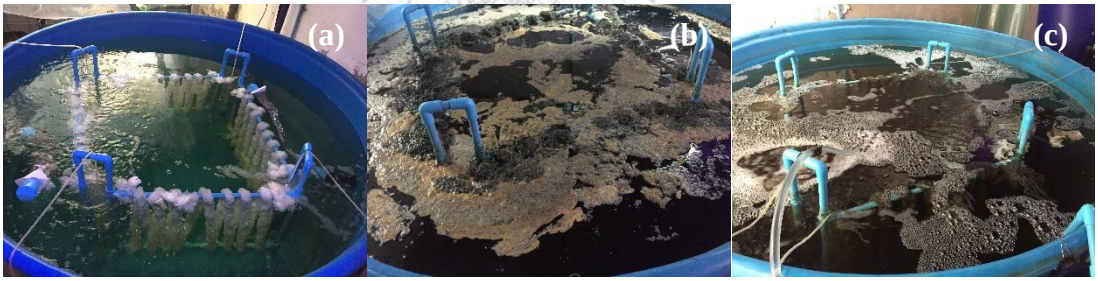

Figure 36. Experimental marine RAS for long-term operation of (a) biofilter acclimation with aerobic shrimp cultivation, (b) denitrification (anoxic, no shrimps) and (c) re-oxygenation, without water exchange.

- **Water quality in marine RAS**

The water quality parameters in long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in table 17.

Results of the water quality showed that all parameters during the entire period of aerobic condition were appropriate for the normal growth of an aquatic animal as well as an aerobic microorganism. The DO concentration (figure 37) were between 8 and 9 mg-O_2 L^{-1} which encouraged the growth of healthy shrimp (Nonwachai et al. 2011) and was sufficient for complete nitrification (Ward 2008). The temperature

values were within the range of 25.9 to 29.1°C and the pH levels (figure 38) were between 7.3 and 8.0. The gradual decrease of alkalinity during aerobic period (figure 39) was directly related to nitrification (Gujer and Jenkins 1975). For the phases of biofilter acclimation (60 days) and first crop of shrimp cultivation (60 days), the supplement of bicarbonate was periodically performed to maintain the alkalinity within the range of 100 to 150 mg-$CaCO_3$ L^{-1}. Meanwhile, in the second (60 days) and third (15 days) crops of cultivation, there was no additional bicarbonate related to the elevated concentration of remaining alkalinity resulted from previous denitrification, as started from 320.0±14.1 and 400.0±56.6 mg-$CaCO_3$ L^{-1}, respectively. This was related to research study by Li et al. (2008) that the nitrification and denitrification co-processes contributed to reduce the chemical supply to maintain alkalinity in the system. The ORP values in aquaculture water zone (figure 40) were within the recommendation for nitrification as between 62.9 and 162.9 mV (Li and Irvin 2007) while the lower range of –27.1 to 65.2 mV was observed inside fibrous biofilter structure due to the partial lack of dissolved oxygen.

For the whole period of denitrification, the sudden decline of DO to lower than 0.5 mg-O_2 L^{-1} occurred after methanol addition. With the low oxygen condition, the growth of heterotroph was promoted instead of autotrophic microorganisms (Spietz et al. 2015; van Niel et al. 1993). The water temperatures ranged from 27.4 to 29.7°C and the pH dropped from 7.51±0.09 to 7.36±0.01 and from 7.44±0.04 to 6.41±0.17, for first and second rounds of denitrification, respectively. Nitrate removal via denitrification normally increases both pH and alkalinity. In this study, a small decrease in pH was possibly resulted from the production of H_2S in some dead-zones (Kim and Bae 2000). The alkalinity increased clearly as from 160.0±0.0 to 320.0±14.1 mg-$CaCO_3$ L^{-1} and from 150.0±14.1 to 400.0±56.6 mg-$CaCO_3$ L^{-1}, for two rounds of replication, respectively, related to the role of denitrifying microorganisms (van Rijn et al. 2006). The immediate decrease in ORP to minus values as from 18.2±7.5 to −137.9±3.4 mV and from −11.1±2.2 to −200.5±12.2 mV were observed in both aquaculture water and biofilter core, respectively. These ranges were little higher than the criteria for denitrification of between −200 and −400 mV (Lee et al. 2000); nevertheless, they were clearly indicated the prevalence of anoxic condition in experimental system. The COD concentration was only monitored after methanol supplement, as shown in figure 41. During the reduction of nitrate, the external organic carbon is utilized as an energy source for metabolism, growth and cell synthesis (Hamlin et al. 2008), resulted in the rapid decline from 355.56±31.43 to 22.22±0.00 mg-COD L^{-1} and from 352.78±3.93 to 33.33±15.71 mg-COD L^{-1}, for two rounds of replication, respectively.

Table 17. Water quality parameters measured in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps), without water exchange. Data are shown as the mean ± 1SD.

Parameters	**Acclimation** (Oxic; 60 days)	**Cultivation #1** (Oxic; 60 days)	**Denitrification #1** (Anoxic; 7 days)	**Cultivation #2** (Oxic; 60 days)	**Denitrification #2** (Anoxic; 7 days)	**Cultivation #3** (Oxic; 15 days)
DO ($mg\text{-}O_2\ L^{-1}$)	8.50±0.22	8.50±0.24	0.70±1.05	8.50±0.24	1.60±1.98	8.30±0.18
Temperature (ºC)	27.30±0.54	27.40±0.23	27.80±0.50	27.00±1.07	28.90±0.89	28.10±0.25
pH	7.70±0.15	7.70±0.13	7.50±0.20	7.60±0.16	7.10±0.73	7.80±0.08
Alkalinity ($mg\text{-}CaCO_3\ L^{-1}$)	138.4±22.60	144.00±13.98	248.80±81.28	224.60±48.65	280.00±90.42	321.80±49.66
ORP in water (mV)	122.10±14.05	123.30±7.85	–22.60±71.43	128.10±14.77	–66.00±78.76	83.10±9.56
ORP in biofilter (mV)	30.60±23.93	40.30±8.98	–91.00±83.07	40.10±7.30	–107.60±94.76	33.50±7.42
COD ($mg\text{-}COD\ L^{-1}$)	Nm	Nm	338.90±69.39	Nm	247.20±99.34	Nm
SS ($mg\text{-}SS\ L^{-1}$)	67.70±47.74	121.40±46.82	100.40±0.00	125.00±28.15	Nm	Nm

Nm = not measure

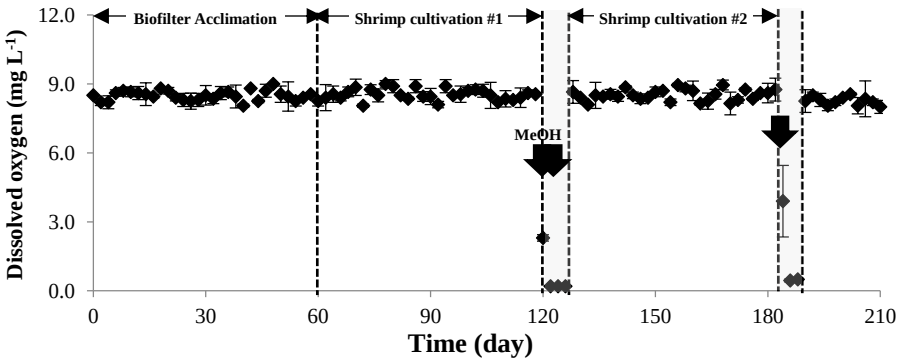

Figure 37. Dissolved oxygen in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps). Black arrows (⬇) indicate the addition of methanol.

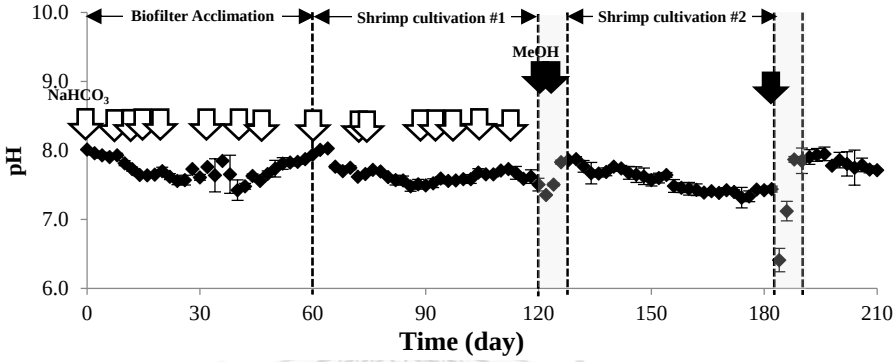

Figure 38. The pH in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps). White arrows (⇩) indicate the addition of sodium bicarbonate and black arrows (⬇) indicate the addition of methanol.

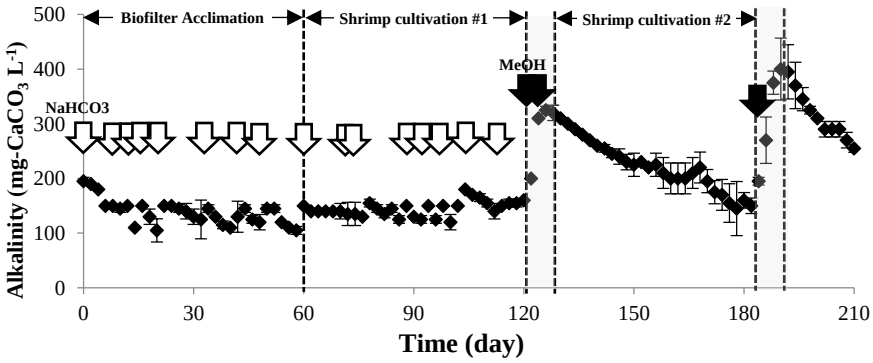

Figure 39. Alkalinity in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps). White arrows (⇩) indicate the addition of sodium bicarbonate and black arrows (⬇) indicate the addition of methanol.

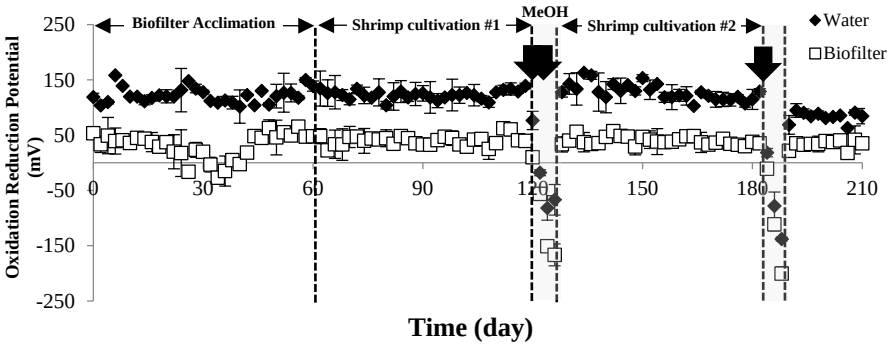

Figure 40. Oxidation-reduction potential in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).
Black arrows (⬇) indicate the addition of methanol.

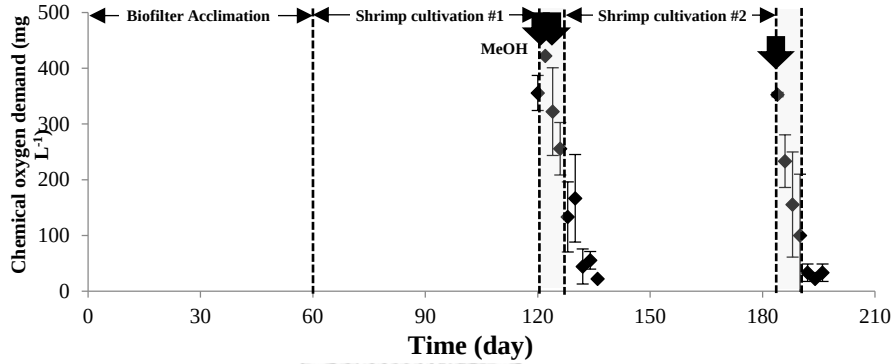

Figure 41. Chemical oxygen demand in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).
Black arrows (⬇) indicate the addition of methanol.

Suspended solid in long-term operation of shrimp culture system is shown in figure 42. The concentration of suspended solid continuously increased to 108.5±31.82 mg-SS L^{-1} during the biofilter acclimation period, though the aquaculture water was periodically exchanged. To begin the first crop of shrimp cultivation, the marine water was completely 100% changed; however, the rise in concentration was still found. The levels of suspended solid were fluctuated between 55.0 and 184.0 mg-SS L^{-1} which were higher than the tolerance value of lower than 40 mg-SS L^{-1} (Muir, 1982). Related to the previous study, nonetheless, the shrimp culture could still succeed in the RAS with suspended solid concentration in range of 100 to 300 mg-SS L^{-1} (Gaona et al. 2015).

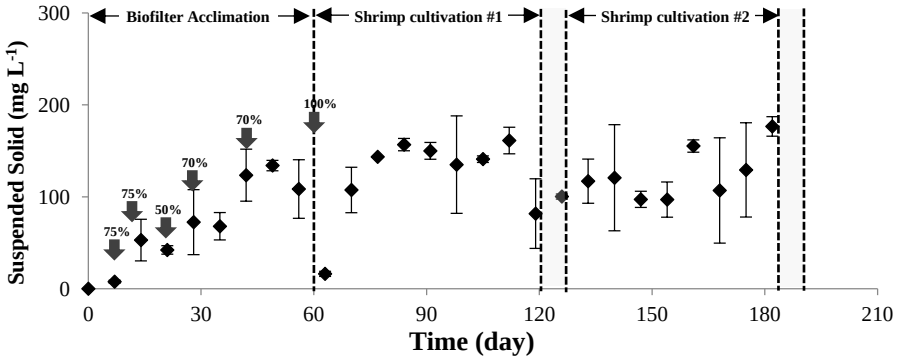

Figure 42. Suspended solid in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).
Grey arrows (⬇) indicate the percentage of water exchange.

- **Nitrogen mass balance**

The percentage of organic compounds (carbon, hydrogen and nitrogen) and the nitrogen mass balance in long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in table 18 and 19, respectively.

Almost all of nitrogen input was derived from protein in shrimp feed as equal to 64.12% (in biofilter acclimation), 68.70% (in first crop of shrimp cultivation) and 69.68% (in second crop of shrimp cultivation), respectively. During the operation of RAS, the nitrogen was transferred to accumulate in shrimp as equal to 68.33, 68.67 and 49.36% on the last day of the experiment in each period. For dissolved inorganic nitrogen (DIN), the percentage of 3.39 was found during biofilter acclimation related to the accumulation of remaining nitrate from the development of complete nitrification activity. With the co-processes of nitrification and denitrification using fibrous biofilter that could remove DIN, the lower percentages of 1.12 and 0.06% were observed in two crops of shrimp cultivation. The remaining sediments of 1.48, 1.32 and 1.55% were still deposited in aquaculture system during biofilter acclimation, shrimp cultivation crop 1 and 2, respectively, while the higher proportions of solid particle (2.88, 8.45 and 4.87%) were removed by using the solid capture device and the cleaning of biofilter. For the unidentified nitrogen, during biofilter acclimation, the percentage of 23.92 was observed. The loss of nitrogen was probably caused by the water exchange that was periodically performed to prevent the toxicity of ammonia and nitrite on shrimp when biofilter was not activated. In first and second crop of shrimp cultivation, the 20.44 and 43.57% of unidentified nitrogen were probably mainly related to the denitrification process that could reduce nitrate to nitrogen gas.

Table 18. Percentages of carbon, hydrogen and nitrogen in artificial feed, white shrimp and sediment in marine RAS, measured by CHNS elemental analyzer.

Sample	Percentage		
	Carbon	**Hydrogen**	**Nitrogen**
Artificial feed	41.05	6.25	9.71
White shrimp	38.82	6.39	14.70
Sediment	20.83	3.67	6.99

Table 19. Nitrogen budget in long-term operation (210 days) of marine RAS.

Period	Composition	Nitrogen (g)	Nitrogen (%)
Biofilter acclimation	**Input**		
	Feed	647.45	64.12
	Shrimp	342.49	34.62
	Dissolved inorganic nitrogen (DIN)	12.46	1.26
	Sediment	0.00	0.00
	Total	1002.40	100.00
	Remain		
	Shrimp	679.66	68.33
	Dissolved inorganic nitrogen (DIN)	35.18	3.39
	Sediment	14.84	1.48
	Solid removal	28.35	2.88
	Unidentified	244.37	23.92
	Total	1002.40	100.00
Shrimp cultivation #1	**Input**		
	Feed	748.79	68.70
	Shrimp	334.93	30.73
	Dissolved inorganic nitrogen (DIN)	3.91	0.36
	Sediment	2.30	0.21
	Total	1089.92	100.00
	Remain		
	Shrimp	748.52	68.67
	Dissolved inorganic nitrogen (DIN)	12.18	1.12
	Sediment	14.38	1.32
	Solid removal	92.17	8.45
	Unidentified	222.67	20.44
	Total	1089.92	100.00

Table 19. Nitrogen budget in long-term operation (210 days) of marine RAS (continued).

Shrimp cultivation #2	**Input**		
	Feed	1113.21	69.68
	Shrimp	465.20	29.21
	Dissolved inorganic nitrogen (DIN)	2.87	0.18
	Sediment	16.58	1.03
	Total	1597.86	100.00
	Remain		
	Shrimp	787.89	49.36
	Dissolved inorganic nitrogen (DIN)	10.52	0.66
	Sediment	24.76	1.55
	Solid removal	77.80	4.87
	Unidentified	696.89	43.57
	Total	1597.86	100.00

- **Growth of white shrimp in marine RAS**

Growth of white shrimp cultured in long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in table 20.

Shrimp were started to culture at the average weight of 3.64±0.93 and 3.56±0.55 g and at the average length of 8.46±0.80 and 8.66±0.58 cm, for the biofilter acclimation and first crop of cultivation, respectively. While the bigger size shrimp with average weight of 7.03±1.26 g and average length of 9.83±1.00 cm was used in the second crop of cultivation. According to the statistical results in figure 43, the increase of shrimp size during two months of cultivation period in the first crop of cultivation was more than in biofilter acclimation which was probably related to the water quality, especially the concentrations of ammonia and nitrite in RAS (Gutierrez-Wing and Malone 2006). Also, the average DWG of shrimp in the first (0.08±0.01 g-shrimp day^{-1}) and second (0.08±0.02 g-shrimp day^{-1}) crops of cultivation was slightly higher than in biofilter acclimation period (0.06±0.00 g-shrimp day^{-1}). With the survival rate, the results clearly indicated that the shrimp could survive better in RAS with acclimated biofilter with the survival rate of 54.35±6.14% and 76.67±3.46% for the first and second crops of cultivation than during the biofilter acclimation period (23.44±6.63). Compared with another RAS (Ray and Lotz 2017), the percentages of shrimp survival in this study were close to the rate in aquaculture with a moving bed bioreactor (MBBR) as 61±0.0%, whereas the rate in biofloc (BF) treatment was 43±14%. Finally, for shrimp density, the initial and final (at 2 months) density of shrimp cultured during biofilter acclimation and first crop of cultivation were from 1.16±0.09 to 2.31±0.02 kg m^{-3} and from 1.14±0.00 to 2.55±0.14 kg m^{-3}, respectively. Meanwhile, the operation of marine RAS

for second crop of cultivation had the efficient ability to culture shrimp at high stocking density as from 1.58±0.07 to 2.68±0.18 kg m^{-3}, respectively.

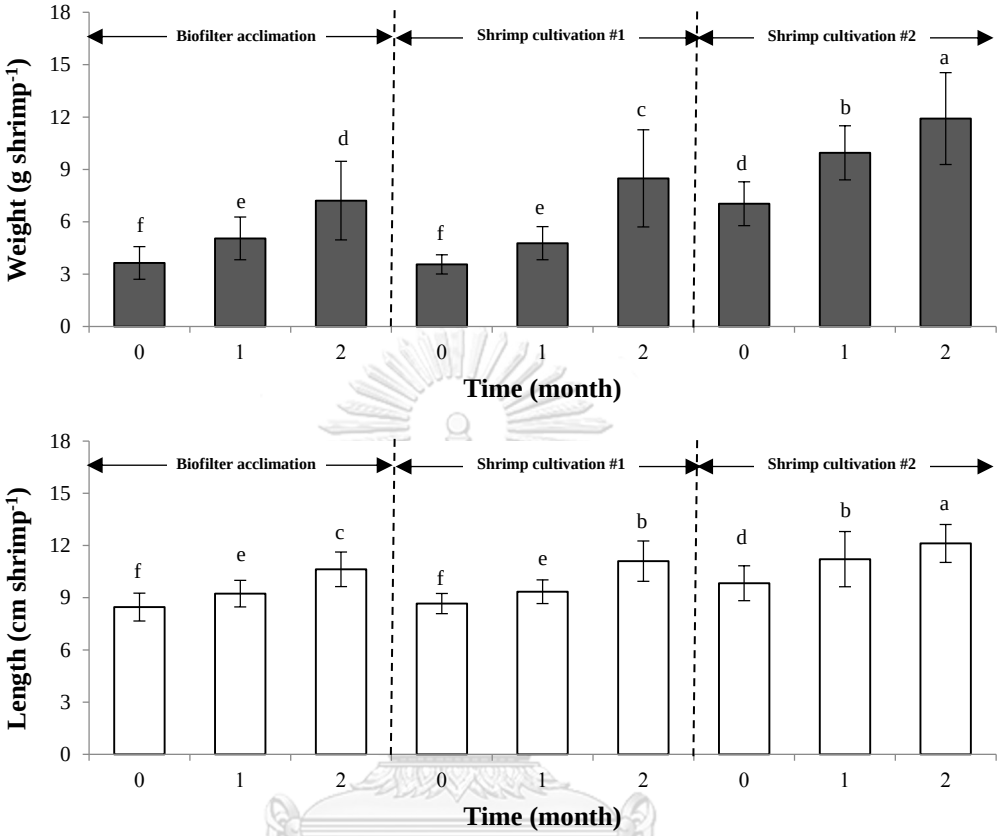

Figure 43. Comparison of shrimp average weight and length which cultured in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days.
Data are shown as the mean ± 1SD. Means with a different letter are significantly different ($P < 0.05$ ANOVA and Duncan's multiple range test).

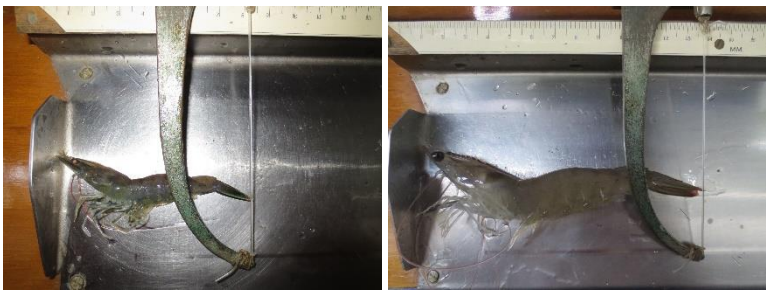

Figure 44. Experimental shrimp size for (a) zero and (b) two months of culture period in long-term operation of marine RAS.

Table 20. Growth of shrimp cultured in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days. Data are shown as the mean ± 1SD, derived from two repeats.

Period	Time (month)	DWG (g day^{-1})	Avg. weight (g shrimp^{-1})	Avg. length (cm shrimp^{-1})	Survival rate (%)	FCR	Density (kg m^{-3})
Biofilter acclimation	0	-	3.64±0.93	8.46±0.80	100.00±0.00	-	1.16±0.09
	1	0.05±0.01	5.05±1.23	9.23±0.76	Nm	2.35±0.22	1.61±0.16
	2	0.07±0.02	7.21±2.25	10.63±0.99	23.44±6.63	2.19±0.79	2.31±0.02
Shrimp cultivation #1	0	-	3.56±0.55	8.66±0.58	100.00±0.00	-	1.14±0.00
	1	0.04±0.00	4.77±0.95	9.35±0.68	Nm	3.53±0.42	1.43±0.03
	2	0.12±0.01	8.49±2.78	11.10±1.16	54.35±6.14	1.16±0.09	2.55±0.14
Shrimp cultivation #2	0	-	7.03±1.26	9.83±1.00	100.00±0.00	-	1.58±0.07
	1	0.10±0.02	9.95±1.55	11.21±1.59	Nm	2.21±0.49	2.24±0.04
	2	0.07±0.02	11.91±2.63	12.12±1.09	76.67±3.46	4.79±1.38	2.68±0.18

Nm = not measured

4.2.2 Nitrogen removal efficiency of fibrous biofilter

This part was focused on the evaluation of nitrification and denitrification efficiencies of fibrous Biocord^{TM} biofilter during long-term operation in RAS. The experimental results are as follows:

- **Nitrification efficiency of fibrous biofilter**

Nitrification rate of fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 45.

The nitrification efficiency of fibrous biofilter increased significantly during the biofilter acclimation period and reached to the maximum rate of 123.00±37.63 mg-N m^{-2} day^{-1} on week 4. The increase of nitrification efficiency was resulted from the attachment and growth of natural microorganisms on material surface. According to the previous study, the development of ammonia-oxidation microorganisms can be completed within one month of acclimation (Kuhn et al. 2010; Sesuk et al. 2009). The efficiency was still constant on week 6 (115.04±17.38 mg-N m^{-2} day^{-1}) before decreasing to 23.12±10.09 mg-N m^{-2} day^{-1} on week 8 which probably resulted from the excessive removal of solid deposited on biofilter (figure 47). In the first crop of shrimp cultivation, the ammonia removal rates of acclimated biofilters were no significant difference throughout two months of aerobic operation period. The rate slightly higher on week 11 (42.76±4.72 mg-N m^{-2} day^{-1}) and became constant before increasing to 63.10±8.47 mg-N m^{-2} day^{-1} on week 17. Under anoxic condition, the nitrifying microorganisms could still play the role in ammonia removal with the remaining rate of 59.87±7.44 mg-N m^{-2} day^{-1}. Related to previous studies, the function of nitrifying organisms could be observed under the absence of oxygen (Mortimer et al. 2004; Schmidt et al. 2002). The statistically significant increase of nitrification efficiency was observed in the second crop of shrimp cultivation. These results were supported by Keuter et al. (2017) that the activity of nitrifying biofilter correlated to the operation period (Keuter et al. 2017), with the maximum rate of 112.30±50.22 mg-N m^{-2} day^{-1} on week 24. Also, the acclimated biofilter could still perform nitrification with the rate of 67.59±6.04 mg-N m^{-2} day^{-1} even though the system was operated under anoxic condition for a week. In the evaluation of the probability for third crop cultivation (week 29), the acclimated biofilter was used effectively indicated by the rather constant rate of 74.33±12.51 mg-N m^{-2} day^{-1}.

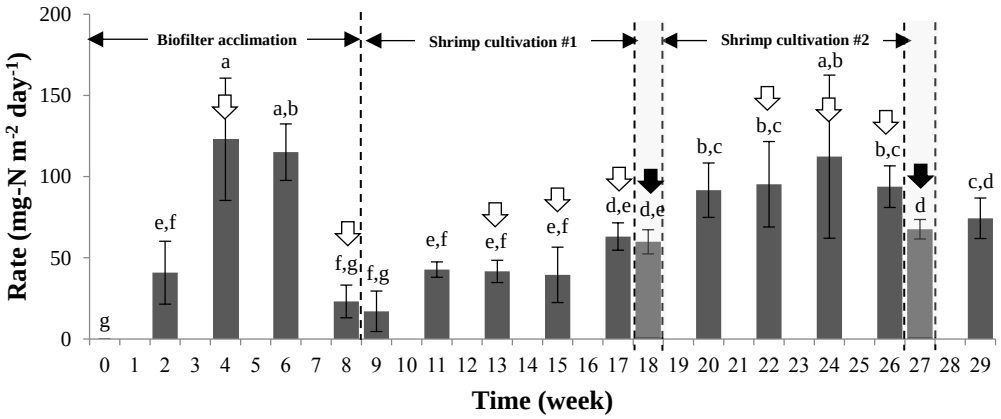

Figure 45. Nitrification rate of Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps). White arrows (⇩) indicate the cleaning of biofilter and black arrows (⬇) indicate the addition of methanol. Data are shown as the mean ± 1SD, derived from six repeats. Means with a different letter are significantly different ($P < 0.05$ ANOVA and Duncan's multiple range test).

- **Denitrification efficiency of fibrous biofilter**

Denitrification rate of fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 46.

Similar to the denitrification rate in study 1, the efficiencies during biofilter acclimation and aerobic shrimp cultivation were represented the ability of biofilter to reduce nitrate via anoxic denitrification process. The results showed that denitrification efficiency increased significantly with the extension of biofilter acclimation period and reached to 21.23±4.35 mg-N m^{-2} day^{-1} on week 8. In the first crop of shrimp cultivation, the water in RAS was 100% changed, resulting in the decline of denitrification rate as 12.59±4.84 mg-N m^{-2} day^{-1} on week 9. The high rate of 20.25±4.55 mg-N m^{-2} day^{-1} was found on week 13 before continuously decreasing to 9.80±2.23 mg-N m^{-2} day^{-1} on week 17 which probably related to the quality of solid deposited on biofilter. The maximum rate of 66.80±15.45 mg-N m^{-2} day^{-1} occurred on week 18 when the system was switched to anoxic condition with methanol supplement at COD: Nitrate-N ratio of 5:1 (Pungrasmi et al. 2016). Same as nitrification, there were the rather constant denitrification rates presented throughout the second crop of shrimp cultivation with slightly higher rate than the first crop of cultivation. Under the anoxic condition, similarly, the highest rate of 67.68±4.74 mg-N m^{-2} day^{-1} was observed on week 27. This results clearly indicated that there was no statistically significant different between denitrification efficiencies of first and second crops of shrimp cultivation. In the evaluation of the probability for third crop cultivation (week 29), the acclimated

biofilter was used effectively indicated by the rather constant rate of 16.79±2.60 mg-N m^{-2} day^{-1}.

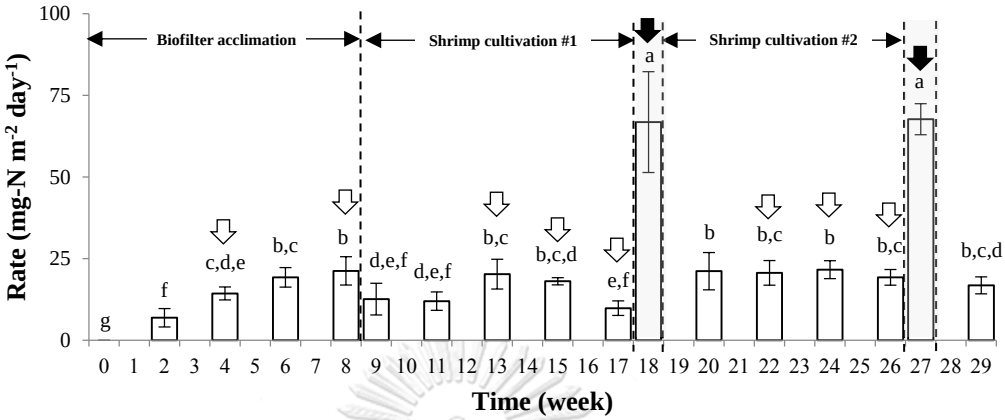

Figure 46. Deitrification rate of Biocord™ biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps). White arrows (⇩) indicate the cleaning of biofilter and black arrows (⬇) indicate the addition of methanol. Data are shown as the mean ± 1SD, derived from six repeats. Means with a different letter are significantly different (P < 0.05 ANOVA and Duncan's multiple range test).

- **Solid deposited on fibrous biofilter**

Solid deposited on fibrous Biocord™ biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 47.

According to the characteristic of Biocord™ biofilter, the excess solid can be captured within the fibrous filter structure which contributes to control the level of suspended solids in RAS (Khammi et al. 2015). In this experiment, the quantity of solid deposited on biofilter increased significantly from 0.49±0.25 g-biomass m^{-1} on week 0 to 28.62±5.10 g-biomass m^{-1} on week 4. The cleaning of biofilter was applied to remove the excess solid deposited on filter material, resulted in the decrease of biomass on biofilter on week 6 and 8. In the first and second crops of shrimp cultivation, the excess solid deposit was cleaned more often (as on week 4, 6 and 8 of each crop) to maintain the quantity of biomass. Related to the research study conducted by Nootong et al (2013), however, the over excess reduces the efficiency of nitrification resulted from the insufficient oxygen inside filter core (Nootong et al. 2013). The elimination of excess solid, approx. 6.61±1.68 g/m per time, was conductive to maintain the biomass quantity on material throughout the period.

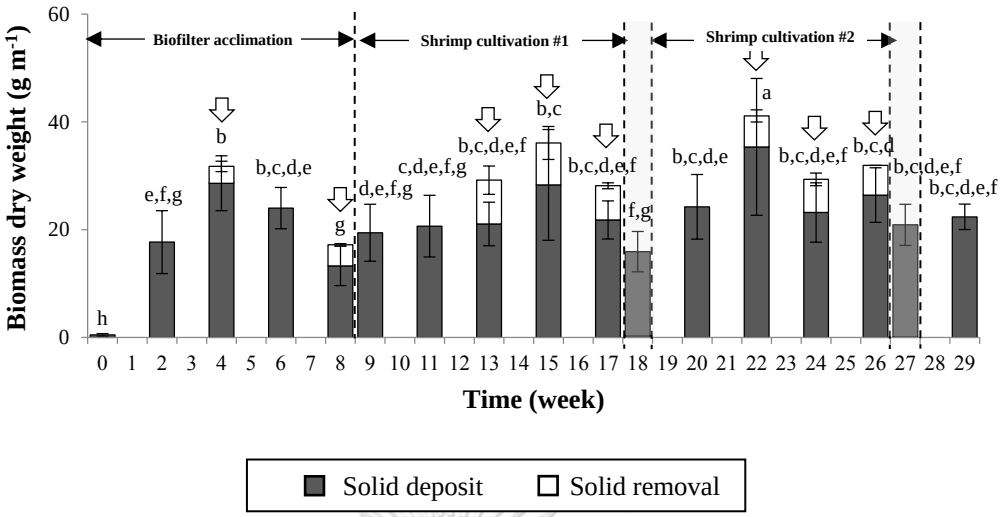

Figure 47. Dry weight of solid deposited on Biocord™ biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps). White arrows (⇩) indicate the cleaning of biofilter and black arrows (⬇) indicate the addition of methanol. Data are shown as the mean ± 1SD, derived from six repeats. Means with a different letter are significantly different ($P < 0.05$ ANOVA and Duncan's multiple range test).

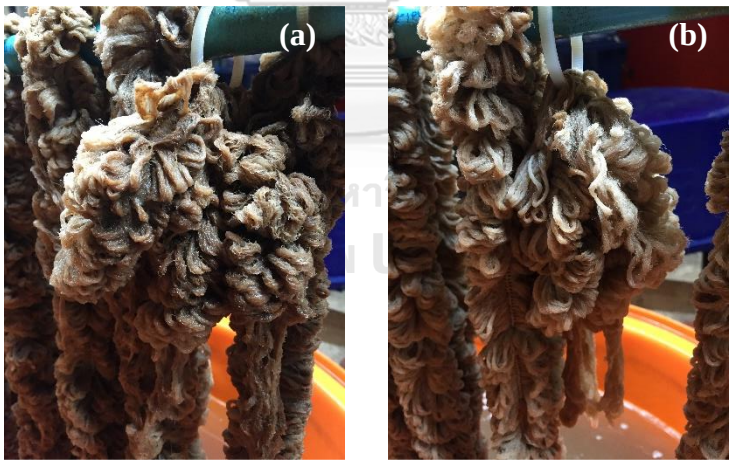

Figure 48. (a) Before and (b) after cleaning of fibrous Biocord™ biofilter during long-term operation of marine RAS.

4.2.3 Microbial diversity in long-term operation of aquaculture system

This part was focused on the microbial diversity on fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system. The experimental results are as follows:

- **Microbial community structure at the taxonomic levels**

The relative abundances of microbial phyla and class observed on the fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 49 and 50, respectively.

The seventeen different microbial phylum with the relative abundance >1% in at least one sample were found in this study. Almost all analyzed sequences belonged to seven phyla of *Proteobacteria* (48.66%), *Bacteroidetes* (17.46%), *Chloroflexi* (14.88%), *Planctomycetes* (6.42%), *Actinobacteria* (3.79%), *Verrucomicrobia* (1.68%), *Gemmatimonadetes* (1.12%) and *Firmicutes* (1.10%), while unassigned phyla and phylum with low relative abundance were included in others. At class level, there were thirty bacterial class (>1% in at least one sample) and almost sequences belonged to *Alphaproteobacteria* (24.08%), *Gammaproteobacteria* (17.86%), *Anaerolineae* (14.18%), *Flavobacteriia* (10.68%), *Deltaproteobacteria* (5.06%), [*Saprospirae*] (4.26%), *Planctomycetia* (3.46%), *Actinobacteria* (1.92%) *Phycisphaerae* (1.88%) and *Acidmicrobiia* (1.71%).

Similar to study 1, *Proteobacteria (Alpha-, Delta- and Gamma-)* were the most abundant bacterial phylum, as ranging from 25.84 to 72.35% under aerobic condition (biofilter acclimation and shrimp cultivation periods) and from 57.25 to 79.62% under anoxic condition. The next phylum was *Bacteroidetes* (mainly dominated by the class *Flavobacteriia a*nd [*Saprospirae*]) with the relative abundances of between 7.14 and 30.29% under aerobic condition, and between 7.74 and 16.02% under anoxic condition. These results were related to many studies that *Proteobacteria* and *Bacteriodetes* are the predominant heterotrophic organisms in marine RAS (Martins et al. 2013; Wietz et al. 2009; Rud et al. 2017). *Chloroflexi* mainly dominated by the class *Anaerolineae*) are another group that has been isolated in a few RASs (Schreier et al. 2010), as appeared under both aerobic (from 0.36 to 45.99%) and anoxic (from 1.54 to 10.92%) conditions. According to the anammox bacteria, it seemed like *Planctomycetes* (mainly dominated by the class *Planctomycetia* and *Phycisphaerae*) were more dominant under aerobic (within 3.02 to 12.87%) than anoxic (within 0.89 to 5.64%) condition. And importantly, *Nitrospirae* in associated with nitrifying bacteria were accounted for 0.24% of total sequences. The nearly relative abundance of approximately 0.40% had been discovered in another marine RAS (Rud et al. 2017), but cannot be found in the anoxic biofiltration (Brailo et al. 2019). In this study, *Nitrospirae* were accounted for

0.00 to 1.87% under aerobic condition and were still detected at low level as 0.06 to 0.18% under anoxic condition.

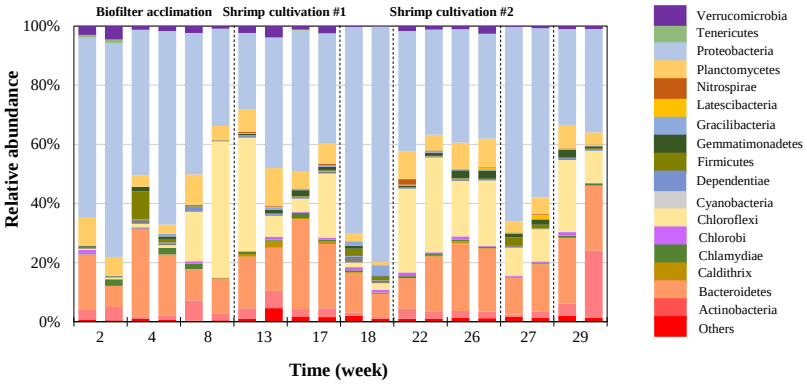

Figure 49. Relative abundances (%) of bacterial phyla, as determined by Miseq pyrosequencing, observed on fibrous Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

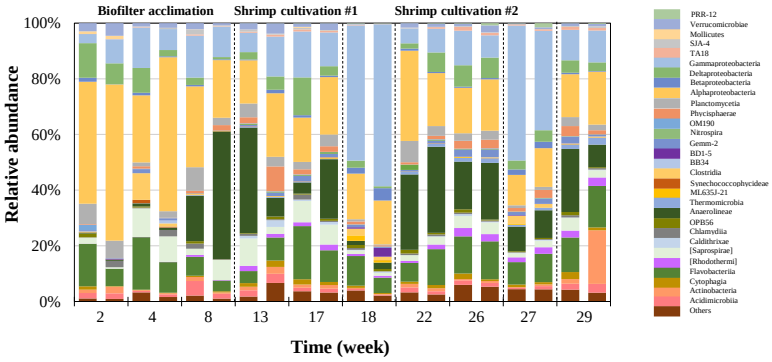

Figure 50. Relative abundances (%) of bacterial class, as determined by Miseq pyrosequencing, observed on fibrous Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

Beta diversity analysis of phyla diversity among biofilters during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 51.

The PCoA 3D plot demonstrated that the diversity pattern was more influenced by system condition (aerobic and anoxic) than operational time. The visible differences in microbial community on filter material used under aerobic and anoxic were observed. The microbial diversity analyzed from filters used under anoxic denitrification (week

18 and 27) were grouped together and clearly separated. And not only related to the operating condition, the diversity isolated during the first period of acclimation (from week 2 to 4) were also spit form the others.

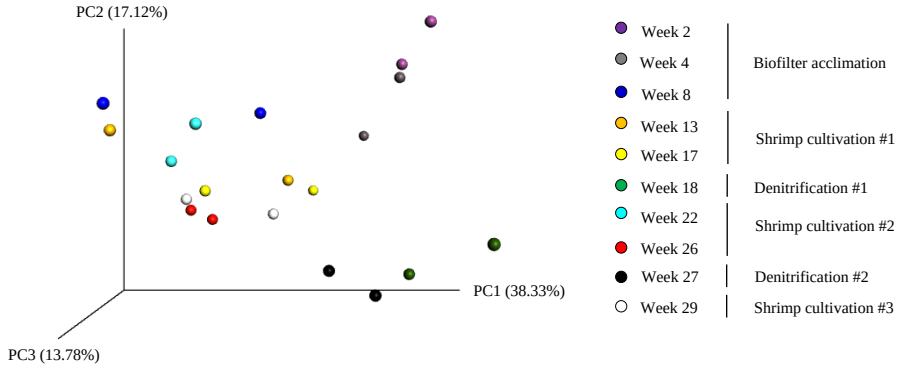

Figure 51. Dissimilarity (beta diversity) among bacterial communities observed on fibrous Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps), calculated as Principal Coordinates Analysis (PCoA) 3D plot of the weighted UniFrac distance matrix.

- **Nitrifying community structure**

The richness heat-map of the nitrifying archaea and bacteria observed on the fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 52.

In this study, there were 5 OTUs of AOB belonged to family *Nitrosomonadaceae*. The uncultured bacterium clone PI1AB88 (GenBank: HQ276072.1) was the most predominant AOB, as form 0.00 to 0.35%, which was promoted after two months of acclimation. Related to the previous study, this partial sequence 16S rRNA gene has also been observed in the salt marsh sediments (Martiny et al. 2011). Next, the uncultured *Nitrosomonas sp*. clone W3-12 (GenBank: FN394311.1) was another abundant AOB, as form 0.00 to 0.12%, which has also been isolated from seawater, marine sediment and nitrification reactor (Sudarno et al. 2010). The presence of AOB were found during the whole period of aerobic shrimp cultivation. Similar to study 1, the sudden decrease of their numbers occurred when the filter samples were submerged under anoxic condition due to the lack of oxygen (van Niel et al. 1993) and the inhibition of methanol (Munz et al. 2011). These two members of AOB, nevertheless, could recover within 2 to 4 weeks after re-oxygenation. Aside from bacteria, the AOA are considered as the additional group involved in ammonia removal through nitritation (Konneke et al. 2005). Archaea have normally been discovered in ubiquitous

environments, including fresh waters, marine waters, coral reefs, estuaries, sediments and soils (Francis et al. 2005). Unfortunately, only 2 OTUs of AOA in the phylum *Crenarchaeota* were identified with the low relative abundances of below 0.01%. For NOB, there was only one main dominance form 8 OTUs of family *Nitrospiraceae* observed in this experiment. Uncultured bacterium clone SF_NOB_Cd08 (GenBank: HM345625.1) became outstanding NOB after three months of submerging in marine RAS. Related to previous study, this partial sequence 16S rRNA gene had also been observed in low-density shrimp cultivation at salinity level of 25 PSU (Brown et al. 2013). Same as AOB, the decrease of expression level was detected under anoxic denitrification and then their growths could recover after re-oxygenation.

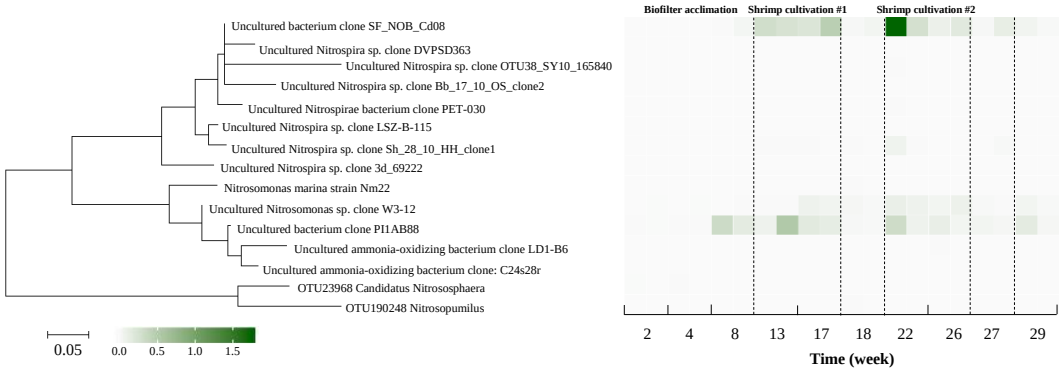

Figure 52. Richness heat-map of the nitrifying archaea and bacteria observed on fibrous Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

- **Denitrifying community structure**

The richness heat-map of the denitrifying bacteria at genus level observed on the fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 53.

In this study, the twelve genus of denitrifiers were promoted in this experiment. Almost members could survive during aerobic shrimp cultivation; however, their expression levels were obviously higher when fibrous filters were used under anoxic condition. Similar to previous studies (Rissanen et al. 2016; Auclair et al. 2012), the genus *Methylophaga* were greatly predominant with the relative abundances between 27.38 and 48.33% in the marine methanol-fed denitrification systems. In the same cycle of methanol in denitrification, the genus *Methylotenera* were another abundant group, as ranging from 1.6 to 4.28%, which have also been isolated from freshwater sediments (Kalyuhznaya et al. 2009; Kalyuzhnaya et al. 2012; Kalyuzhnaya et al. 2009) and low-salinity RAS (Satanwat et al. 2019). Additionally, the genus

Marinobacter belonged to *Gammaproteobacteria* are normally dominant in marine denitrifiers (Mrkonjic Fuka et al. 2007) and accounted for 0.03 to 0.38% under anoxic denitrification in this present study while the number of genus *Paracoccus* belonged to *Alphaproteobacteria* expressed for 0.03 to 0.53%.

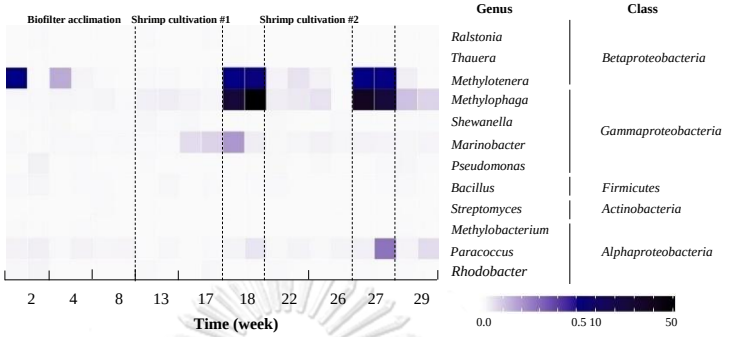

Figure 53. Richness heat-map of the denitrifying bacteria observed on fibrous Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

- **Anaerobic ammonia-oxidizing (anammox) bacterial group**

The relative abundances of anammox bacteria observed on the fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 54.

There were 16 most abundant OTUs (from 116 OTUs) of *Planctomyces* with the relative abundance >0.1% in at least one sample. The OTU141 related to the uncultured *planctomycete* MERTZ_21CM_342 16S (GenBank: AF424500.1) was the outstanding group with the maximum expression level of 1.11% under aerobic shrimp cultivation and 0.09% under anoxic denitrification. The OTU5957 related to the uncultured *planctomycete*, clone IPI_1463-1559-1655 (GenBank: FR714368.1) was also predominant with the maximum level of 1.03 and 0.01% under aerobic and anoxic condition, respectively. Similar to study 1 and previous study (Brailo et al. 2019), it seemed like the *Planctomyces* preferred to live with oxygen availability while the number of abundance obviously decreased when the RAS was switched to anoxic condition with methanol addition.

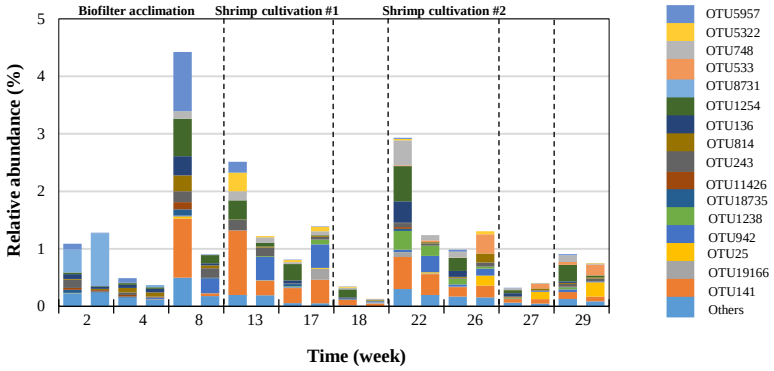

Figure 54. Relative abundances (%) of the anammox bacteria, as determined by Miseq pyrosequencing, observed on fibrous Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

- **Vibrio bacterial group**

The relative abundances of *Vibrio* bacteria anammox bacteria observed on the fibrous Biocord^{TM} biofilter during the long-term operation of shrimp culture system which adjusted the salinity level of 25 PSU and initial shrimp stocking density of 1 kg m^{-3} is shown in figure 55.

There were 29 OTUs of genus *Vibrio* observed during long-term operation of marine RAS. The most predominant group belonged to OTU165 with the relative abundances as ranging from 0.22 to 3.77% under aerobic nitrification and from 0.23 to 0.40% under anoxic denitrification. According to NCBI blast, this sequence is closed to *Vibrio parahaemolyticus* strain 160807 (GenBank: CP033142.1) which has been isolated from shrimp with the acute hepatopancreatic necrosis disease (AHPND), previously named the early mortality syndrome (EMS). While the OTU75150 related to *Vibrio parahaemolyticus* strain NIORKP316 (GenBank: MH767383.1) was also found but with the low level of only 0.09% of maximum abundance under aerobic and was absent under anoxic condition. These results were similar to study 1 in which the reduction of their numbers occurred when the filter samples were submerged under anoxic condition due to the lack of oxygen.

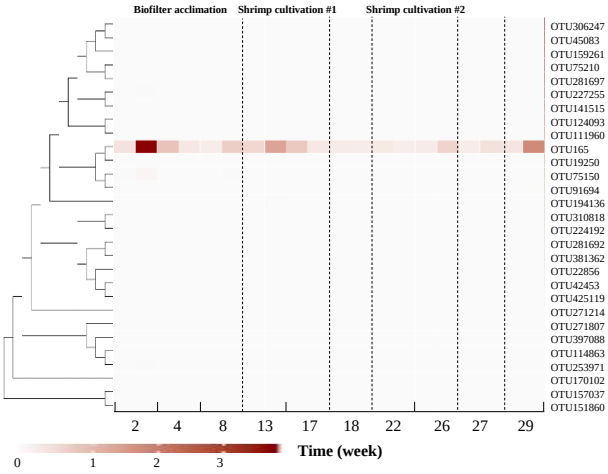

Figure 55. Richness heat-map of the Vibrio bacteria observed on fibrous Biocord^{TM} biofilter during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

Chapter 5

Conclusions and suggestions

5.1 Conclusions

Study 1: Effects of salinity, stocking density, and acclimation period on nitrogen removal efficiency and microbial community

Within a single tank, the biofilter acclimation could proceed in parallel with shrimp cultivation in aquaculture tanks which were varied the salinity levels at 5, 15 and 25 PSU, and the stocking densities at semi-intensive (50 shrimp m^{-2}) and intensive (100 shrimp m^{-2}). Complete nitrification without nitrite accumulation was achieved after 1 month of the experiment while denitrification was performed after shrimp harvest under anoxic condition with methanol supplement at COD:Nitrate-N of 5:1. The maximum ammonia removal rate occurred in the intensive system at 5 PSU as 100.42±5.97 mg-N m^{-2} day^{-1} (for fibrous Biocord™ biofilter) and 145.43±1.17 mg-N m^{-2} day^{-1} (for Japanese filter mat). For denitrification, the highest efficiencies were also found in the intensive system as 81.86±4.40 mg-N m^{-2} day^{-1} at 25 PSU (for fibrous biofilter) and 165.80±50.17 mg-N m^{-2} day^{-1} at 5 PSU (for filter mat).

The sequencing results showed that *Proteobacteria (Alpha-, Beta-, Delta- and Gamma-)* and *Bacteroidetes* (dominated by the class *Cytophagia, Flavobacteriia and Saprospirae*) were the outstanding groups in all experimental systems. However, the predominant AOB in low-salinity system (uncultured *Nitrosomonadaceae* bacterium clone 2d_95589) was different from that under medium- and high-salinity conditions. Likewise, the growth of uncultured *Nitrospira sp.* clone Bb_17_10_HH_clone55 was promoted at 5 PSU while the relative abundance of uncultured *Nitrospira sp.* clone Bb_28_10_HH_clone81 increased at 25 PSU. Under anoxic denitrification, furthermore, the growth of *Methylotenera* was promoted in low-salinity system while *Methylophaga* were the outstanding groups under other salinity conditions.

Study 2: Application of biofilter in marine RAS for long-term operation

Throughout 7 months of the experiment, the fibrous Biocord™ biofilter was used in marine (25 PSU) RAS at the initial shrimp density of 1 kg-shrimp m^{-3}. Complete nitrification without nitrite accumulation was achieved after approximately 2 months of biofilter acclimation in parallel with shrimp cultivation. Fibrous biofilters with the initial nitrification and denitrification rates of 17.05±12.44 and 12.59±4.84 mg-N m^{-2} day^{-1}, respectively, were then applied for two rounds replication of aerobic nitrification with shrimp cultivation followed by anoxic denitrification. For long-term operation, ammonia and nitrite were controlled within the acceptable levels (less than 1 mg-N L^{-}

^{1}) while nitrate was then remove from more than 50 mg-N L^{-1} to lower than 10 mg-N L^{-1} within 1 week.

Similar to study 1, *Proteobacteria (Alpha-, Delta- and Gamma-) and Bacteroidetes* (mainly dominated by the class *Flavobacteriia a*nd *Saprospirae)* were the most abundant bacterial groups. The uncultured bacterium clone PI1AB88 and the uncultured bacterium clone SF_NOB_Cd08 were the main players in ammonia and nitrite oxidation, respectively, while *Methylophaga* and *Methylotenera* were the predominant denitrifying bacteria in anoxic denitrification.

Overall conclusion

This research provided the simple RAS for shrimp cultivation which could perform nitrification and denitrification co-processes within a single aquaculture tank using biological filter system. Even though the efficiency of nitrification was highest under low-salinity (5 PSU), the high-salinity (25 PSU) system was applied to evaluate the possibility of using biofilter for nitrogen removal for long-term operation of RAS due to the suitable salinity level that provided the maximum survival rate of white shrimp. And although Japanese filter mat had higher nitrification and denitrification efficiencies, the fibrous Biocord^{TM} biofilter was used in long-term marine RAS related to the better system management without the clogging of solid particle inside biofilter structure that allowed hydrogen sulfide production.

The rather rapid development of nitrogen removal activity on biofilter was successful when acclimated in aquaculture tank. During two months of aerobic shrimp cultivation, the fibrous biofilter could perform efficient nitrification while heterotrophic denitrifiers that multiplied inside the media could reduce some of nitrate. The rest of nitrate was completely removed via denitrification after shrimp cultivation by switching the system from aerobic to anoxic condition with methanol supplement at COD:Nitrate-N of 5:1. With achieving nitrogen removal, the RAS was ready to culture the next crop of shrimp after reoxygenation.

To apply this system for farm use, however, the biofilter acclimation should be started with either extensive or semi-intensive stocking density of shrimp to prevent the effect of elevated concentrations of ammonia and nitrite on aquatic organisms. While the cultivation at intensive or super intensive density can be done after biofilter activation. The cultivation period can be extended more than two months (e.g. for three or four months); nevertheless, the accumulation of nitrate at dangerous levels should beware. Moreover, this system is possible to apply in a large-scale RAS with higher density of aquatic animals by calculating the length of fibrous biofilter based on the nitrogen removal rate of material and the nitrogen waste generation rate of shrimp.

5.2 Suggestions

In relevance with the limitations of this study, the suggestions for further research are as follows:

The marine RAS in this study was conducted based on shrimp culture system and used white shrimp as the experimental aquatic animal which are normally reared at the lower density and produce less suspended solid than in fish cultivation. Therefore, in order to apply this system to culture fish, the application of fibrous biofilter for nitrogen removal in the elevated suspended solid system need to be further investigated.

For long-term operation of RAS, the management of solid deposited on fibrous biofilter was performed by monthly cleaning with saline water while the solid collection devices were used to collect the suspended solid in aquaculture tank and replaced with the new tube weekly. According to the results of elevated concentration of suspended solid, however, this method was not suitable for applying in the pilot scale and the efficiency was not satisfied. Therefore, the type of solid removal unit and its efficiency need to be further improved. In addition, to control the pathogens and disease outbreaks in aquatic animals during long-term operation, the disinfection unit (e.g. ozonation) is further required.

The next generation DNA sequencing on Illumina MiSeq using universal primer was used to study the microbial diversity and community dynamics in marine RAS. Related to the limitation of this method that can only recognize classifications down to the levels of family or genus, the deep-sequencing of microorganisms need to be further identified by applying other methods with specific primers.

REFERENCES

Abeliovich, A. 2006. The Nitrite-Oxidizing Bacteria. *Prokaryotes* 5:861.

Achuthan, C., V. J. R. Kumar, N. J. Manju, R. Philip, and I. S. B. Singh. 2006. Development of nitrifying bacterial consortia for immobilizing in nitrifying bioreactors designed for penaeid and non-penaeid larval rearing systems in the tropics. *Indian Journal of Marine Sciences* 35 (3):240-248.

Ambus, P., and S. Zechmeister-Boltenstern. 2007. Chapter 22 - Denitrification and N-Cycling in Forest Ecosystems A2 - Bothe, Hermann. In *Biology of the Nitrogen Cycle*, edited by S. J. Ferguson and W. E. Newton. Amsterdam: Elsevier, 343-358.

APHA, AWWA, and WEF. 1998. *Standard Methods for the Examination of Water and Wastewater*. Washington DC: American Public Health Association.

APHA, AWWA, and WPCF. 2005. *Standard Methods for the Examination of Water and Wastewater*. edited by 21st. Washington DC: American Public Health Association.

ASTM. 1995. *Standard Test Methods for Chemical Oxygen Demand (Dichromate Oxygen Demand) of Water*: American Society for Testing and Materials, Philadelphia, PA.

Auclair, J., S. Parent, and R. Villemur. 2012. Functional Diversity in the Denitrifying Biofilm of the Methanol-Fed Marine Denitrification System at the Montreal Biodome. *Microbial Ecology* 63 (4):726-735.

Avnimelech, Y. 2006. Bio-filters: The need for an new comprehensive approach. *Aquacultural Engineering* 34 (3):172-178.

Azim, M. E., and D. C. Little. 2008. The biofloc technology (BFT) in indoor tanks: Water quality, biofloc composition, and growth and welfare of Nile tilapia (Oreochromis niloticus). *Aquaculture* 283 (1–4):29-35.

Bakunina, I., O. Nedashkovskaya, L. Balabanova, T. Zvyagintseva, V. Rasskasov, and V. Mikhailov. 2013. Comparative Analysis of Glycoside Hydrolases Activities from Phylogenetically Diverse Marine Bacteria of the Genus Arenibacter. *Marine Drugs* 11 (6):1977-1998.

Barbieri, E. 2010. Acute toxicity of ammonia in white shrimp (Litopenaeus schmitti) (Burkenroad, 1936, Crustacea) at different salinity levels. *Aquaculture* 306 (1-4):329-333.

Borea, L., V. Naddeo, and V. Belgiorno. 2017. An Electro Moving Bed Membrane Bioreactor (eMB-MBR) as a Novel Technology for Wastewater Treatment and Reuse. In *Frontiers in Wastewater Treatment and Modelling: FICWTM 2017*, edited by G. Mannina. Cham: Springer International Publishing, 159-164.

Bower, C. E., and T. Holm-Hansen. 1980. A Salicylate - Hypochlorite Method for Determining Ammonia in Seawater. *Canadian Journal of Fisheries and Aquatic Sciences* 37:794-798.

Brailo, M., H. J. Schreier, R. McDonald, J. Maršić-Lučić, A. Gavrilović, M. Pećarević, and J. Jug-Dujaković. 2019. Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum. *Aquaculture (Amsterdam, Netherlands)* 503:198-206.

Brown, M. N., A. Briones, J. Diana, and L. Raskin. 2013. Ammonia-oxidizing archaea and nitrite-oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system. *Federation of European Microbiological Societies* 83:17-25.

Camargo, J. A., A. Alonso, and A. Salamanca. 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. *Chemosphere* 58 (9):1255-1267.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* 6:1621.

Chapentier, J., G. Martin, H. Wacheux, and P. Gilles. 1998. ORP regulation and activated sludge: 15 years of experience. *Water Science and Technology* 38 (3):197-208.

Cobo, M. a. d. L., S. Sonnenholzner, M. Wille, and P. Sorgeloos. 2014. Ammonia tolerance of Litopenaeus vannamei (Boone) larvae. *Aquaculture Research* 45 (3):470-475.

Cortés-Lorenzo, C., M. Rodríguez-Díaz, D. Sipkema, B. Juárez-Jiménez, B. Rodelas, H. Smidt, and J. González-López. 2015. Effect of salinity on nitrification efficiency and structure of ammonia-oxidizing bacterial communities in a submerged fixed bed bioreactor. *Chemical Engineering Journal* 266:233-240.

Coughlan, L. M., P. D. Cotter, C. Hill, and A. Alvarez-Ordóñez. 2016. New Weapons to Fight Old Enemies: Novel Strategies for the (Bio)control of Bacterial Biofilms in the Food Industry. *Frontiers in Microbiology* 7 (1641).

Crab, R., Y. Avnimelech, T. Defoirdt, P. Bossier, and W. Verstraete. 2007. Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture* 270 (1–4):1-14.

Daims, H. 2014. The Family Nitrospiraceae. In *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*, edited by E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson. Berlin, Heidelberg: Springer Berlin Heidelberg, 733-749.

Daniel, L. M. C., E. Pozzi, E. Foresti, and F. A. Chinalia. 2009. Removal of ammonium via simultaneous nitrification–denitrification nitrite-shortcut in a single packed-bed batch reactor. *Bioresource Technology* 100 (3):1100-1107.

Das, S., and H. Dash. 2015. *Microbial Biotechnology- A Laboratory Manual for Bacterial Systems*.

Dincer, A. R., and F. Kargi. 1999. Salt inhibition of nitrification and denitrification in saline wastewater. *Environmental Technology* 20:1147-1153.

Dogsa, I., M. Kriechbaum, D. Stopar, and P. Laggnerz. 2005. Structure of Bacterial Extracellular Polymeric Substances at Different pH Values as Determined by SAXS. *Biophysical Journal* 89:2711-2720.

El-Shafai, S. A., F. A. El-Gohary, F. A. Nasr, N. P. van der Steen, and H. J. Gijzen. 2004. Chronic ammonia toxicity to duckweed-fed tilapia (Oreochromis niloticus). *Aquaculture* 232 (1–4):117-127.

Flint, S. H., P. J. Bremer, and J. D. Brooks. 1997. Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling* 11 (1):81-97.

Francis, C. A., K. J. Roberts, J. M. Beman, A. E. Santoro, and B. B. Oakley. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and

sediments of the ocean. *Proceedings of the National Academy of Sciences of the United States of America* 102 (41):14683-14688.

Furtado, P. S., B. R. Campos, F. P. Serra, M. Klosterhoff, L. A. Romano, and W. Wasielesky. 2015. Effects of nitrate toxicity in the Pacific white shrimp, Litopenaeus vannamei, reared with biofloc technology (BFT). *Aquaculture International* 23 (1):315-327.

Gao, W., L. Tian, T. Huang, M. Yao, W. Hu, and Q. Xu. 2016. Effect of salinity on the growth performance, osmolarity and metabolism-related gene expression in white shrimp Litopenaeus vannamei. *Aquaculture Reports* 4:125-129.

Gaona, C., M. Souza de Almeida, V. Viau, L. Poersch, and W. Wasielesky. 2015. *Effect of different total suspended solids levels on a Litopenaeus vannamei (Boone, 1931) BFT culture system during biofloc formation*.

Gross, A., S. Abutbul, and D. Zil. 2004. Acute and Chronic Effects of Nitrite on White Shrimp, Litopenaeus vannamei, Cultured in Low-Salinity Brackish Water. *World Aquaculture Society* 35 (3):315-321.

Gujer, W., and D. Jenkins. 1975. A nitrification model for the contact stabilization activated sludge process. *Water Research* 9 (5):561-566.

Gutierrez-Wing, M. T., and R. F. Malone. 2006. Biological filters in aquaculture: Trends and research directions for freshwater and marine applications. *Aquacultural Engineering* 34 (3):163-171.

Hagopian, D. S., and J. G. Riley. 1998. A closer look at the bacteriology of nitrification. *Aquacultural Engineering* 18 (4):223-244.

Hamlin, H. J., J. T. Michaels, C. M. Beaulaton, W. F. Graham, W. Dutt, P. Steinbach, T. M. Losordo, K. K. Schrader, and K. L. Main. 2008. Comparing denitrification rates and carbon sources in commercial scale upflow denitrification biological filters in aquaculture. *Aquacultural Engineering* 38 (2):79-92.

Hammer, B. K., and B. L. Bassler. 2003. Quorum sensing controls biofilm formation in Vibrio cholerae. *Molecular Microbiology* 50 (1):101-114.

Herlemann, D. P., M. Labrenz, K. Jürgens, S. Bertilsson, J. J. Waniek, and A. F. Andersson. 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME journal* 5 (10):1571-1579.

Itoi, S., A. Niki, and H. Sugita. 2006. Changes in microbial communities associated with the conditioning of filter material in recirculating aquaculture systems of the pufferfish Takifugu rubripes. *Aquaculture* 256 (1–4):287-295.

Kalyuhznaya, M. G., W. Martens-Habbena, T. Wang, M. Hackett, S. M. Stolyar, D. A. Stahl, M. E. Lidstrom, and L. Chistoserdova. 2009. Methylophilaceae link methanol oxidation to denitrification in freshwater lake sediment as suggested by stable isotope probing and pure culture analysis. *Environmental Microbiology Reports* 1 (5):385-392.

Kalyuzhnaya, M. G., D. A. C. Beck, D. Suciu, A. Pozhitkov, M. E. Lidstrom, and L. Chistoserdova. 2009. Functioning in situ: gene expression in Methylotenera mobilis in its native environment as assessed through transcriptomics. *The ISME journal* 4:388.

Kalyuzhnaya, M. G., D. A. C. Beck, A. Vorobev, N. Smalley, D. D. Kunkel, M. E. Lidstrom, and L. Chistoserdova. 2012. Novel methylotrophic isolates from lake sediment, description of Methylotenera versatilis sp. nov. and emended

description of the genus Methylotenera. *International Journal of Systematic and Evolutionary Microbiology* 62 (1):106-111.

Keuter, S. 2011. Characterization of nitrifying bacteria in marine recirculation aquaculture systems with regard to process optimization, Department of Biology, University of Hamburg.

Keuter, S., S. Beth, G. Quantz, C. Schulz, and E. Spieck. 2017. Longterm Monitoring of Nitrification and Nitrifying Communities during Biofilter Activation of Two Marine Recirculation Aquaculture Systems (RAS). *International Journal of Aquaculture and Fishery Sciences* 3 (3):051-061.

Khammi, A., M. Kutako, C. Sangwichien, and K. Nootong. 2015. Development and evaluation of compact aquaculture system for the application of zero water-exchange inland aquacultures. *Engineering Journal* 19 (2):15-27.

Kim, E.-W., and J.-H. Bae. 2000. Alkalinity requirements and the possibility of simultaneous heterotrophic denitrification during sulfur-utilizing autotrophic denitrification. *Water Science and Technology* 42 (3-4):233-238.

Kirkpatrick, J., B. Oakley, C. Fuchsman, S. Srinivasan, J. T. Staley, and J. W. Murray. 2006. Diversity and distribution of Planctomycetes and related bacteria in the suboxic zone of the Black Sea. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 72 (4):3079-3083.

Kladivko, E. J., and D. R. Keeney. 1987. Soil nitrogen mineralization as affected by water and temperature interactions. *Biology and Fertility of Soils* 5 (3):248-252.

Klotz, M. G., and L. Y. Stein. 2011. Genomics of ammonia-oxidizing bacteria and insights to their evolution. In *Nitrification*, edited by B. B. Ward, M. G. Klotz and D. J. Arp. Washington, DC., 57-94.

Koga, Y. 2012. Thermal Adaptation of the Archaeal and Bacterial Lipid Membranes. *Archaea* 2012:6.

Konneke, M., A. E. Bernhard, J. R. de la Torre, C. B. Walker, J. B. Waterbury, and D. A. Stahl. 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437 (7058):543-546.

Kuhn, D. D., D. D. Drahos, L. Marsh, and G. J. Flick. 2010. Evaluation of nitrifying bacteria product to improve nitrification efficacy in recirculating aquaculture systems. *Aquacultural Engineering* 43 (2):78-82.

Lawson, T. B. 1995. Aquaculture in open Systems. In *Fundamentals of Aquacultural Engineering*. Boston, MA: Springer.

Lee, P. G., R. N. Lea, E. Dohmann, W. Prebilsky, P. E. Turk, H. Ying, and J. L. Whitson. 2000. Denitrification in aquaculture systems: an example of a fuzzy logic control problem. *Aquacultural Engineering* 23 (1–3):37-59.

Lewis, W. M. J., and D. P. Morris. 1986. Toxicity of Nitrite to Fish: A Review. *The American Fisheries Society* 115:183-195.

Li, B., and S. Irvin. 2007. The comparison of alkalinity and ORP as indicators for nitrification and denitrification in a sequencing batch reactor (SBR). *Biochemical Engineering Journal* 34 (3):248-255.

Li, Y. Z., Y. L. He, D. G. Ohandja, J. Ji, J. F. Li, and T. Zhou. 2008. Simultaneous nitrification–denitrification achieved by an innovative internal-loop airlift MBR: Comparative study. *Bioresource Technology* 99 (13):5867-5872.

Magalhães, C. M., S. B. Joye, R. M. Moreira, W. J. Wiebe, and A. A. Bordalo. 2005. Effect of salinity and inorganic nitrogen concentrations on nitrification and

denitrification rates in intertidal sediments and rocky biofilms of the Douro River estuary, Portugal. *Water Research* 39 (9):1783-1794.

Martins, P., D. F. R. Cleary, A. C. C. Pires, A. M. Rodrigues, V. Quintino, R. Calado, and N. C. M. Gomes. 2013. Molecular Analysis of Bacterial Communities and Detection of Potential Pathogens in a Recirculating Aquaculture System for Scophthalmus maximus and Solea senegalensis. *Plos ONE* 8 (11):e80847.

Martiny, J. B. H., J. A. Eisen, K. Penn, S. D. Allison, and M. C. Horner-Devine. 2011. Drivers of bacterial β-diversity depend on spatial scale. *Proceedings of the National Academy of Sciences* 108 (19):7850-7854.

Merbt, S. N., D. A. Stahl, E. O. Casamayor, E. Martí, G. W. Nicol, and J. I. Prosser. 2012. Differential photoinhibition of bacterial and archaeal ammonia oxidation. *FEMS Microbiology Letters* 327 (1):41-46.

Monroe, D. 2007. Looking for Chinks in the Armor of Bacterial Biofilms. *PLoS Biology* 5 (11):2458-2461.

Mortimer, R. J. G., S. J. Harris, M. D. Krom, T. E. Freitag, J. I. Prosser, J. Barnes, P. Anschutz, P. J. Hayes, and I. M. Davies. 2004. Anoxic nitrification in marine sediments. *Marine Ecology Progress Series* 276:37-51.

Mrkonjic Fuka, M., S. H. Gesche Braker, and L. Philippot. 2007. Chapter 20 - Molecular Tools to Assess the Diversity and Density of Denitrifying Bacteria in Their Habitats A2 - Bothe, Hermann. In *Biology of the Nitrogen Cycle*, edited by S. J. Ferguson and W. E. Newton. Amsterdam: Elsevier, 313-330.

Munz, G., C. Lubello, and J. A. Oleszkiewicz. 2011. Modeling the decay of ammonium oxidizing bacteria. *Water Research* 45 (2):557-564.

Nakano, M., T. Inagaki, S. Okunishi, R. Tanaka, and H. Maeda. 2010. Effect of salinity on denitrification under limited single carbon source by Marinobacter sp. isolated from marine sediment. *Journal of Basic Microbiology* 50:285-289.

Nonwachai, T., W. Purivirojkul, N. Chuchird, and C. Limsuwan. 2011. Effects of dissolved oxygen levels on growth, survival and immune response of juvenile pacific white shrimp Litopenaeus vannamei. *Journal of Fisheries and Environment* 35 (3):1-10.

Nootong, K., S. Nurit, and S. Powtongsook. 2013. Control of Inorganic Nitrogen and Suspended Solids Concentrations in a Land-Based Recirculating Aquaculture System. *Engineering Journal* 17 (1):49-59.

Percival, S. L., and D. W. Williams. 2014. Chapter Twelve - Vibrio. In *Microbiology of Waterborne Diseases (Second Edition)*, edited by S. L. Percival, M. V. Yates, D. W. Williams, R. M. Chalmers and N. F. Gray. London: Academic Press, 237-248.

Pester, M., T. Rattei, S. Flechl, A. Gröngröft, A. Richter, J. Overmann, B. Reinhold-Hurek, A. Loy, and M. Wagner. 2012. amoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions. *Environmental Microbiology* 14 (2):525–539.

Pester, M., C. Schleper, and M. Wagner. 2011. The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology. *Curr Opin Microbiol* 14 (3):300-306.

Prosser, J. I., I. M. Head, and L. Y. Stein. 2014. The Family Nitrosomonadaceae. In *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*, edited by E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson. Berlin, Heidelberg: Springer Berlin Heidelberg, 901-918.

Prosser, J. I., and G. W. Nicol. 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends in Microbiology* 20 (11):523-531.

Pungrasmi, W., P. Phinitthanaphak, and S. Powtongsook. 2016. Nitrogen removal from a recirculating aquaculture system using a pumice bottom substrate nitrification-denitrification tank. *Ecological Engineering* 95:357-363.

Pungrasmi, W., C. Playchoom, and S. Powtongsook. 2013. Optimization and evaluation of a bottom substrate denitrification tank for nitrate removal from a recirculating aquaculture system. *Journal of Environmental Sciences* 25 (8):1557-1564.

Puttaswamygowda, G. H., S. Olakkaran, A. Antony, and A. Kizhakke Purayil. 2019. Chapter 22 - Present Status and Future Perspectives of Marine Actinobacterial Metabolites. In *Recent Developments in Applied Microbiology and Biochemistry*, edited by V. Buddolla: Academic Press, 307-319.

Randall, D. J., and T. K. N. Tsui. 2002. Ammonia toxicity in fish. *Marine Pollution Bulletin* 45 (1–12):17-23.

Ray, A. J., and J. M. Lotz. 2017. Shrimp (Litopenaeus vannamei) production and stable isotope dynamics in clear-water recirculating aquaculture systems versus biofloc systems. *Aquaculture Research* 48 (8):4390-4398.

Rissanen, A. J., A. Ojala, M. Dernjatin, J. Jaakkola, and M. Tiirola. 2016. Methylophaga and Hyphomicrobium can be used as target genera in monitoring saline water methanol-utilizing denitrification. *Journal of Industrial Microbiology & Biotechnology* 43 (12):1647-1657.

Rocher, V., A. M. Laverman, J. Gasperi, S. Azimi, S. Guérin, S. Mottelet, T. Villières, and A. Pauss. 2015. Nitrite accumulation during denitrification depends on the carbon quality and quantity in wastewater treatment with biofilters. *Environmental Science and Pollution Research* 22 (13):10179-10188.

Rud, I., J. Kolarevic, A. B. Holan, I. Berget, S. Calabrese, and B. F. Terjesen. 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. *Aquacultural Engineering* 78:50-62.

Ruppert, E. E., R. S. Fox, and R. B. Barnes. 2004. *Invertebrate Zoology*. edited by Seven. Belmont, California: Brooks Cole Thomson.

Sakami, T., T. Andoh, T. Morita, and Y. Yamamoto. 2012. Phylogenetic diversity of ammonia-oxidizing archaea and bacteria in biofilters of recirculating aquaculture systems. *Marine Genomics* 7:27-31.

Satanwat, P., W. Pungrasmi, and S. Powtongsook. 2019. Effects of Salinity and Immobilization Period on the Nitrification and Denitrification Co-processes during Biofilter Acclimation in a Marine Recirculating Aquaculture System. *Journal of Water and Environment Technology* 17 (2):89-99.

Sauder, L. A., K. Engel, J. C. Stearns, A. P. Masella, R. Pawliszyn, and J. D. Neufeld. 2011. Aquarium Nitrification Revisited: Thaumarchaeota Are the Dominant Ammonia Oxidizers in Freshwater Aquarium Biofilters. *Plos ONE* 6 (8):e23281.

Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies. 2002. Pseudomonas aeruginosa Displays Multiple Phenotypes during Development as a Biofilm. *Journal of bacteriology* 184 (4):1140-1154.

Schimel, J. P., and J. Bennett. 2004. NITROGEN MINERALIZATION: CHALLENGES OF A CHANGING PARADIGM. *Ecology* 85 (3):591-602.

Schmidt, I., O. Sliekers, M. Schmid, I. Cirpus, M. Strous, E. Bock, J. G. Kuenen, and M. S. Jetten. 2002. Aerobic and anaerobic ammonia oxidizing bacteria--competitors or natural partners? *FEMS Microbiol Ecol* 39 (3):175-181.

Schramm, A., D. D. Beer, A. Gieseke, and R. Amann. 2000. Microenvironments and distribution of nitrifying bacteria in a membrane-bound biofilm. *Environmental Microbiology* 2 (6):680-686.

Schreier, H. J., N. Mirzoyan, and K. Saito. 2010. Microbial diversity of biological filters in recirculating aquaculture systems. *Current Opinion in Biotechnology* 21 (3):318-325.

Schuler, D. J. 2008. Acute Toxicity of Ammonia and Nitrite to White Shrimp (L. vannamei) at Low Salinities, Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Sesuk, T., S. Powtongsook, and K. Nootong. 2009. Inorganic nitrogen control in a novel zero-water exchanged aquaculture system integrated with airlift-submerged fibrous nitrifying biofilters. *Bioresource Technology* 100 (6):2088-2094.

Sharma, B., and R. C. Ahlert. 1977. Nitrification and nitrogen removal. *Water Research* 11 (10):897-925.

Sirivedhin, T., and K. A. Gray. 2006. Factors affecting denitrification rates in experimental wetlands: Field and laboratory studies. *Ecological Engineering* 26 (2):167-181.

Spietz, R. L., C. M. Williams, G. Rocap, and M. C. Horner-Devine. 2015. A Dissolved Oxygen Threshold for Shifts in Bacterial Community Structure in a Seasonally Hypoxic Estuary. *Plos ONE* 10 (8):e0135731-e0135731.

Stewart, N. T., G. D. Boardman, and L. A. Helfrich. 2006. Characterization of nutrient leaching rates from settled rainbow trout (Oncorhynchus mykiss) sludge. *Aquacultural Engineering* 35 (2):191-198.

Strickland, J. D. H., and T. R. Parsons. 1972. *A Practical Handbook of Seawater Analysis*. edited by 2nd. Ottawa: Fisheries research board of Canada.

Sudarno, U., S. Bathe, J. Winter, and C. Gallert. 2010. Nitrification in fixed-bed reactors treating saline wastewater. *Applied Microbiology and Biotechnology* 85 (6):2017-2030.

Sylvia, D. M., J. J. Fuhrmann, P. G. Hartel, and D. A. Zuberer. 2005. *Principles and application of soil microbiology*. edited by second. Pearson. New Jersey.

Tharavathy, N. C. 2014. Water quality management in shrimp culture *Acta Biologica Indica* 3 (1):536-540.

Timmons, M. B., J. M. Ebeling, F. W. Wheaton, S. T. Summerfelt, and B. J. Vinci. 2002. *Recirculating aquaculture system*. edited by Second. New York: Cayaga Aqua Ventures, Ithaca.

Uhrig, B. 2017. *Environmental Microbiology*. Morrisville, United States: Lulu.com.

van Niel, E. W. J., L. A. Robertson, and J. G. Kuenen. 1993. A mathematical description of the behaviour of mixed chemostat cultures of an autotrophic nitrifier and a heterotrophic nitrifier/aerobic denitrifier; a comparison with experimental data. *FEMS Microbiology Ecology* 11 (2):99-108.

van Rijn, J. 2013. Waste treatment in recirculating aquaculture systems. *Aquacultural Engineering* 53:49-56.

van Rijn, J., Y. Tal, and H. J. Schreier. 2006. Denitrification in recirculating systems: Theory and applications. *Aquacultural Engineering* 34 (3):364-376.

Wang, Y., H.-F. Sheng, Y. He, J.-Y. Wu, Y.-X. Jiang, N. F.-Y. Tam, and H.-W. Zhou. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 78 (23):8264-8271.

Ward, B. B. 2008. Nitrification. In *Encyclopedia of Ecology*, edited by S. E. Jørgensen and B. D. Fath. Oxford: Academic Press, 2511-2518.

Wietz, M., M. R. Hall, and L. Høj. 2009. Effects of seawater ozonation on biofilm development in aquaculture tanks. *Systematic and Applied Microbiology* 32 (4):266-277.

Wu, Y., X. Ke, M. Hernández, W. Baozhan, M. G. Dumont, Z. Jia, and R. Conradb. 2013. Autotrophic Growth of Bacterial and Archaeal Ammonia Oxidizers in Freshwater Sediment Microcosms Incubated at Different Temperatures. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 79:3076–3084.

Yang, G., L. Song, X. Lu, N. Wang, and Y. Li. 2017. Effect of the exposure to suspended solids on the enzymatic activity in the bivalve Sinonovacula constricta. *Aquaculture and Fisheries* 2 (1):10-17.

Yang, P. Y., S. Nitisoravut, and J. S. Wu. 1995. Nitrate removal using a mixed-culture entrapped microbial cell immobilization process under high salt conditions. *Water Research* 29 (6):1525-1532.

Yu, D.-s., Y.-z. Peng, and K. Zhang. 2004. Effects of seawater salinity on nitrite accumulation in short-range nitrification to nitrite as end product. *Journal of Environmental Sciences* 16 (2):247-251.

Yuan, X., X. Qian, R. Zhang, R. Ye, and W. Hu. 2012. Performance and microbial community analysis of a novel bio-cord carrier during treatment of a polluted river. *Bioresource Technology* 117:33-39.

Yun, H.-J., and D.-J. Kim. 2003. Nitrite accumulation characteristics of high strength ammonia wastewater in an autotrophic nitrifying biofilm reactor. *Chemical Technology and Biotechnology* 78 (4):377-383.

Zhang, R., X. Qian, H. Li, X. Yuan, and R. Ye. 2012. Selection of optimal river water quality improvement programs using QUAL2K: A case study of Taihu Lake Basin, China. *Science of The Total Environment* 431:278-285.

Zhu, S., J. Shen, Y. Ruan, X. Guo, Z. Ye, Y. Deng, and M. Shi. 2016. The effects of different seeding ratios on nitrification performance and biofilm formation in marine recirculating aquaculture. *Environmental Science and Pollution Research* 23:14540-14548.

APPENDIX

Appendix A

Method for water analysis

A.1 Ammonia analysis, according to Salicylate-Hypochlorite Method (Bower and Holm-Hansen 1980)

Reagents

1. Salicylate-catalyst solution:
   Dissolve 440 g of sodium salicylate ($C_6H_4(OH)COONa$) and 0.28 g of sodium nitroprusside dehydrate ($Na_2Fe(CN)_5NO.2H_2O$) in de-ionized water (D.I.) and adjust the volume to 1 L. Store in an amber bottle at 5ºC. This solution is stable for 3 month.
2. Alkaline-citrate solution:
   Dissolve 18.5 g of sodium hydroxide (NaOH) and 100 g of sodium citrate dehydrate ($Na_3C_6H_5O_7.2H_2O$) in de-ionized water (D.I.) and adjust the volume to 1 L. Store in an amber bottle at 5ºC.
3. Sodium hypochlorite solution:
   Use 1.5 N commercial hypochlorite.
4. Alkaline-hypochlorite solution:
   Mix alkaline-citrate and sodium hypochlorite solution at 9:1 ratio. This solution is stable for 1 h.

Procedure

Add 0.6 ml of salicylate-catalyst solution and 1.0 ml of alkaline-hypochlorite solution in 5 ml of sample (use D.I. as sample in blank), mix and allow the reagent to react for 1 to 3 h. Read the absorbance at 640 nm in a spectrophotometer and compare the ammonia concentration to the standard calibration curve as figure A-1.

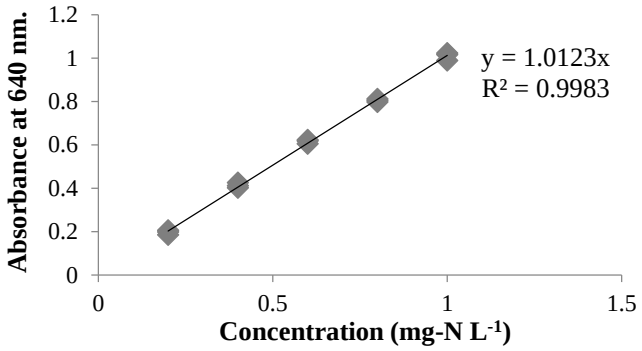

Figure A-1. Standard calibration curve for ammonia.

A.2 Nitrite analysis, according to Colorimetric and Spectrophotometric Method (Strickland and Parsons 1972)

Reagents

1. Sulfanilamide solution:
   Dissolve 5 g of sulphanilamide ($C_6H_8N_2O_2S$) in 50 ml of hydrochloric acid (HCl) and adjust the volume to 1 L. Store in an amber bottle at 5ºC.
2. Naphthylethylenediamine solution:
   Dissolve 0.5 g of N-(1-Naphthyl)-Ethylenediamine Dihydrochloride (NNED) in D.I. and adjust the volume to 500 ml. Store in an amber bottle at 5ºC. This solution is stable for 1 month.

Procedure

Add 0.1 ml of sulfanilamide solution in 5 ml of sample (use D.I. as sample in blank), mix and allow the reagent to react for 2 to 10 min. Add the 0.1 ml of NNED, mix and allow the reagent to react for 0.5 to 2 h. Read the absorbance at 543 nm in a spectrophotometer and compare the nitrite concentration to the standard calibration curve as figure A-2.

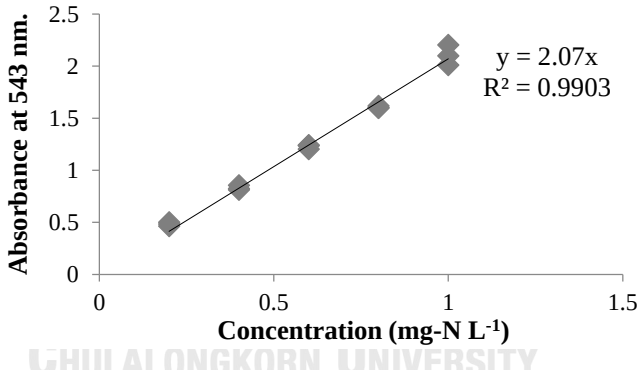

Figure A-2. Standard calibration curve for nitrite.

A.3 Nitrate analysis, according to Ultraviolet Spectrophotometric Method (APHA et al. 2005)

Procedure

Read the 5 ml of sample (use D.I. as sample in blank) at absorbance of 220 and 275 nm in a spectrophotometer and use the difference value to compare the nitrate concentration to the standard calibration curve as figure A-3. Subtract the concentration with nitrite concentration to get the final nitrate concentration.

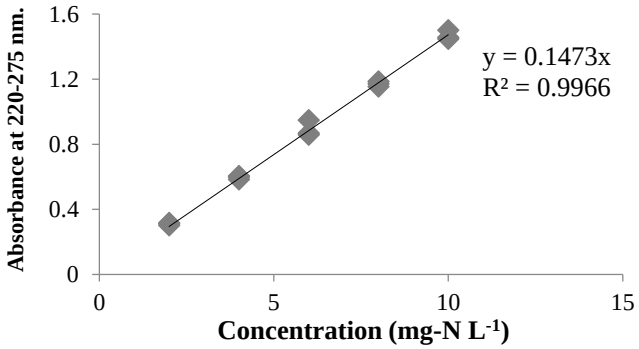

Figure A-3. Standard calibration curve for nitrate.

A.4 Alkalinity analysis, modified from Titration method (Strickland and Parsons 1972)

Procedure

Titrate 5 m of sample with 0.01 M H_2SO_4 until reach the end point of pH 4.5. The alkalinity can be calculated as the following:

$$\text{Alkalinity (mg-CaCO}_3\text{ L}^{-1}) = \frac{\text{Volume of H}_2\text{SO}_4\text{ (ml)} \times \text{Normality} \times 50{,}000}{\text{Volume of sample (ml)}} \quad \text{Equation A-1}$$

A.5 Suspended solid analysis, according to the Filtration Method (APHA et al. 2005)

Procedure

Filter the sample through a pre-weighed filter and dry the residue retained on the filter in an oven at 103–105°C until the weight of the filter no longer changes. The SS can be calculated as the following:

$$\text{Suspended solid (mg-SS L}^{-1}) = \frac{\text{Difference of filter weight (g)} \times 10^6}{\text{Volume of sample (ml)}} \quad \text{Equation A-2}$$

A.6 Chemical oxygen demand analysis, according to the Closed Reflux, Titrimetric Method (ASTM 1995)

Reagents

1. 0.1 N standard potassium dichromate solution:
   Dilute 4.913 g of potassium dichromate ($K_2Cr_2O_7$) in 500 ml of D.I. Add 167 ml of H_2SO_4 and 33.3 g of mercury (II) sulfate ($HgSO_4$), mix and adjust the volume to 1 L.
2. Sulfuric acid with silver sulfate solution:
   Dilute 22 g of silver sulfate ($AgSO_4$) in 2.5 L of H_2SO_4, and allow the reagent to react for 1 to 2 d.
3. 0.1 N ferrous ammonium sulfate (FAS) solution:
   Dilute 39 g of ferrous ammonium hexahydrate ($Fe(NH_4)_2(SO_4)_2.6H_2O$) in 500 ml of D.I. Add 20 ml of H_2SO_4 and adjust the volume to 1 L.
4. Ferroin indicator:
   Dilute 1.485 g of 1,10-phenanthroline monohydrate ($C_{12}H_8N_2.H_2O$) and 0.695 g ferrous sulfate ($FeSO_4.7H_2O$) in D.I. and adjust the volume to 100 ml.

Procedure

Add 3 ml of 0.1 N of standard potassium dichromate solution and 7 ml of sulfuric acid with silver sulfate solution in 5 ml of sample (use D.I. as sample in blank), mix and heat in an oven at 150ºC for 2 h. Add 2–3 drops of ferroin indicator and titrate with 0.1 N FAS until reach the end point of red-brown color. The COD can be calculated as the following:

$$\text{Chemical oxygen demand (mg-COD } L^{-1}) = \frac{(A - B) \times N \times 8{,}000}{\text{Volume of sample (ml)}} \qquad \text{Equation A-3}$$

Where: A is volume of FAS for blank titration (ml)

B is volume of FAS for sample titration (ml)

N is normality of FAS (N)

To calculate the concentration of FAS, add 5 ml of 0.1 N standard potassium dichromate solution and 15 ml of H_2SO_4 in 50 ml of D.I. Add 2–3 drops of ferroin indicator and titrate with 0.1 N FAS until reach the end point of red-brown color. The normality of FAS can be calculated as the following:

$$\text{Normality of FAS (N)} = \frac{\text{Volume of } K_2Cr_2O_7 \text{ (ml)} \times 0.1}{\text{Volume of FAS (ml)}} \qquad \text{Equation A-4}$$

Appendix B

Data results in the study 1: effects of salinity, stocking density, and acclimation period on nitrogen removal efficiency and microbial community.

Table B-1. Water quality parameters in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation, followed by 10 days of denitrification (anoxic, no shrimps), without water exchange.

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-$CaCO_3$ L^{-1})	ORP (mV)	COD (mg L^{-1})
50 shrimps m^{-2}; 5 PSU							
2/27/2017 13:00	0	6.9	28.1	7.71	150.0	142.6	
2/28/2017 13:00	1	7.3	28.3	7.65	150.0	158.7	
3/1/2017 13:00	2	7.1	27.9	7.68	150.0	135.1	
3/2/2017 13:00	3	7.4	27.7	7.75	150.0	144.8	
3/3/2017 13:00	4	6.3	28.2	7.81	150.0	152.6	
3/4/2017 13:00	5	6.9	28.3	7.77	150.0	131.7	
3/5/2017 13:00	6	6.8	28.5	7.64	160.0	152.8	
3/6/2017 13:00	7	5.5	29.2	7.53	160.0	140.0	
3/7/2017 13:00	8	5.9	28.8	8.26	150.0	139.8	
3/8/2017 13:00	9	6.3	28.8	7.92	140.0	120.6	
3/9/2017 13:00	10	6.1	28.9	7.84	130.0	131.4	
3/10/2017 13:00	11	5.5	28.6	8.06	120.0	123.8	
3/11/2017 13:00	12	6.3	28.7	7.96	125.0	108.9	
3/12/2017 13:00	13	7.8	28.8	7.89	130.0	132.5	
3/13/2017 13:00	14	4.0	29.2	8.10	110.0	129.9	
3/14/2017 13:00	15	5.5	29.5	8.33	110.0	138.6	
3/15/2017 13:00	16	6.2	29.1	7.99	115.0	135.4	
3/16/2017 13:00	17	7.1	28.3	7.91	115.0	139.8	
3/17/2017 13:00	18	6.5	27.9	7.95	120.0	131.6	
3/18/2017 13:00	19	7.0	27.7	7.89	120.0	145.0	
3/19/2017 13:00	20	7.2	28.2	7.93	125.0	148.8	
3/20/2017 13:00	21	7.1	28.7	7.94	125.0	140.1	
3/21/2017 13:00	22	6.8	29.3	7.98	130.0	141.5	
3/22/2017 13:00	23	7.1	29.5	7.82	130.0	148.7	
3/23/2017 13:00	24	7.5	29.8	7.76	130.0	134.2	
3/24/2017 13:00	25	7.9	29.5	7.72	120.0	115.6	
3/25/2017 13:00	26	8.1	28.5	7.73	115.0	120.0	
3/26/2017 13:00	27	7.6	27.5	7.74	110.0	131.6	
3/27/2017 13:00	28	8.1	26.6	8.10	140.0	145.0	
3/28/2017 13:00	29	9.5	27.4	8.21	120.0	128.8	
3/29/2017 13:00	30	9.0	27.8	8.12	120.0	135.2	
3/30/2017 13:00	31	7.9	28.5	7.99	115.0	144.8	
3/31/2017 13:00	32	6.8	28.7	7.91	120.0	135.3	
4/1/2017 13:00	33	7.2	28.0	7.78	110.0	149.6	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
4/2/2017 13:00	34	7.5	27.2	7.65	120.0	135.8	
4/3/2017 13:00	35	7.3	27.7	7.71	110.0	126.8	
4/4/2017 13:00	36	7.4	28.0	7.76	100.0	136.8	
4/5/2017 13:00	37	6.5	28.1	7.71	90.0	135.5	
4/6/2017 13:00	38	6.2	28.3	7.65	95.0	130.0	
4/7/2017 13:00	39	7.1	27.9	7.68	90.0	145.0	
4/8/2017 13:00	40	6.5	27.7	8.22	150.0	167.3	
4/9/2017 13:00	41	7.0	28.2	8.17	140.0	135.8	
4/10/2017 13:00	42	7.2	29.6	8.15	130.0	122.3	
4/11/2017 13:00	43	7.9	29.5	8.15	130.0	152.4	
4/12/2017 13:00	44	8.5	29.4	8.15	120.0	134.6	
4/13/2017 13:00	45	8.2	29.3	8.14	110.0	145.3	
4/14/2017 13:00	46	7.2	29.0	8.04	100.0	151.2	
4/15/2017 13:00	47	7.5	29.0	8.01	95.0	138.9	
4/16/2017 13:00	48	6.0	29.0	7.98	90.0	132.6	
4/17/2017 13:00	49	7.3	28.4	8.13	160.0	141.6	
4/18/2017 13:00	50	7.4	28.9	8.05	155.0	142.5	
4/19/2017 13:00	51	7.5	29.4	7.99	150.0	149.8	
4/20/2017 13:00	52	6.9	29.4	7.87	150.0	152.3	
4/21/2017 13:00	53	6.6	29.5	7.85	150.0	150.6	
4/22/2017 13:00	54	6.8	29.6	7.92	150.0	146.5	
4/23/2017 13:00	55	6.9	29.6	8.04	150.0	130.0	
4/24/2017 13:00	56	7.3	29.7	8.11	150.0	142.2	
4/25/2017 13:00	57	6.8	29.9	8.13	140.0	152.1	
4/26/2017 13:00	58	5.2	30.1	8.15	140.0	153.9	
4/27/2017 13:00	59	5.6	30.0	8.05	130.0	142.9	
4/28/2017 13:00	60	6.2	29.7	7.75	140.0	135.9	
4/29/2017 13:00	61	0.2	30.2	6.89	136.6	-143.5	265.05
4/30/2017 13:00	62	0.1	29.8	6.92	150.0	-125.8	177.78
5/1/2017 13:00	63	0.2	30.5	6.95	160.0	-138.6	105.05
5/2/2017 13:00	64	0.1	30.9	6.98	170.0	-143.5	64.65
5/3/2017 13:00	65	0.1	31.5	7.06	210.0	-45.1	65.31
5/4/2017 13:00	66	0.5	30.9	7.25	210.0	-68.4	72.73
5/5/2017 13:00	67	2.0	30.4	7.49	205.0	33.5	62.59
5/6/2017 13:00	68	1.7	29.9	7.43	195.0	42.4	56.57
5/7/2017 13:00	69	2.2	29.7	7.45	190.0	63.8	56.57
5/8/2017 13:00	70	3.4	29.7	7.63	178.1	79.0	105.05
5/9/2017 13:00	71	0.1	30.6	7.21	190.0	-28.9	72.73
5/10/2017 13:00	72	0.5	28.9	7.54	205.0	-4.6	56.57
5/11/2017 13:00	73	1.6	28.2	7.81	205.0	7.2	51.70
5/12/2017 13:00	74	2.3	30.3	7.79	200.0	14.2	48.48
5/13/2017 13:00	75	2.8	31.5	7.75	200.0	21.3	46.26
5/14/2017 13:00	76	3.9	30.1	7.82	205.0	72.9	70.00

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
5/15/2017 13:00	77	3.3	30.8	7.64	200.0	54.2	40.82
5/16/2017 13:00	78	2.9	29.7	7.76	205.0	14.2	35.37
5/17/2017 13:00	79	1.8	30.6	7.77	200.0	10.8	24.24
5/18/2017 13:00	80	2.2	31.8	7.79	200.0	2.9	29.73
100 shrimps m^{-2}; 5 PSU							
2/27/2017 13:00	0	7.2	28.2	7.62	150.0	141.9	
2/28/2017 13:00	1	7.1	28.4	7.57	150.0	132.5	
3/1/2017 13:00	2	7.2	28.3	7.64	150.0	150.8	
3/2/2017 13:00	3	7.3	28.4	7.73	150.0	143.4	
3/3/2017 13:00	4	6.1	28.5	7.76	150.0	150.6	
3/4/2017 13:00	5	6.5	28.6	7.69	160.0	136.5	
3/5/2017 13:00	6	5.0	28.8	7.63	170.0	160.0	
3/6/2017 13:00	7	4.1	28.9	7.51	150.0	152.2	
3/7/2017 13:00	8	5.3	28.9	7.96	140.0	162.1	
3/8/2017 13:00	9	6.2	28.8	7.72	130.0	143.9	
3/9/2017 13:00	10	6.1	28.8	7.46	120.0	152.9	
3/10/2017 13:00	11	4.6	28.5	8.00	120.0	155.9	
3/11/2017 13:00	12	5.6	28.6	7.91	115.0	145.8	
3/12/2017 13:00	13	6.3	28.6	7.83	110.0	149.6	
3/13/2017 13:00	14	4.0	28.8	7.96	130.0	139.8	
3/14/2017 13:00	15	5.4	29.3	8.03	120.0	130.6	
3/15/2017 13:00	16	5.2	28.9	8.04	130.0	141.9	
3/16/2017 13:00	17	6.1	28.2	7.99	125.0	123.8	
3/17/2017 13:00	18	6.5	27.9	8.03	135.0	138.5	
3/18/2017 13:00	19	7.4	27.6	8.05	140.0	142.5	
3/19/2017 13:00	20	7.2	28.5	8.03	145.0	139.9	
3/20/2017 13:00	21	7.6	28.9	8.07	150.0	128.8	
3/21/2017 13:00	22	7.9	29.5	8.14	160.0	115.4	
3/22/2017 13:00	23	7.6	29.6	8.09	160.0	129.8	
3/23/2017 13:00	24	7.7	29.8	8.12	160.0	120.2	
3/24/2017 13:00	25	8.0	29.4	8.13	155.0	110.9	
3/25/2017 13:00	26	8.3	28.3	8.11	160.0	105.3	
3/26/2017 13:00	27	7.6	27.6	8.10	160.0	115.4	
3/27/2017 13:00	28	7.9	26.7	8.02	140.0	128.6	
3/28/2017 13:00	29	9.1	27.4	8.14	150.0	125.9	
3/29/2017 13:00	30	8.7	27.9	8.06	145.0	132.8	
3/30/2017 13:00	31	7.5	28.4	7.95	140.0	141.5	
3/31/2017 13:00	32	6.8	28.6	7.83	130.0	152.9	
4/1/2017 13:00	33	6.6	27.9	7.72	130.0	159.5	
4/2/2017 13:00	34	6.4	27.2	7.60	120.0	150.8	
4/3/2017 13:00	35	6.5	27.7	7.68	120.0	148.4	
4/4/2017 13:00	36	6.8	28.2	7.71	120.0	142.1	
4/5/2017 13:00	37	6.9	28.1	7.66	100.0	152.4	
4/6/2017 13:00	38	6.5	28.5	7.62	90.0	131.3	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
4/7/2017 13:00	39	6.9	27.7	7.61	80.0	135.8	
4/8/2017 13:00	40	6.2	27.5	8.25	150.0	142.0	
4/9/2017 13:00	41	7.0	28.9	8.16	140.0	126.9	
4/10/2017 13:00	42	6.9	29.5	8.08	130.0	115.6	
4/11/2017 13:00	43	7.3	29.5	8.12	120.0	145.9	
4/12/2017 13:00	44	7.5	29.4	8.06	110.0	111.1	
4/13/2017 13:00	45	7.8	29.4	8.08	100.0	129.6	
4/14/2017 13:00	46	6.5	28.8	7.99	110.0	138.5	
4/15/2017 13:00	47	6.2	29.5	7.95	90.0	153.9	
4/16/2017 13:00	48	5.8	29.8	7.94	90.0	140.2	
4/17/2017 13:00	49	7.2	28.5	8.70	130.0	160.4	
4/18/2017 13:00	50	7.1	29.1	8.26	130.0	135.8	
4/19/2017 13:00	51	6.9	29.3	7.94	130.0	145.9	
4/20/2017 13:00	52	6.8	29.5	7.89	130.0	150.0	
4/21/2017 13:00	53	6.9	29.6	7.81	130.0	146.7	
4/22/2017 13:00	54	7.1	29.6	7.92	120.0	148.2	
4/23/2017 13:00	55	7.2	29.6	7.99	115.0	142.3	
4/24/2017 13:00	56	7.5	29.6	8.04	110.0	115.6	
4/25/2017 13:00	57	6.2	29.8	8.06	110.0	125.6	
4/26/2017 13:00	58	4.3	29.9	8.09	100.0	111.1	
4/27/2017 13:00	59	5.7	30.2	7.98	100.0	122.1	
4/28/2017 13:00	60	6.2	30.0	7.67	95.0	122.4	
4/29/2017 13:00	61	0.1	30.3	6.89	131.2	-86.9	297.98
4/30/2017 13:00	62	0.1	30.8	6.68	120.0	-143.2	202.42
5/1/2017 13:00	63	0.1	31.2	6.75	115.0	-128.6	177.78
5/2/2017 13:00	64	0.1	31.4	6.81	112.2	-115.7	161.62
5/3/2017 13:00	65	0.1	31.8	7.03	150.0	-120.3	145.45
5/4/2017 13:00	66	0.2	31.2	7.25	170.0	-108.6	105.05
5/5/2017 13:00	67	0.2	30.7	7.44	180.0	-109.6	72.73
5/6/2017 13:00	68	0.4	30.5	7.32	210.0	56.0	24.24
5/7/2017 13:00	69	1.3	29.8	7.35	200.0	73.6	24.24
5/8/2017 13:00	70	2.0	30.0	7.66	152.2	90.0	105.05
5/9/2017 13:00	71	0.1	30.7	7.33	150.0	-19.2	35.37
5/10/2017 13:00	72	6.8	30.1	8.32	148.6	64.7	8.08
5/11/2017 13:00	73	6.7	27.8	8.34	180.0	78.3	0.00
5/12/2017 13:00	74	7.3	29.9	8.41	211.3	120.0	0.00
5/13/2017 13:00	75	6.5	30.7	8.49	200.0	155.4	8.08
5/14/2017 13:00	76	6.3	29.0	8.38	195.0	142.3	0.00
5/15/2017 13:00	77	6.8	30.5	8.45	200.0	145.0	0.00
5/16/2017 13:00	78	6.5	29.3	8.36	170.0	158.5	8.08
5/17/2017 13:00	79	7.2	30.1	8.39	171.8	143.9	8.08
5/18/2017 13:00	80	7.1	31.3	8.41	180.0	150.2	0.00
50 shrimps m^{-2}; 15 PSU							
2/27/2017 13:00	0	7.5	28.1	7.41	160.0	115.3	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
2/28/2017 13:00	1	7.8	28.4	7.50	170.0	135.7	
3/1/2017 13:00	2	8.0	28.3	7.76	170.0	152.8	
3/2/2017 13:00	3	8.7	28.3	7.97	190.0	128.9	
3/3/2017 13:00	4	6.3	28.2	7.71	180.0	135.4	
3/4/2017 13:00	5	6.4	28.2	7.69	175.0	129.6	
3/5/2017 13:00	6	6.7	28.3	7.67	170.0	138.4	
3/6/2017 13:00	7	7.1	28.7	7.81	170.0	142.0	
3/7/2017 13:00	8	5.9	28.9	7.97	160.0	148.5	
3/8/2017 13:00	9	6.0	28.7	7.72	150.0	113.5	
3/9/2017 13:00	10	5.3	28.5	7.53	140.0	128.6	
3/10/2017 13:00	11	4.3	28.4	7.81	140.0	150.0	
3/11/2017 13:00	12	5.6	28.6	7.78	125.0	124.8	
3/12/2017 13:00	13	6.7	28.7	7.76	110.0	158.9	
3/13/2017 13:00	14	4.9	29.0	7.81	120.0	143.2	
3/14/2017 13:00	15	5.8	29.2	7.86	120.0	123.6	
3/15/2017 13:00	16	6.2	28.8	7.79	125.0	142.1	
3/16/2017 13:00	17	6.1	28.3	7.82	120.0	147.6	
3/17/2017 13:00	18	6.5	27.8	7.84	125.0	135.0	
3/18/2017 13:00	19	6.4	27.5	7.89	130.0	142.8	
3/19/2017 13:00	20	6.2	28.3	7.83	125.0	134.7	
3/20/2017 13:00	21	6.6	29.1	7.81	130.0	129.4	
3/21/2017 13:00	22	6.4	29.4	7.79	130.0	135.6	
3/22/2017 13:00	23	6.9	29.6	7.68	130.0	142.8	
3/23/2017 13:00	24	7.4	29.7	7.60	130.0	153.3	
3/24/2017 13:00	25	7.5	29.6	7.69	135.0	164.9	
3/25/2017 13:00	26	7.9	28.1	7.78	140.0	135.2	
3/26/2017 13:00	27	7.6	27.2	7.88	150.0	152.1	
3/27/2017 13:00	28	7.7	26.9	7.96	150.0	142.7	
3/28/2017 13:00	29	9.4	27.5	8.01	120.0	122.9	
3/29/2017 13:00	30	8.7	27.7	7.95	120.0	123.7	
3/30/2017 13:00	31	7.9	28.3	7.80	120.0	131.2	
3/31/2017 13:00	32	7.9	28.8	7.83	120.0	125.7	
4/1/2017 13:00	33	7.8	28.1	7.75	110.0	152.9	
4/2/2017 13:00	34	7.2	27.1	7.68	120.0	146.3	
4/3/2017 13:00	35	7.0	27.5	7.72	110.0	164.1	
4/4/2017 13:00	36	6.7	28.0	7.69	100.0	152.9	
4/5/2017 13:00	37	6.5	28.3	7.70	100.0	162.3	
4/6/2017 13:00	38	6.8	28.5	7.62	100.0	141.2	
4/7/2017 13:00	39	6.9	27.8	7.69	100.0	158.9	
4/8/2017 13:00	40	7.3	27.6	8.12	150.0	148.0	
4/9/2017 13:00	41	7.0	28.7	8.07	140.0	152.6	
4/10/2017 13:00	42	7.0	29.7	8.00	130.0	153.8	
4/11/2017 13:00	43	7.9	29.6	7.96	125.0	132.7	
4/12/2017 13:00	44	8.5	29.4	7.92	125.0	122.1	
4/13/2017 13:00	45	8.4	29.2	7.98	120.0	118.5	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
4/14/2017 13:00	46	7.4	28.5	7.92	100.0	131.9	
4/15/2017 13:00	47	6.9	28.5	7.95	100.0	143.2	
4/16/2017 13:00	48	6.1	28.4	7.90	100.0	142.1	
4/17/2017 13:00	49	7.6	28.3	8.05	160.0	158.6	
4/18/2017 13:00	50	7.2	29.1	7.95	150.0	145.0	
4/19/2017 13:00	51	6.4	29.4	7.86	140.0	138.5	
4/20/2017 13:00	52	6.5	29.5	7.80	150.0	153.9	
4/21/2017 13:00	53	6.3	29.7	7.84	170.0	140.2	
4/22/2017 13:00	54	6.4	29.8	7.81	165.0	160.4	
4/23/2017 13:00	55	6.5	29.9	7.90	160.0	141.9	
4/24/2017 13:00	56	6.6	29.9	7.93	160.0	150.0	
4/25/2017 13:00	57	6.2	30.0	7.85	150.0	149.5	
4/26/2017 13:00	58	6.8	30.2	7.91	130.0	163.2	
4/27/2017 13:00	59	4.9	30.3	7.83	140.0	154.8	
4/28/2017 13:00	60	5.6	30.1	7.65	135.0	156.9	
4/29/2017 13:00	61	0.1	30.8	6.74	101.9	-126.9	257.53
4/30/2017 13:00	62	0.1	30.9	6.75	105.0	-113.2	181.95
5/1/2017 13:00	63	0.2	31.2	6.78	105.0	-98.5	145.45
5/2/2017 13:00	64	0.1	31.5	6.94	140.0	-85.7	113.13
5/3/2017 13:00	65	0.1	31.9	7.55	240.0	-55.6	88.89
5/4/2017 13:00	66	0.2	31.5	7.58	295.0	-0.7	72.73
5/5/2017 13:00	67	0.4	30.7	7.61	290.0	3.7	64.65
5/6/2017 13:00	68	0.5	30.3	7.57	270.0	54.2	56.57
5/7/2017 13:00	69	1.0	30.2	7.62	260.0	52.3	51.70
5/8/2017 13:00	70	1.5	29.9	7.49	257.8	51.7	88.89
5/9/2017 13:00	71	0.1	30.8	7.53	265.0	-20.9	51.70
5/10/2017 13:00	72	6.9	29.6	8.28	300.0	122.6	40.40
5/11/2017 13:00	73	6.7	27.9	8.31	190.0	138.5	29.73
5/12/2017 13:00	74	7.2	29.5	8.26	162.4	140.8	16.16
5/13/2017 13:00	75	6.3	30.2	8.34	190.0	153.5	21.77
5/14/2017 13:00	76	6.1	29.1	8.37	185.0	141.2	16.16
5/15/2017 13:00	77	6.2	30.3	8.36	190.0	143.9	21.77
5/16/2017 13:00	78	6.8	29.4	8.31	185.0	152.4	16.16
5/17/2017 13:00	79	6.9	30.2	8.38	192.7	142.8	24.24
5/18/2017 13:00	80	6.8	30.9	8.35	190.0	151.5	24.24
100 shrimps m^{-2}; 15 PSU							
2/27/2017 13:00	0	7.4	28.1	7.59	160.0	133.5	
2/28/2017 13:00	1	7.5	27.9	7.57	160.0	115.4	
3/1/2017 13:00	2	7.9	28.0	7.75	160.0	127.1	
3/2/2017 13:00	3	8.5	27.9	7.99	160.0	132.3	
3/3/2017 13:00	4	6.3	27.9	7.80	160.0	142.8	
3/4/2017 13:00	5	6.4	28.2	7.70	160.0	158.9	
3/5/2017 13:00	6	6.2	28.5	7.73	160.0	128.9	
3/6/2017 13:00	7	6.5	29.0	7.78	180.0	140.6	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
3/7/2017 13:00	8	5.0	29.1	7.97	160.0	123.7	
3/8/2017 13:00	9	5.8	29.2	7.82	150.0	131.2	
3/9/2017 13:00	10	5.6	28.8	7.67	140.0	125.7	
3/10/2017 13:00	11	4.7	28.6	7.88	110.0	152.9	
3/11/2017 13:00	12	6.2	28.8	7.78	115.0	146.3	
3/12/2017 13:00	13	7.0	28.9	7.80	120.0	164.1	
3/13/2017 13:00	14	4.9	29.4	7.85	110.0	152.9	
3/14/2017 13:00	15	5.6	29.6	7.91	120.0	162.3	
3/15/2017 13:00	16	5.2	29.0	7.84	130.0	141.2	
3/16/2017 13:00	17	6.1	28.5	7.82	130.0	158.9	
3/17/2017 13:00	18	6.5	27.7	7.76	135.0	143.7	
3/18/2017 13:00	19	7.1	27.4	7.71	140.0	131.2	
3/19/2017 13:00	20	6.9	28.5	7.79	145.0	145.7	
3/20/2017 13:00	21	6.5	29.5	7.77	150.0	152.9	
3/21/2017 13:00	22	6.1	29.7	7.80	160.0	146.3	
3/22/2017 13:00	23	6.5	29.7	7.71	150.0	154.1	
3/23/2017 13:00	24	7.1	29.8	7.63	150.0	152.9	
3/24/2017 13:00	25	7.0	29.6	7.72	145.0	161.8	
3/25/2017 13:00	26	7.3	28.7	7.88	140.0	141.2	
3/26/2017 13:00	27	7.6	27.3	7.91	140.0	158.9	
3/27/2017 13:00	28	7.7	27.2	7.96	120.0	142.0	
3/28/2017 13:00	29	9.2	27.7	8.04	120.0	148.5	
3/29/2017 13:00	30	8.5	28.0	7.93	120.0	113.5	
3/30/2017 13:00	31	7.3	28.9	7.82	120.0	128.6	
3/31/2017 13:00	32	5.4	29.2	7.71	120.0	120.0	
4/1/2017 13:00	33	5.8	28.7	7.70	140.0	145.0	
4/2/2017 13:00	34	6.0	27.5	7.68	130.0	138.5	
4/3/2017 13:00	35	6.5	28.0	7.68	120.0	133.9	
4/4/2017 13:00	36	6.4	28.5	7.68	110.0	130.2	
4/5/2017 13:00	37	6.8	28.4	7.65	100.0	150.4	
4/6/2017 13:00	38	6.2	28.2	7.61	95.0	141.9	
4/7/2017 13:00	39	6.1	27.7	7.60	90.0	150.0	
4/8/2017 13:00	40	6.5	28.0	8.08	150.0	149.5	
4/9/2017 13:00	41	6.0	28.9	8.01	140.0	143.3	
4/10/2017 13:00	42	5.6	29.7	7.98	130.0	132.8	
4/11/2017 13:00	43	5.9	29.6	7.95	125.0	118.1	
4/12/2017 13:00	44	6.5	29.4	7.93	120.0	125.3	
4/13/2017 13:00	45	6.4	29.4	7.92	110.0	128.6	
4/14/2017 13:00	46	5.5	29.1	7.80	110.0	116.9	
4/15/2017 13:00	47	5.9	28.9	7.72	100.0	121.3	
4/16/2017 13:00	48	4.8	28.6	7.68	90.0	125.8	
4/17/2017 13:00	49	6.0	28.5	7.89	130.0	134.8	
4/18/2017 13:00	50	6.2	28.9	7.87	130.0	136.5	
4/19/2017 13:00	51	5.9	29.5	7.85	130.0	140.0	
4/20/2017 13:00	52	6.1	29.7	7.84	140.0	152.1	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
4/21/2017 13:00	53	5.7	29.9	7.82	150.0	139.2	
4/22/2017 13:00	54	6.2	29.9	7.89	140.0	142.1	
4/23/2017 13:00	55	7.1	29.9	7.85	130.0	145.6	
4/24/2017 13:00	56	6.7	29.9	7.94	120.0	152.0	
4/25/2017 13:00	57	6.0	29.9	7.92	115.0	148.6	
4/26/2017 13:00	58	5.9	29.9	7.91	110.0	126.3	
4/27/2017 13:00	59	5.3	30.0	7.83	100.0	122.1	
4/28/2017 13:00	60	5.9	29.8	7.72	95.0	143.4	
4/29/2017 13:00	61	0.2	30.7	6.89	82.2	-138.5	298.88
4/30/2017 13:00	62	0.2	30.5	6.91	90.0	-146.9	275.37
5/1/2017 13:00	63	0.2	31.0	6.92	100.0	-132.4	262.16
5/2/2017 13:00	64	0.1	31.5	7.01	120.0	-149.3	242.42
5/3/2017 13:00	65	0.1	31.8	7.62	220.0	0.3	161.62
5/4/2017 13:00	66	0.3	31.1	7.68	246.0	-1.6	88.89
5/5/2017 13:00	67	0.4	30.7	7.62	260.0	19.9	62.59
5/6/2017 13:00	68	0.7	30.1	7.56	260.0	46.2	56.57
5/7/2017 13:00	69	0.9	30.5	7.55	260.0	50.8	56.57
5/8/2017 13:00	70	1.0	29.8	7.49	268.9	53.3	137.37
5/9/2017 13:00	71	0.1	30.9	7.57	275.0	-50.6	56.57
5/10/2017 13:00	72	6.1	29.8	7.64	278.8	108.2	48.48
5/11/2017 13:00	73	6.4	28.1	7.80	220.0	111.4	56.57
5/12/2017 13:00	74	6.7	29.3	8.42	215.0	120.8	64.65
5/13/2017 13:00	75	6.9	30.2	8.45	220.0	153.5	56.57
5/14/2017 13:00	76	6.1	29.2	8.39	215.0	151.2	62.59
5/15/2017 13:00	77	6.5	30.2	8.42	220.0	133.9	56.57
5/16/2017 13:00	78	7.2	29.8	8.36	220.0	152.4	51.70
5/17/2017 13:00	79	7.1	30.9	8.37	220.0	142.8	56.57
5/18/2017 13:00	80	7.3	31.2	8.38	220.0	151.5	56.57
50 shrimps m^{-2}; 25 PSU							
2/27/2017 13:00	0	7.2	28.1	7.62	160.0	134.6	
2/28/2017 13:00	1	7.4	27.9	7.65	170.0	121.5	
3/1/2017 13:00	2	7.8	27.9	7.86	170.0	125.8	
3/2/2017 13:00	3	8.5	27.9	8.04	170.0	139.5	
3/3/2017 13:00	4	6.6	28.4	7.80	200.0	114.8	
3/4/2017 13:00	5	6.4	28.5	7.70	190.0	127.9	
3/5/2017 13:00	6	6.4	28.5	7.62	190.0	121.9	
3/6/2017 13:00	7	7.1	29.0	7.76	180.0	128.3	
3/7/2017 13:00	8	5.7	29.3	7.88	160.0	145.6	
3/8/2017 13:00	9	5.8	28.9	7.75	150.0	152.3	
3/9/2017 13:00	10	5.5	28.8	7.63	140.0	120.0	
3/10/2017 13:00	11	4.8	28.5	7.83	120.0	133.8	
3/11/2017 13:00	12	5.6	28.7	7.85	120.0	145.2	
3/12/2017 13:00	13	7.2	28.9	7.89	120.0	154.6	
3/13/2017 13:00	14	4.0	29.3	7.77	150.0	135.8	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
3/14/2017 13:00	15	5.6	29.6	7.77	140.0	162.3	
3/15/2017 13:00	16	6.2	29.1	7.84	140.0	163.9	
3/16/2017 13:00	17	6.1	28.5	7.92	135.0	164.8	
3/17/2017 13:00	18	6.4	27.8	7.83	130.0	152.6	
3/18/2017 13:00	19	6.9	27.5	7.85	130.0	143.9	
3/19/2017 13:00	20	6.5	28.3	7.84	125.0	150.0	
3/20/2017 13:00	21	6.5	29.2	7.76	120.0	151.6	
3/21/2017 13:00	22	6.3	29.5	7.70	120.0	138.9	
3/22/2017 13:00	23	6.9	29.7	7.64	125.0	135.6	
3/23/2017 13:00	24	7.4	29.8	7.60	130.0	121.7	
3/24/2017 13:00	25	7.0	29.5	7.64	135.0	108.4	
3/25/2017 13:00	26	7.2	28.6	7.76	140.0	111.8	
3/26/2017 13:00	27	7.3	27.2	7.79	140.0	144.6	
3/27/2017 13:00	28	7.1	26.7	7.85	120.0	129.4	
3/28/2017 13:00	29	8.4	27.5	7.91	120.0	132.5	
3/29/2017 13:00	30	8.0	27.9	7.85	115.0	135.7	
3/30/2017 13:00	31	7.8	28.4	7.74	115.0	132.6	
3/31/2017 13:00	32	6.7	28.7	7.62	110.0	119.8	
4/1/2017 13:00	33	6.5	28.5	7.59	100.0	121.5	
4/2/2017 13:00	34	6.6	27.1	7.58	110.0	133.9	
4/3/2017 13:00	35	6.7	27.8	7.59	100.0	125.8	
4/4/2017 13:00	36	6.6	28.2	7.61	90.0	124.2	
4/5/2017 13:00	37	6.5	28.3	7.58	90.0	146.9	
4/6/2017 13:00	38	6.2	28.5	7.54	90.0	121.4	
4/7/2017 13:00	39	6.9	27.9	7.61	90.0	136.1	
4/8/2017 13:00	40	6.5	28.1	7.96	150.0	142.5	
4/9/2017 13:00	41	6.1	29.0	7.92	135.0	154.8	
4/10/2017 13:00	42	6.3	29.5	7.88	120.0	150.6	
4/11/2017 13:00	43	6.9	29.6	7.85	110.0	143.3	
4/12/2017 13:00	44	7.1	29.5	7.84	105.0	132.8	
4/13/2017 13:00	45	7.4	29.5	7.82	100.0	128.1	
4/14/2017 13:00	46	6.7	28.9	7.72	110.0	135.3	
4/15/2017 13:00	47	5.9	28.8	7.70	105.0	138.6	
4/16/2017 13:00	48	5.6	28.7	7.71	100.0	116.9	
4/17/2017 13:00	49	7.4	28.6	7.88	140.0	121.3	
4/18/2017 13:00	50	6.5	29.5	7.84	140.0	103.4	
4/19/2017 13:00	51	6.0	29.7	7.84	140.0	104.9	
4/20/2017 13:00	52	6.1	29.8	7.85	140.0	114.6	
4/21/2017 13:00	53	6.3	29.9	7.86	140.0	121.5	
4/22/2017 13:00	54	6.5	30.0	7.88	140.0	125.8	
4/23/2017 13:00	55	7.0	29.9	7.87	140.0	134.6	
4/24/2017 13:00	56	7.2	29.9	7.89	140.0	135.8	
4/25/2017 13:00	57	6.4	29.8	7.85	130.0	142.5	
4/26/2017 13:00	58	4.2	29.7	7.81	130.0	151.9	
4/27/2017 13:00	59	5.5	30.0	7.72	130.0	138.6	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
4/28/2017 13:00	60	6.1	29.8	7.63	115.0	143.5	
4/29/2017 13:00	61	0.1	30.9	7.38	100.0	-149.9	262.16
4/30/2017 13:00	62	0.1	30.8	6.91	115.0	-139.5	202.42
5/1/2017 13:00	63	0.1	31.1	6.92	130.0	-133.3	177.78
5/2/2017 13:00	64	0.1	31.5	7.03	140.0	-121.9	145.45
5/3/2017 13:00	65	0.1	31.6	7.39	190.0	5.8	113.13
5/4/2017 13:00	66	0.3	30.9	7.36	204.0	16.2	80.81
5/5/2017 13:00	67	0.5	30.6	7.34	200.0	27.7	72.73
5/6/2017 13:00	68	0.4	30.0	7.28	170.0	45.7	64.65
5/7/2017 13:00	69	1.2	30.2	7.35	175.0	41.8	62.59
5/8/2017 13:00	70	2.4	29.8	7.43	173.8	72.3	177.78
5/9/2017 13:00	71	0.1	30.8	7.30	210.0	-48.8	113.13
5/10/2017 13:00	72	0.3	29.7	7.32	215.0	-2.6	88.89
5/11/2017 13:00	73	0.5	28.6	7.31	220.0	-6.4	72.73
5/12/2017 13:00	74	1.6	28.9	7.36	225.0	33.8	80.81
5/13/2017 13:00	75	2.4	29.9	7.41	220.0	42.3	72.73
5/14/2017 13:00	76	3.1	30.2	7.59	215.0	84.2	77.85
5/15/2017 13:00	77	2.7	30.5	7.47	220.0	46.9	72.73
5/16/2017 13:00	78	2.6	30.7	7.45	225.0	56.4	62.59
5/17/2017 13:00	79	2.8	30.6	7.48	225.0	33.8	80.81
5/18/2017 13:00	80	3.2	31.0	7.47	225.0	42.3	77.85
100 shrimps m^{-2}; 25 PSU							
2/27/2017 13:00	0	7.4	27.9	7.70	160.0	141.9	
2/28/2017 13:00	1	7.6	28.2	7.72	170.0	152.5	
3/1/2017 13:00	2	8.2	28.3	7.85	170.0	150.7	
3/2/2017 13:00	3	8.8	28.5	8.04	180.0	145.5	
3/3/2017 13:00	4	6.3	28.7	7.82	170.0	152.6	
3/4/2017 13:00	5	6.4	28.7	7.85	180.0	146.5	
3/5/2017 13:00	6	6.3	28.8	7.89	190.0	153.1	
3/6/2017 13:00	7	7.8	29.1	7.96	220.0	156.9	
3/7/2017 13:00	8	5.9	29.4	7.92	210.0	124.5	
3/8/2017 13:00	9	5.8	29.2	7.72	205.0	142.6	
3/9/2017 13:00	10	5.5	29.0	7.63	200.0	145.3	
3/10/2017 13:00	11	4.5	28.5	7.82	100.0	144.2	
3/11/2017 13:00	12	6.1	28.9	7.74	105.0	153.2	
3/12/2017 13:00	13	5.6	29.2	7.66	110.0	158.1	
3/13/2017 13:00	14	4.7	29.7	7.76	110.0	154.0	
3/14/2017 13:00	15	5.2	28.9	7.76	140.0	135.6	
3/15/2017 13:00	16	5.8	28.9	7.69	135.0	120.2	
3/16/2017 13:00	17	6.1	28.4	7.61	135.0	123.5	
3/17/2017 13:00	18	6.2	27.9	7.75	140.0	134.9	
3/18/2017 13:00	19	5.9	27.6	7.59	135.0	112.6	
3/19/2017 13:00	20	5.5	28.4	7.63	135.0	128.7	
3/20/2017 13:00	21	6.3	29.4	7.64	135.0	122.9	

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
3/21/2017 13:00	22	6.0	29.8	7.67	140.0	127.3	
3/22/2017 13:00	23	6.8	30.0	7.62	140.0	143.6	
3/23/2017 13:00	24	7.4	30.1	7.58	140.0	152.8	
3/24/2017 13:00	25	6.6	29.9	7.64	145.0	153.9	
3/25/2017 13:00	26	6.9	28.5	7.72	145.0	141.7	
3/26/2017 13:00	27	7.0	27.1	7.79	150.0	138.9	
3/27/2017 13:00	28	7.5	26.8	7.83	120.0	140.5	
3/28/2017 13:00	29	9.0	27.7	7.90	120.0	149.7	
3/29/2017 13:00	30	8.4	27.9	7.81	120.0	151.2	
3/30/2017 13:00	31	7.3	28.6	7.74	120.0	160.0	
3/31/2017 13:00	32	6.8	29.0	7.62	120.0	159.5	
4/1/2017 13:00	33	7.5	28.1	7.58	130.0	139.8	
4/2/2017 13:00	34	7.2	27.1	7.54	110.0	136.4	
4/3/2017 13:00	35	6.9	27.7	7.55	100.0	149.3	
4/4/2017 13:00	36	6.7	28.1	7.57	90.0	152.1	
4/5/2017 13:00	37	7.0	28.2	7.56	85.0	168.6	
4/6/2017 13:00	38	6.9	28.5	7.60	80.0	132.2	
4/7/2017 13:00	39	7.1	27.8	7.60	80.0	145.8	
4/8/2017 13:00	40	6.8	28.3	7.82	150.0	143.2	
4/9/2017 13:00	41	7.0	29.0	7.78	130.0	139.8	
4/10/2017 13:00	42	6.8	29.8	7.74	110.0	162.8	
4/11/2017 13:00	43	7.2	29.7	7.72	105.0	159.8	
4/12/2017 13:00	44	7.5	29.6	7.71	100.0	169.3	
4/13/2017 13:00	45	8.0	29.6	7.68	100.0	153.9	
4/14/2017 13:00	46	7.1	29.0	7.57	90.0	138.6	
4/15/2017 13:00	47	6.5	29.0	7.50	90.0	165.6	
4/16/2017 13:00	48	5.6	29.0	7.59	90.0	152.3	
4/17/2017 13:00	49	7.2	28.8	7.73	140.0	121.0	
4/18/2017 13:00	50	6.5	29.5	7.70	135.0	153.9	
4/19/2017 13:00	51	5.1	29.9	7.74	130.0	151.7	
4/20/2017 13:00	52	5.7	30.1	7.69	135.0	148.1	
4/21/2017 13:00	53	5.9	30.2	7.65	140.0	168.3	
4/22/2017 13:00	54	6.1	30.2	7.69	135.0	139.7	
4/23/2017 13:00	55	6.8	30.1	7.73	130.0	140.4	
4/24/2017 13:00	56	6.6	30.2	7.77	120.0	151.8	
4/25/2017 13:00	57	5.8	30.1	7.75	110.0	172.4	
4/26/2017 13:00	58	4.2	30.1	7.74	120.0	149.3	
4/27/2017 13:00	59	5.9	30.2	7.70	100.0	152.5	
4/28/2017 13:00	60	6.3	30.0	7.60	100.0	158.7	
4/29/2017 13:00	61	0.1	30.8	7.43	110.0	-98.5	295.37
4/30/2017 13:00	62	0.2	31.2	7.22	180.0	-133.5	202.42
5/1/2017 13:00	63	0.1	31.3	7.29	190.0	-125.6	177.78
5/2/2017 13:00	64	0.1	31.3	7.35	200.0	-131.7	145.45
5/3/2017 13:00	65	0.1	31.5	7.67	295.0	-114.8	137.37
5/4/2017 13:00	66	0.4	31.2	7.66	300.0	-23.6	129.29

Date/Time	Day	DO (mg L^{-1})	Temp (°C)	pH	Alkalinity (mg-CaCO3 L^{-1})	ORP (mV)	COD (mg L^{-1})
5/5/2017 13:00	67	0.5	30.4	7.54	290.0	24.7	88.89
5/6/2017 13:00	68	0.5	29.7	7.48	283.1	34.5	64.65
5/7/2017 13:00	69	0.6	29.9	7.41	260.0	30.4	56.57
5/8/2017 13:00	70	1.0	29.7	7.43	245.0	39.1	145.45
5/9/2017 13:00	71	5.8	29.8	8.22	240.0	66.2	72.73
5/10/2017 13:00	72	6.5	28.3	8.19	178.4	116.5	48.48
5/11/2017 13:00	73	6.6	27.5	8.26	175.0	154.2	35.37
5/12/2017 13:00	74	7.3	28.6	8.31	180.0	150.9	56.57
5/13/2017 13:00	75	7.1	30.1	8.39	175.0	129.8	51.70
5/14/2017 13:00	76	6.2	28.9	8.37	170.0	155.7	40.82
5/15/2017 13:00	77	7.1	29.7	8.33	170.0	143.6	51.70
5/16/2017 13:00	78	7.0	30.3	8.38	170.0	152.1	35.37
5/17/2017 13:00	79	6.7	30.5	8.29	163.6	118.2	48.48
5/18/2017 13:00	80	6.9	29.9	8.24	170.0	148.4	42.26

Table B-2. Growth of shrimp cultured in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}) during 60 days of biofilter acclimation with aerobic shrimp cultivation.

Start experiment								
50 shrimps m^{-2}; 5 PSU			50 shrimps m^{-2}; 15 PSU			50 shrimps m^{-2}; 25 PSU		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	4.30	8.0	1	3.51	7.5	1	6.00	9.0
2	3.51	7.5	2	4.30	8.0	2	6.00	9.0
3	6.00	9.0	3	5.02	8.5	3	3.51	7.5
4	3.51	7.5	4	3.51	7.5	4	3.51	7.5
5	4.30	8.0	5	5.02	8.5	5	2.91	7.0
6	3.51	7.5	6	3.51	7.5	6	3.51	7.5
7	3.51	7.5	7	2.33	6.5	7	3.51	7.5
8	3.51	7.5	8	3.51	7.5	8	2.91	7.0
9	3.51	7.5	9	1.88	6.0	9	3.51	7.5
10	4.30	8.0	10	4.30	8.0	10	4.30	8.0
11	2.91	7.0	11	3.51	7.5	11	2.91	7.0
12	4.30	8.0	12	2.33	6.5	12	3.51	7.5
13	3.51	7.5	13	2.33	6.5	13	4.30	8.0
14	5.02	8.5	14	2.33	6.5	14	3.51	7.5
15	3.51	7.5	15	2.91	7.0	15	4.30	8.0
100 shrimps m^{-2}; 5 PSU			100 shrimps m^{-2}; 15 PSU			100 shrimps m^{-2}; 25 PSU		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	3.51	7.5	1	4.30	8.0	1	2.91	7.0
2	3.51	7.5	2	2.91	7.0	2	2.91	7.0
3	4.30	8.0	3	2.91	7.0	3	3.51	7.5
4	3.51	7.5	4	3.51	7.5	4	2.91	7.0
5	2.91	7.0	5	4.30	8.0	5	4.30	8.0
6	1.88	6.0	6	3.51	7.5	6	4.30	8.0
7	2.91	7.0	7	3.51	7.5	7	2.91	7.0
8	4.30	8.0	8	3.51	7.5	8	3.51	7.5

No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
9	3.51	7.5	9	3.51	7.5	9	3.51	7.5
10	4.30	8.0	10	2.91	7.0	10	3.51	7.5
11	2.91	7.0	11	4.30	8.0	11	3.51	7.5
12	2.91	7.0	12	2.91	7.0	12	2.91	7.0
13	2.33	6.5	13	5.65	8.0	13	3.51	7.5
14	4.30	8.0	14	3.51	7.5	14	4.30	8.0
15	4.30	8.0	15	4.30	8.0	15	2.91	7.0
1 month of experiment								
50 shrimps m^{-2}; 5 PSU			50 shrimps m^{-2}; 15 PSU			50 shrimps m^{-2}; 25 PSU		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	5.14	8.00	1	4.51	8.00	1	3.48	7.00
2	3.85	8.00	2	4.94	8.00	2	4.56	8.50
3	5.60	8.50	3	4.30	6.50	3	6.93	9.00
4	4.56	7.50	4	4.94	7.50	4	5.40	8.50
5	3.40	8.50	5	5.37	9.00	5	5.77	8.50
6	6.20	9.00	6	3.76	6.50	6	4.68	8.00
7	5.68	8.50	7	5.13	7.50	7	5.84	9.00
8	4.01	7.50	8	4.16	9.00	8	3.56	7.00
9	6.51	9.00	9	5.05	8.00	9	4.25	7.50
10	4.94	9.00	10	4.79	8.50	10	4.70	7.50
11	4.82	8.00	11	4.08	8.00	11	4.99	8.00
12	5.78	9.00	12	5.77	9.00	12	5.96	8.50
13	5.60	8.00	13	3.41	6.00	13	4.80	8.00
14	5.36	9.00	14	5.43	8.50	14	3.46	7.00
15	5.10	8.50	15	5.50	8.50	15	4.97	8.00
100 shrimps m^{-2}; 5 PSU			100 shrimps m^{-2}; 15 PSU			100 shrimps m^{-2}; 25 PSU		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	5.43	8.50	1	6.30	8.00	1	6.22	8.00
2	5.30	7.00	2	5.89	7.50	2	5.14	8.00
3	4.51	8.00	3	7.69	10.00	3	5.93	8.00
4	5.83	8.50	4	4.35	8.00	4	6.88	8.50
5	5.34	8.00	5	4.13	8.50	5	4.15	8.00
6	5.60	8.50	6	5.43	9.00	6	5.00	8.00
7	4.81	8.50	7	5.09	8.50	7	6.32	9.00
8	6.46	9.00	8	5.43	9.50	8	5.98	8.00
9	6.32	9.50	9	4.77	7.50	9	5.72	8.00
10	5.27	9.00	10	4.91	8.00	10	4.46	7.50
11	7.19	9.00	11	5.69	8.50	11	5.09	8.00
12	5.71	9.00	12	4.62	7.00	12	6.99	8.50
13	6.02	9.50	13	5.65	9.00	13	5.52	8.00
14	6.14	9.00	14	4.02	8.00	14	4.18	8.50
15	6.13	8.50	15	6.29	9.00	15	5.83	8.00
2 months of experiment								
50 shrimps m^{-2}; 5 PSU			50 shrimps m^{-2}; 15 PSU			50 shrimps m^{-2}; 25 PSU		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	5.15	9.50	1	12.09	13.00	1	6.16	9.50
2	5.01	9.50	2	5.12	10.00	2	5.63	10.00
3	4.39	9.00	3	5.63	9.00	3	6.17	9.50
4	4.11	8.00	4	4.83	9.00	4	7.29	10.50
5	5.77	9.50	5	8.66	11.00	5	8.05	10.50

No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
6	6.66	10.00	6	2.56	7.00	6	8.05	10.00
7	10.50	8.86	7	6.01	10.00	7	9.04	12.00
8	9.50	6.26	8	6.60	10.50	8	8.62	11.00
9	8.12	10.50	9	8.57	10.50	9	5.50	10.00
10	7.15	10.50	10	3.58	8.00	10	7.88	11.00
100 shrimps m^{-2}; 5 PSU			100 shrimps m^{-2}; 15 PSU			100 shrimps m^{-2}; 25 PSU		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	7.65	10.50	1	5.07	9.50	1	8.10	10.50
2	8.23	11.50	2	4.08	9.00	2	8.07	11.50
3	11.26	12.00	3	6.47	10.00	3	10.88	11.00
4	5.78	9.50	4	9.85	10.50	4	5.44	9.50
5	10.36	13.50	5	5.39	9.50	5	6.33	10.00
6	7.80	10.00	6	6.28	9.50	6	7.97	10.50
7	5.97	9.50	7	9.52	10.50	7	5.16	9.50
8	5.48	5.50	8	4.24	9.00	8	5.23	9.50
9	4.60	9.00	9	5.97	9.50	9	6.36	10.00
10	9.60	11.00	10	8.12	10.50	10	10.08	11.50

Table B-3. Nitrification rate of fibrous Biocord™ biofilter acclimated in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}).

50 shrimps m^{-2}; 5 PSU			50 shrimps m^{-2}; 15 PSU			50 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	50.16	0.94	2	39.58	2.03	2	25.40	6.56
4	67.72	4.23	4	3.7.02	3.15	4	35.26	3.59
6	62.64	5.50	6	21.89	4.11	6	37.41	1.66
8	54.00	0.83	8	18.29	3.63	8	29.53	11.15
Anoxic	32.78	14.72	Anoxic	15.31	3.12	Anoxic	25.62	1.68
100 shrimps m^{-2}; 5 PSU			100 shrimps m^{-2}; 15 PSU			100 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	100.42	5.97	2	43.54	0.53	2	38.99	3.64
4	77.53	7.46	4	95.16	5.52	4	71.34	5.87
6	75.77	6.96	6	28.29	4.41	6	53.38	12.43
8	69.58	4.91	8	20.50	1.19	8	50.49	1.26
Anoxic	31.32	13.89	Anoxic	20.83	2.88	Anoxic	19.89	2.68

Table B-4. Nitrification rate of Japanese filter mat acclimated in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}).

50 shrimps m^{-2}; 5 PSU			50 shrimps m^{-2}; 15 PSU			50 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	62.60	28.21	2	47.61	1.63	2	31.34	4.76
4	75.74	4.17	4	48.65	3.81	4	57.00	21.28
6	144.40	4.37	6	62.41	4.09	6	114.20	19.15

Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
8	114.59	6.31	8	46.53	7.22	8	95.80	3.45
Anoxic	36.90	3.09	Anoxic	34.87	9.40	Anoxic	40.76	5.88
100 shrimps m^{-2}; 5 PSU			100 shrimps m^{-2}; 15 PSU			100 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	103.84	4.57	2	93.85	13.14	2	38.90	4.28
4	85.24	4.37	4	84.65	7.16	4	97.24	2.61
6	145.43	1.17	6	101.51	35.54	6	114.33	3.98
8	128.58	2.69	8	88.89	14.99	8	91.11	4.71
Anoxic	40.44	9.95	Anoxic	26.44	10.46	Anoxic	42.15	13.43

Table B-5. Denitrification rate of fibrous Biocord^{TM} biofilter acclimated in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}).

50 shrimps m^{-2}; 5 PSU			50 shrimps m^{-2}; 15 PSU			50 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	7.26	1.09	2	5.02	1.49	2	2.38	0.76
4	12.06	1.70	4	19.40	1.39	4	0.39	
6	16.04	3.86	6	8.74	1.40	6	0.65	
8	18.49	1.51	8	17.15	3.97	8	6.88	
Anoxic	67.24	11.80	Anoxic	21.93	1.55	Anoxic	10.92	
100 shrimps m^{-2}; 5 PSU			100 shrimps m^{-2}; 15 PSU			100 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	15.08	1.52	2	5.17	0.90	2	10.80	1.96
4	11.46	1.90	4	9.74	1.84	4	9.74	2.02
6	13.69	0.87	6	7.90	1.16	6	9.67	1.90
8	22.21	2.67	8	30.36	4.10	8	17.15	0.71
Anoxic	54.83	12.76	Anoxic	39.72	6.04	Anoxic	81.86	4.40

Table B-6. Denitrification rate of Japanese filter mat acclimated in aquaculture systems varied salinity levels (5, 15 and 25 PSU) and stocking densities (50 and 100 shrimp m^{-2}).

50 shrimps m^{-2}; 5 PSU			50 shrimps m^{-2}; 15 PSU			50 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	17.78	2.09	2	13.40	1.49	2	6.35	2.27
4	24.24	1.12	4	26.63	6.60	4	10.24	0.38
6	42.68	7.27	6	14.41	1.12	6	16.11	3.54
8	36.58	1.37	8	91.58	11.00	8	28.26	4.83
Anoxic	94.21	15.38	Anoxic	100.03	20.14	Anoxic	118.23	16.64
100 shrimps m^{-2}; 5 PSU			100 shrimps m^{-2}; 15 PSU			100 shrimps m^{-2}; 25 PSU		
Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
2	20.19	3.41	2	13.18	3.19	2	8.24	2.14
4	26.81	3.17	4	27.74	3.95	4	20.96	5.07
6	49.44	0.21	6	22.05	3.75	6	31.86	5.87
8	94.09	1.04	8	83.90	7.64	8	34.72	5.13

Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD	Week	Rate (mg-N m^{-2} d^{-1})	SD
Anoxic	165.80	50.17	Anoxic	95.96	8.93	Anoxic	124.37	25.63

Table B-7. Relative abundances (%) of bacterial phyla observed on fibrous Biocord™ biofilter acclimated under low-, medium- and high-salinity levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

Phylum	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
Salinity of 5 PSU						
Others	2.50	4.03	8.37	14.65	0.26	0.10
Acidobacteria	0.00	0.00	0.00	0.09	0.00	0.00
Actinobacteria	0.30	1.02	7.56	5.81	0.01	0.01
Bacteroidetes	72.56	40.71	33.62	21.10	3.62	3.23
Caldithrix	0.00	0.00	0.00	0.00	0.00	0.00
Chloroflexi	0.01	0.08	1.16	3.46	0.01	0.00
Cyanobacteria	0.00	0.00	0.84	0.41	0.01	0.00
Dependentiae	0.00	0.00	0.08	0.03	0.00	0.01
Firmicutes	0.01	0.04	0.21	0.33	0.00	0.02
Gemmatimonadetes	0.00	0.05	2.17	4.20	0.01	0.01
Nitrospirae	0.00	0.01	1.07	1.97	0.00	0.00
Planctomycetes	0.61	2.15	2.81	2.79	0.00	0.01
Proteobacteria	23.50	51.20	41.26	44.68	95.72	96.14
Saccharibacteria	0.00	0.00	0.42	0.00	0.36	0.22
Verrucomicrobia	0.51	0.73	0.42	0.49	0.01	0.24
	100	100	100	100	100	100
Phylum	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
Salinity of 15 PSU						
Others	0.52	0.70	7.73	5.44	0.03	0.54
Acidobacteria	0.02	0.14	0.37	1.45	0.01	0.03
Actinobacteria	0.23	0.29	5.16	10.56	0.01	0.24
Bacteroidetes	26.95	30.76	25.26	31.66	3.33	8.33
Caldithrix	0.00	0.00	0.05	0.03	0.00	0.00
Chloroflexi	0.04	0.02	1.22	1.96	0.00	0.07
Cyanobacteria	0.00	0.00	1.66	0.70	0.00	0.01
Dependentiae	0.00	0.00	0.25	1.48	0.00	0.38
Firmicutes	0.01	0.03	0.29	2.26	0.00	0.12
Gemmatimonadetes	0.01	0.01	1.74	2.14	0.00	0.01
Nitrospirae	0.00	0.00	0.71	1.49	0.00	0.04
Planctomycetes	0.15	0.41	1.21	3.29	0.00	0.01
Proteobacteria	71.98	66.94	53.13	36.06	96.60	90.17
Saccharibacteria	0.01	0.00	0.49	0.54	0.02	0.05
Verrucomicrobia	0.09	0.70	0.75	0.93	0.00	0.02
	100	100	100	100	100	100
Phylum	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
Salinity of 25 PSU						
Others	1.13	2.02	5.83	12.02	0.46	1.24
Acidobacteria	0.00	0.00	0.37	0.35	0.04	0.32
Actinobacteria	0.18	0.66	13.94	7.89	0.00	1.34
Bacteroidetes	14.12	17.59	15.93	23.74	2.05	16.82
Caldithrix	0.02	1.04	4.73	4.23	0.01	0.03
Chloroflexi	0.01	0.04	2.59	4.62	0.00	0.17

Phylum	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
Cyanobacteria	0.00	0.00	0.49	1.35	0.00	0.11
Dependentiae	0.01	0.06	0.91	0.66	0.00	0.16
Firmicutes	0.16	0.20	5.80	3.24	0.00	4.48
Gemmatimonadetes	0.14	0.51	1.76	1.71	0.00	0.00
Nitrospirae	0.00	0.00	0.73	1.70	0.00	0.02
Planctomycetes	0.24	1.34	0.61	1.79	0.00	0.03
Proteobacteria	83.47	76.36	45.03	34.44	97.45	75.09
Saccharibacteria	0.00	0.00	0.88	1.09	0.00	0.17
Verrucomicrobia	0.51	0.18	0.40	1.15	0.00	0.01
	100	100	100	100	100	100

Table B-8. Relative abundances (%) of bacterial class observed on fibrous Biocord™ biofilter acclimated under low-, medium- and high-salinity levels in semi-intensive (S; 50 shrimp m^{-2}) and intensive (I; 100 shrimp m^{-2}) aquaculture systems during biofilter acclimation with aerobic shrimp cultivation (1st and 8th weeks), followed by anoxic denitrification.

Class	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
Salinity of 5 PSU						
Other	2.62	5.38	10.57	15.99	0.62	0.59
Sva0725	0.00	0.00	0.00	0.00	0.00	0.00
Acidimicrobiia	0.04	0.10	3.00	1.19	0.00	0.01
Actinobacteria	0.26	0.92	4.24	4.55	0.01	0.00
Cytophagia	1.88	1.02	8.54	5.81	0.20	0.02
Flavobacteriia	25.71	30.12	18.77	11.72	3.03	3.20
Sphingobacteriia	1.30	0.16	0.33	0.10	0.00	0.00
[Saprospirae]	43.55	8.36	5.69	3.35	0.38	0.01
Caldithrixae	0.00	0.00	0.00	0.00	0.00	0.00
Anaerolineae	0.01	0.08	1.16	3.24	0.01	0.00
4C0d-2	0.00	0.00	0.84	0.41	0.01	0.00
SJA-4	0.00	0.00	0.08	0.03	0.00	0.00
Clostridia	0.01	0.04	0.21	0.33	0.00	0.02
Gemm-1	0.00	0.04	0.67	1.32	0.01	0.01
Gemm-2	0.00	0.01	1.50	2.88	0.00	0.00
Nitrospira	0.00	0.01	1.07	1.97	0.00	0.00
028H05-P-BN-P5	0.56	1.75	0.00	0.10	0.00	0.00
OM190	0.02	0.04	1.97	1.70	0.00	0.00
Planctomycetia	0.03	0.06	0.47	0.48	0.00	0.00
Alphaproteobacteria	12.85	30.99	22.02	15.83	3.05	9.69
Betaproteobacteria	4.30	8.13	8.00	2.39	68.32	79.03
Deltaproteobacteria	1.88	2.04	4.62	8.68	0.33	0.05
Epsilonproteobacteria	0.00	0.07	0.01	0.02	0.38	0.18
Gammaproteobacteria	4.46	9.96	5.81	17.49	23.63	7.19
Verrucomicrobiae	0.51	0.71	0.42	0.42	0.01	0.00
	100	100	100	100	100	100
Class	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
Salinity of 15 PSU						
Other	0.85	1.13	10.06	9.14	0.06	1.06
Sva0725	0.00	0.00	0.11	1.17	0.00	0.00
Acidimicrobiia	0.13	0.18	2.43	5.15	0.00	0.09
Actinobacteria	0.11	0.11	2.71	5.33	0.01	0.14
Cytophagia	0.11	0.28	1.64	1.88	0.00	0.06
Flavobacteriia	25.32	28.48	18.93	20.62	3.28	8.21
Sphingobacteriia	0.00	0.05	0.16	0.51	0.00	0.00

Class	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
[Saprospirae]	1.22	1.79	4.03	7.43	0.06	0.05
Caldithrixae	0.00	0.00	0.05	0.03	0.00	0.00
Anaerolineae	0.04	0.02	1.22	1.70	0.00	0.06
4C0d-2	0.00	0.00	1.66	0.70	0.00	0.01
SJA-4	0.00	0.00	0.25	1.48	0.00	0.02
Clostridia	0.01	0.03	0.29	2.26	0.00	0.12
Gemm-1	0.00	0.00	0.01	0.00	0.00	0.00
Gemm-2	0.01	0.01	1.73	2.14	0.00	0.01
Nitrospira	0.00	0.00	0.71	1.49	0.00	0.04
028H05-P-BN-P5	0.00	0.03	0.00	0.02	0.00	0.00
OM190	0.01	0.05	0.10	0.86	0.00	0.00
Planctomycetia	0.14	0.21	0.57	1.29	0.00	0.01
Alphaproteobacteria	28.23	23.76	21.41	15.95	7.17	7.87
Betaproteobacteria	0.45	0.77	0.52	1.01	1.14	3.49
Deltaproteobacteria	34.38	36.64	20.41	3.98	0.10	4.29
Epsilonproteobacteria	0.00	0.00	0.01	0.03	0.03	0.06
Gammaproteobacteria	8.93	5.77	10.25	14.94	88.16	74.42
Verrucomicrobiae	0.09	0.69	0.75	0.87	0.00	0.00
	100	100	100	100	100	100
Class	S-1wk	I-1wk	S-8wk	I-8wk	S-anox	I-anox
Salinity of 25 PSU						
Other	2.45	3.77	8.12	14.78	0.49	1.77
Sva0725	0.00	0.00	0.00	0.00	0.00	0.00
Acidimicrobiia	0.18	0.43	4.35	4.60	0.00	0.36
Actinobacteria	0.00	0.23	9.59	3.29	0.00	0.98
Cytophagia	0.01	0.05	5.94	1.14	0.00	0.01
Flavobacteriia	12.23	11.14	5.07	11.65	1.97	16.34
Sphingobacteriia	0.00	0.02	0.02	0.36	0.00	0.00
[Saprospirae]	1.37	5.33	4.65	9.72	0.08	0.46
Caldithrixae	0.02	1.04	4.73	4.23	0.01	0.03
Anaerolineae	0.01	0.04	2.59	4.62	0.00	0.17
4C0d-2	0.00	0.00	0.49	1.35	0.00	0.11
SJA-4	0.01	0.06	0.91	0.66	0.00	0.16
Clostridia	0.16	0.20	5.80	3.24	0.00	4.48
Gemm-1	0.00	0.00	0.00	0.00	0.00	0.00
Gemm-2	0.14	0.51	1.76	1.71	0.00	0.00
Nitrospira	0.00	0.00	0.73	1.70	0.00	0.02
028H05-P-BN-P5	0.00	0.01	0.00	0.00	0.00	0.00
OM190	0.00	0.61	0.05	0.03	0.00	0.01
Planctomycetia	0.24	0.17	0.14	1.43	0.00	0.01
Alphaproteobacteria	26.82	17.81	24.31	17.98	2.84	8.41
Betaproteobacteria	0.29	0.73	0.67	0.96	0.50	0.66
Deltaproteobacteria	51.95	52.48	11.24	7.79	0.41	0.24
Epsilonproteobacteria	0.00	0.00	0.00	0.05	0.00	1.33
Gammaproteobacteria	3.61	5.18	8.44	7.58	93.70	64.46
Verrucomicrobiae	0.51	0.18	0.40	1.14	0.00	0.01
	100	100	100	100	100	100

Appendix C

Data results in the study 2: application of biofilter in marine RAS for long-term operation.

Table C-1. Ammonia nitrite and nitrate concentration in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

Date/Time	Day	Ammonia (mg-N L^{-1})		Nitrite (mg-N L^{-1})		Nitrate (mg-N L^{-1})	
		Average	SD	Average	SD	Average	SD
Biofilter acclimation							
8/27/2018 10:00	0	0.56	0.43	0.08	0.03	5.59	0.31
8/28/2018 10:00	1	0.72	0.16	0.08	0.02	5.83	0.16
8/29/2018 10:00	2	1.05	0.23	0.07	0.01	5.55	0.54
8/30/2018 10:00	3	1.36	0.17	0.07	0.01	5.97	1.40
8/31/2018 10:00	4	1.70	0.45	0.08	0.01	5.50	0.82
9/1/2018 10:00	5	2.20	1.24	0.09	0.00	5.20	0.56
9/2/2018 10:00	6	2.60	1.70	0.12	0.03	5.14	0.36
9/3/2018 10:00	7	1.13	0.69	0.07	0.01	2.31	0.32
9/4/2018 10:00	8	1.52	0.93	0.18	0.01	1.88	0.18
9/5/2018 10:00	9	2.15	1.45	0.41	0.08	2.28	0.38
9/6/2018 10:00	10	2.92	0.04	0.59	0.05	2.05	0.15
9/7/2018 10:00	11	1.24	0.07	0.46	0.11	1.27	0.24
9/8/2018 10:00	12	0.51	0.18	1.12	0.27	1.23	0.51
9/9/2018 10:00	13	0.21	0.06	2.48	0.23	0.97	0.58
9/10/2018 10:00	14	0.16	0.03	3.19	0.39	1.55	1.19
9/11/2018 10:00	15	0.18	0.02	3.84	0.69	2.18	0.83
9/12/2018 10:00	16	0.20	0.07	4.55	0.38	1.73	0.31
9/13/2018 10:00	17	0.23	0.10	5.08	0.15	2.17	1.23
9/14/2018 10:00	18	0.20	0.14	4.44	1.63	2.41	1.04
9/15/2018 10:00	19	0.21	0.13	4.20	1.27	0.31	1.47
9/16/2018 10:00	20	0.28	0.19	5.15	0.32	2.05	2.40
9/17/2018 10:00	21	0.10	0.04	4.72	0.09	1.56	1.83
9/18/2018 10:00	22	0.20	0.07	4.47	0.12	1.33	1.45
9/19/2018 10:00	23	0.23	0.09	3.87	0.53	1.16	0.83
9/20/2018 10:00	24	0.27	0.10	4.07	1.80	1.35	0.61
9/21/2018 10:00	25	0.36	0.17	4.48	1.31	1.89	0.78
9/22/2018 10:00	26	0.71	0.54	4.61	1.13	3.89	0.47
9/23/2018 10:00	27	1.04	0.86	4.84	0.33	3.86	0.38
9/24/2018 10:00	28	0.86	0.61	3.84	1.84	2.84	0.13
9/25/2018 10:00	29	0.49	0.12	3.84	1.71	3.69	1.02
9/26/2018 10:00	30	0.35	0.10	3.79	1.46	4.90	2.86
9/27/2018 10:00	31	0.35	0.13	4.28	0.78	5.16	2.13
9/28/2018 10:00	32	0.34	0.23	4.63	0.20	5.52	2.70
9/29/2018 10:00	33	0.31	0.19	4.88	0.41	4.60	3.49

Date/Time	Day	Ammonia (mg-N L^{-1})		Nitrite (mg-N L^{-1})		Nitrate (mg-N L^{-1})	
		Average	SD	Average	SD	Average	SD
9/30/2018 10:00	34	0.17	0.08	5.53	0.24	5.26	2.93
10/1/2018 10:00	35	0.01	0.01	6.51	0.32	6.09	2.19
10/2/2018 10:00	36	0.07	0.02	6.76	0.23	7.63	2.42
10/3/2018 10:00	37	0.06	0.04	6.96	0.39	9.28	2.70
10/4/2018 10:00	38	0.08	0.03	6.55	0.28	14.42	3.28
10/5/2018 10:00	39	0.08	0.06	6.09	0.22	18.79	2.23
10/6/2018 10:00	40	0.07	0.03	6.42	0.04	8.80	1.11
10/7/2018 10:00	41	0.05	0.04	6.95	0.15	-0.72	0.28
10/8/2018 10:00	42	0.06	0.04	6.48	0.56	1.09	1.59
10/9/2018 10:00	43	0.05	0.03	6.38	0.47	2.52	1.47
10/10/2018 10:00	44	0.04	0.02	6.41	0.30	0.87	0.59
10/11/2018 10:00	45	0.08	0.01	6.62	0.25	0.66	0.98
10/12/2018 10:00	46	0.05	0.02	6.79	0.25	0.25	0.68
10/13/2018 10:00	47	0.06	0.03	5.76	0.69	6.55	1.80
10/14/2018 10:00	48	0.07	0.02	6.06	0.59	5.31	2.26
10/15/2018 10:00	49	0.07	0.03	6.56	0.28	2.85	0.88
10/16/2018 10:00	50	0.06	0.03	5.49	0.95	-0.09	3.01
10/17/2018 10:00	51	0.04	0.03	4.68	1.88	0.07	2.14
10/18/2018 10:00	52	0.07	0.02	5.14	1.76	7.16	6.18
10/19/2018 10:00	53	0.10	0.01	2.42	0.97	9.58	5.07
10/20/2018 10:00	54	0.12	0.01	0.93	0.73	11.59	3.15
10/21/2018 10:00	55	0.14	0.02	0.77	0.49	13.27	4.68
10/22/2018 10:00	56	0.14	0.05	0.30	0.13	16.10	7.10
10/23/2018 10:00	57	0.11	0.03	0.24	0.21	16.21	6.76
10/24/2018 10:00	58	0.07	0.03	0.22	0.07	16.34	7.65
10/25/2018 10:00	59	0.06	0.02	0.07	0.07	17.46	8.72
Shrimp cultivation #1							
10/26/2018 10:00	60	0.06	0.03	0.01	0.01	1.89	0.18
10/27/2018 10:00	61	0.07	0.03	0.04	0.04	2.70	0.62
10/28/2018 10:00	62	0.05	0.04	0.01	0.01	2.70	0.73
10/29/2018 10:00	63	0.11	0.01	0.02	0.01	2.91	0.66
10/30/2018 10:00	64	0.13	0.03	0.05	0.02	2.86	0.81
10/31/2018 10:00	65	0.29	0.09	0.10	0.06	2.74	1.04
11/1/2018 10:00	66	0.45	0.20	0.19	0.03	3.13	0.61
11/2/2018 10:00	67	0.54	0.32	0.32	0.11	3.98	0.53
11/3/2018 10:00	68	0.43	0.21	0.23	0.11	3.97	0.64
11/4/2018 10:00	69	0.23	0.07	0.19	0.12	3.66	0.83
11/5/2018 10:00	70	0.20	0.06	0.18	0.14	4.92	0.13
11/6/2018 10:00	71	0.21	0.04	0.17	0.15	7.80	0.86
11/7/2018 10:00	72	0.24	0.06	0.24	0.24	9.23	0.58
11/8/2018 10:00	73	0.21	0.05	0.28	0.28	10.38	0.65
11/9/2018 10:00	74	0.21	0.07	0.26	0.25	11.55	1.58
11/10/2018 10:00	75	0.22	0.06	0.28	0.25	13.11	3.50
11/11/2018 10:00	76	0.16	0.11	0.24	0.24	12.89	1.35

Date/Time	Day	Ammonia (mg-N L^{-1})		Nitrite (mg-N L^{-1})		Nitrate (mg-N L^{-1})	
		Average	SD	Average	SD	Average	SD
11/12/2018 10:00	77	0.20	0.13	0.17	0.19	14.20	0.94
11/13/2018 10:00	78	0.21	0.11	0.26	0.25	14.05	0.88
11/14/2018 10:00	79	0.22	0.14	0.35	0.32	14.40	0.91
11/15/2018 10:00	80	0.27	0.08	0.22	0.06	14.52	3.04
11/16/2018 10:00	81	0.20	0.02	0.17	0.05	14.29	6.47
11/17/2018 10:00	82	0.20	0.01	0.16	0.03	15.67	4.35
11/18/2018 10:00	83	0.19	0.02	0.15	0.02	16.15	3.47
11/19/2018 10:00	84	0.18	0.02	0.15	0.01	16.93	3.01
11/20/2018 10:00	85	0.20	0.02	0.19	0.01	17.90	2.27
11/21/2018 10:00	86	0.21	0.03	0.21	0.02	20.39	1.48
11/22/2018 10:00	87	0.23	0.01	0.43	0.04	23.36	0.45
11/23/2018 10:00	88	0.24	0.02	0.39	0.05	21.92	0.96
11/24/2018 10:00	89	0.24	0.04	0.30	0.05	19.72	0.11
11/25/2018 10:00	90	0.23	0.07	0.18	0.03	18.07	0.23
11/26/2018 10:00	91	0.21	0.09	0.17	0.03	16.52	1.97
11/27/2018 10:00	92	0.21	0.09	0.26	0.04	22.71	2.77
11/28/2018 10:00	93	0.20	0.10	0.36	0.09	26.46	6.38
11/29/2018 10:00	94	0.16	0.07	0.18	0.02	22.26	5.70
11/30/2018 10:00	95	0.21	0.12	0.20	0.09	19.41	2.50
12/1/2018 10:00	96	0.25	0.15	0.45	0.35	21.34	4.72
12/2/2018 10:00	97	0.12	0.06	0.26	0.18	19.93	3.57
12/3/2018 10:00	98	0.09	0.03	0.14	0.08	21.71	2.42
12/4/2018 10:00	99	0.11	0.06	0.22	0.08	27.18	2.03
12/5/2018 10:00	100	0.11	0.08	0.29	0.12	32.37	2.03
12/6/2018 10:00	101	0.11	0.05	0.18	0.05	24.08	3.58
12/7/2018 10:00	102	0.11	0.03	0.09	0.04	13.03	1.60
12/8/2018 10:00	103	0.12	0.01	0.06	0.01	18.48	4.91
12/9/2018 10:00	104	0.12	0.02	0.07	0.01	22.56	6.57
12/10/2018 10:00	105	0.12	0.03	0.09	0.04	25.92	6.47
12/11/2018 10:00	106	0.12	0.02	0.10	0.05	24.93	0.36
12/12/2018 10:00	107	0.12	0.03	0.11	0.07	21.40	5.99
12/13/2018 10:00	108	0.12	0.02	0.08	0.04	23.43	5.67
12/14/2018 10:00	109	0.11	0.01	0.07	0.05	26.45	6.44
12/15/2018 10:00	110	0.10	0.01	0.02	0.01	23.66	2.07
12/16/2018 10:00	111	0.10	0.01	0.02	0.01	24.63	0.19
12/17/2018 10:00	112	0.09	0.01	0.01	0.00	25.40	2.11
12/18/2018 10:00	113	0.11	0.01	0.07	0.01	31.63	0.40
12/19/2018 10:00	114	0.13	0.02	0.15	0.04	38.46	6.13
12/20/2018 10:00	115	0.14	0.02	0.23	0.01	52.47	7.01
12/21/2018 10:00	116	0.12	0.02	0.06	0.04	53.19	4.41
12/22/2018 10:00	117	0.12	0.03	0.06	0.04	54.32	1.68
12/23/2018 10:00	118	0.10	0.03	0.04	0.03	54.10	1.36
12/24/2018 10:00	119	0.09	0.04	0.03	0.03	54.82	1.59
12/25/2018 10:00	120	0.07	0.06	0.00	0.00	54.72	0.61

Date/Time	Day	Ammonia (mg-N L^{-1})		Nitrite (mg-N L^{-1})		Nitrate (mg-N L^{-1})	
		Average	SD	Average	SD	Average	SD
12/26/2018 10:00	121	0.32	0.06	0.01	0.00	62.09	1.78
12/27/2018 10:00	122	0.24	0.03	0.05	0.01	62.55	2.89
12/28/2018 10:00	123	0.24	0.11	0.66	0.12	52.76	1.84
12/29/2018 10:00	124	0.33	0.07	0.51	0.16	49.43	3.55
12/30/2018 10:00	125	0.36	0.19	0.41	0.10	37.75	7.87
12/31/2018 10:00	126	0.05	0.01	0.26	0.10	24.30	8.67
1/1/2019 10:00	127	0.04	0.06	0.14	0.11	5.91	3.80
Shrimp cultivation #2							
1/2/2019 10:00	128	0.07	0.10	0.02	0.02	2.18	0.09
1/3/2019 10:00	129	0.03	0.02	0.00	0.00	1.75	0.07
1/4/2019 10:00	130	0.00	0.00	0.00	0.00	1.43	0.23
1/5/2019 10:00	131	0.15	0.10	0.02	0.01	1.79	0.12
1/6/2019 10:00	132	0.22	0.10	0.04	0.02	1.72	0.31
1/7/2019 10:00	133	0.27	0.11	0.07	0.04	2.10	0.40
1/8/2019 10:00	134	0.41	0.06	0.30	0.05	5.82	0.29
1/9/2019 10:00	135	0.24	0.01	0.13	0.04	3.67	0.52
1/10/2019 10:00	136	0.23	0.01	0.10	0.03	4.46	0.26
1/11/2019 10:00	137	0.21	0.00	0.08	0.02	5.52	0.30
1/12/2019 10:00	138	0.20	0.01	0.06	0.01	6.23	0.69
1/13/2019 10:00	139	0.16	0.02	0.05	0.01	7.37	0.79
1/14/2019 10:00	140	0.12	0.03	0.04	0.01	8.23	1.05
1/15/2019 10:00	141	0.13	0.03	0.04	0.01	9.75	1.74
1/16/2019 10:00	142	0.14	0.04	0.04	0.02	11.38	2.91
1/17/2019 10:00	143	0.13	0.05	0.08	0.04	17.34	1.19
1/18/2019 10:00	144	0.12	0.05	0.13	0.06	21.28	0.14
1/19/2019 10:00	145	0.14	0.05	0.13	0.05	20.48	0.29
1/20/2019 10:00	146	0.15	0.03	0.12	0.05	19.62	0.75
1/21/2019 10:00	147	0.17	0.03	0.12	0.04	18.30	1.79
1/22/2019 10:00	148	0.18	0.02	0.12	0.03	17.18	2.35
1/23/2019 10:00	149	0.22	0.05	0.20	0.02	16.48	0.92
1/24/2019 10:00	150	0.25	0.09	0.31	0.09	15.21	0.73
1/25/2019 10:00	151	0.22	0.06	0.24	0.05	16.09	0.17
1/26/2019 10:00	152	0.20	0.05	0.19	0.05	16.67	0.81
1/27/2019 10:00	153	0.17	0.03	0.13	0.03	18.02	2.14
1/28/2019 10:00	154	0.17	0.04	0.10	0.03	22.59	1.88
1/29/2019 10:00	155	0.20	0.04	0.18	0.06	24.08	2.33
1/30/2019 10:00	156	0.28	0.11	0.28	0.13	25.39	2.77
1/31/2019 10:00	157	0.36	0.19	0.33	0.22	22.22	1.59
2/1/2019 10:00	158	0.38	0.21	0.40	0.35	20.02	0.59
2/2/2019 10:00	159	0.21	0.04	0.35	0.31	21.72	1.06
2/3/2019 10:00	160	0.16	0.01	0.29	0.27	24.19	3.35
2/4/2019 10:00	161	0.11	0.02	0.29	0.23	27.94	2.35
2/5/2019 10:00	162	0.06	0.04	0.29	0.21	32.14	2.55
2/6/2019 10:00	163	0.08	0.02	0.30	0.21	29.67	3.41

Date/Time	Day	Ammonia (mg-N L^{-1})		Nitrite (mg-N L^{-1})		Nitrate (mg-N L^{-1})	
		Average	SD	Average	SD	Average	SD
2/7/2019 10:00	164	0.09	0.00	0.27	0.18	29.21	0.93
2/8/2019 10:00	165	0.09	0.00	0.18	0.10	30.15	0.51
2/9/2019 10:00	166	0.10	0.01	0.12	0.06	30.87	1.07
2/10/2019 10:00	167	0.10	0.01	0.05	0.02	31.44	0.96
2/11/2019 10:00	168	0.11	0.02	0.03	0.01	31.91	2.14
2/12/2019 10:00	169	0.14	0.03	0.03	0.01	34.12	1.43
2/13/2019 10:00	170	0.16	0.04	0.03	0.01	35.79	4.21
2/14/2019 10:00	171	0.14	0.03	0.03	0.01	36.91	0.33
2/15/2019 10:00	172	0.13	0.02	0.03	0.01	37.52	0.65
2/16/2019 10:00	173	0.11	0.02	0.02	0.01	39.59	0.55
2/17/2019 10:00	174	0.09	0.01	0.02	0.01	42.06	1.86
2/18/2019 10:00	175	0.08	0.01	0.02	0.01	41.04	1.63
2/19/2019 10:00	176	0.09	0.00	0.03	0.01	43.74	5.59
2/20/2019 10:00	177	0.07	0.01	0.03	0.01	45.32	4.14
2/21/2019 10:00	178	0.07	0.02	0.04	0.01	48.06	4.81
2/22/2019 10:00	179	0.00	0.01	0.08	0.01	57.85	1.91
2/23/2019 10:00	180	0.09	0.02	0.07	0.00	61.54	4.77
2/24/2019 10:00	181	0.20	0.06	0.02	0.02	69.62	0.81
2/25/2019 10:00	182	0.32	0.26	0.03	0.01	73.25	2.61
2/26/2019 10:00	183	0.36	0.14	0.09	0.03	71.38	2.38
2/27/2019 10:00	184	0.76	0.13	0.07	0.01	69.32	1.10
2/28/2019 10:00	185	0.76	0.09	0.12	0.01	65.14	5.57
3/1/2019 10:00	186	0.63	0.02	0.20	0.01	65.11	3.23
3/2/2019 10:00	187	0.48	0.03	0.29	0.05	40.58	1.14
3/3/2019 10:00	188	0.36	0.03	0.48	0.10	27.12	5.18
3/4/2019 10:00	189	0.02	0.03	0.67	0.18	8.39	1.83
Shrimp cultivation #3							
3/5/2019 10:00	190	0.12	0.02	0.05	0.01	3.58	0.07
3/6/2019 10:00	191	0.03	0.01	0.07	0.00	5.16	0.59
3/7/2019 10:00	192	0.05	0.01	0.10	0.01	6.02	0.29
3/8/2019 10:00	193	0.03	0.01	0.08	0.01	4.23	0.87
3/9/2019 10:00	194	0.07	0.04	0.06	0.01	3.73	0.58
3/10/2019 10:00	195	0.10	0.08	0.05	0.01	4.19	0.43
3/11/2019 10:00	196	0.14	0.12	0.03	0.00	4.66	0.35
3/12/2019 10:00	197	0.31	0.09	0.04	0.01	5.91	0.68
3/13/2019 10:00	198	0.40	0.15	0.04	0.01	6.43	1.15
3/14/2019 10:00	199	0.13	0.05	0.03	0.01	8.99	1.45
3/15/2019 10:00	200	0.07	0.09	0.02	0.00	10.40	1.88
3/16/2019 10:00	201	0.05	0.02	0.02	0.00	9.93	1.12
3/17/2019 10:00	202	0.10	0.03	0.03	0.01	8.65	1.21
3/18/2019 10:00	203	0.20	0.09	0.03	0.01	8.13	0.59
3/19/2019 10:00	204	0.09	0.05	0.03	0.01	8.69	0.44
3/20/2019 10:00	205	0.11	0.05	0.02	0.01	10.14	0.20
3/21/2019 10:00	206	0.10	0.06	0.01	0.00	11.31	0.60

Date/Time	Day	Ammonia (mg-N L^{-1})		Nitrite (mg-N L^{-1})		Nitrate (mg-N L^{-1})	
		Average	SD	Average	SD	Average	SD
3/22/2019 10:00	207	0.15	0.11	0.01	0.00	11.41	0.13
3/23/2019 10:00	208	0.09	0.04	0.01	0.00	11.79	0.16
3/24/2019 10:00	209	0.07	0.01	0.01	0.00	12.91	0.12
3/25/2019 10:00	210	0.06	0.03	0.01	0.00	14.69	0.82

Table C-2. Water quality parameters in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

Date/Time	Day	DO (mg L^{-1})		Temp (°C)		pH		Alkalinity (mg-$CaCO_3$ L^{-1})		ORP-water (mV)		ORP-biofilter (mV)		SS (mg L^{-1})		COD (mg L^{-1})	
		Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Biofilter acclimation																	
8/27/2018 10:00	0	8.5	0.0	26.6	0.1	8.02	0.01	195.0	7.1	118.9	7.0	53.3	0.7	0.00	0.00		
8/29/2018 10:00	2	8.2	0.1	26.4	0.1	7.96	0.03	190.0	0.0	103.7	1.9	33.4	14.8				
8/31/2018 10:00	4	8.2	0.3	27.1	0.1	7.93	0.04	180.0	0.0	109.9	3.4	49.1	32.9				
9/2/2018 10:00	6	8.6	0.1	27.1	0.2	7.91	0.02	150.0	0.0	158.2	0.5	39.3	23.5				
9/4/2018 10:00	8	8.7	0.1	27.3	0.0	7.93	0.03	150.0	0.0	138.9	0.4	40.7	2.9	7.80	1.56		
9/6/2018 10:00	10	8.7	0.2	26.7	0.1	7.82	0.04	145.0	7.1	119.7	1.1	35.6	4.3				
9/8/2018 10:00	12	8.6	0.3	26.9	0.4	7.73	0.03	150.0	0.0	119.7	2.6	45.0	5.2				
9/10/2018 10:00	14	8.6	0.5	27.4	0.1	7.65	0.02	110.0	0.0	111.1	6.2	43.6	0.8	53.00	22.63		
9/12/2018 10:00	16	8.5	0.1	27.5	0.0	7.64	0.03	150.0	0.0	117.3	5.7	37.4	17.4				
9/14/2018 10:00	18	8.8	0.0	26.4	0.1	7.65	0.00	130.0	14.1	122.0	1.9	28.9	5.4				
9/16/2018 10:00	20	8.7	0.1	27.0	0.1	7.70	0.06	105.0	21.2	120.2	11.9	37.1	8.1				
9/18/2018 10:00	22	8.4	0.1	27.4	0.1	7.62	0.04	150.0	0.0	119.7	1.1	20.0	30.8	42.25	4.60		
9/20/2018 10:00	24	8.3	0.3	27.5	0.4	7.56	0.06	150.0	0.0	131.4	39.2	17.4	42.3				
9/22/2018 10:00	26	8.3	0.4	27.6	0.1	7.57	0.07	145.0	7.1	147.7	0.1	-16.5	5.9				
9/24/2018 10:00	28	8.3	0.3	27.7	0.1	7.73	0.00	140.0	14.1	134.3	7.6	23.4	13.4	72.50	35.36		
9/26/2018 10:00	30	8.5	0.4	27.8	0.0	7.61	0.04	130.0	14.1	127.7	3.5	19.9	0.6				
9/28/2018 10:00	32	8.4	0.2	28.4	0.1	7.76	0.00	125.0	35.4	111.7	1.4	-3.3	13.3				
9/30/2018 10:00	34	8.6	0.3	27.4	0.2	7.64	0.24	145.0	7.1	108.7	3.5	-27.1	3.7				
10/2/2018 10:00	36	8.7	0.2	27.4	0.2	7.85	0.01	130.0	0.0	112.8	7.1	-15.0	27.3	68.00	14.85		
10/4/2018 10:00	38	8.5	0.5	27.1	0.1	7.66	0.28	115.0	7.1	108.8	11.3	4.6	1.0				
10/6/2018 10:00	40	8.1	0.1	27.1	0.0	7.43	0.15	110.0	0.0	101.7	30.1	-2.7	6.3				
10/8/2018 10:00	42	8.8	0.0	27.3	0.1	7.48	0.04	130.0	28.3	122.4	2.1	18.4	8.6	123.50	28.28		

Date/Time	Day	DO (mg L^{-1})		Temp (°C)		pH		Alkalinity (mg-$CaCO_3$ L^{-1})		ORP-water (mV)		ORP-biofilter (mV)		SS (mg L^{-1})		COD (mg L^{-1})	
		Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
10/10/2018 10:00	44	8.3	0.1	26.9	0.1	7.63	0.03	145.0	7.1	103.9	0.6	48.2	4.4				
10/12/2018 10:00	46	8.7	0.3	28.1	0.1	7.56	0.01	125.0	7.1	130.2	1.3	44.9	10.2				
10/14/2018 10:00	48	9.0	0.0	28.1	0.1	7.66	0.04	120.0	14.1	105.3	3.4	62.7	14.9				
10/16/2018 10:00	50	8.6	0.4	28.3	0.0	7.74	0.12	145.0	7.1	120.5	21.4	45.8	31.5	134.00	5.66		
10/18/2018 10:00	52	8.5	0.6	28.3	0.1	7.81	0.08	145.0	7.1	126.7	35.2	55.2	2.5				
10/20/2018 10:00	54	8.3	0.2	27.2	0.1	7.83	0.05	120.0	0.0	126.4	16.2	49.4	6.4				
10/22/2018 10:00	56	8.4	0.0	26.9	0.1	7.84	0.04	110.0	0.0	117.7	3.5	65.2	8.6	108.50	31.82		
10/24/2018 10:00	58	8.6	0.1	27.5	0.0	7.88	0.02	105.0	7.1	149.9	4.3	47.2	7.4				
Shrimp cultivation #1																	
10/26/2018 10:00	60	8.3	0.2	28.1	0.1	7.94	0.01	150.0	0.0	138.7	9.3	47.9	10.8				
10/28/2018 10:00	62	8.4	0.6	28.0	0.0	8.01	0.01	140.0	0.0	134.3	31.7	48.2	13.6				
10/30/2018 10:00	64	8.6	0.4	27.9	0.0	8.03	0.00	140.0	0.0	125.9	24.0	22.3	4.4	16.25	2.83		
11/1/2018 10:00	66	8.4	0.1	26.3	0.0	7.77	0.02	140.0	0.0	127.6	30.3	33.5	25.7				
11/3/2018 10:00	68	8.7	0.2	25.9	0.1	7.70	0.01	140.0	0.0	122.5	9.8	33.0	28.6				
11/5/2018 10:00	70	8.9	0.4	26.7	0.1	7.75	0.03	140.0	14.1	114.9	10.0	46.8	19.1	107.50	24.75		
11/7/2018 10:00	72	8.1	0.1	28.0	0.1	7.62	0.00	135.0	21.2	133.8	6.9	47.2	6.2				
11/9/2018 10:00	74	8.8	0.2	28.0	0.1	7.66	0.01	135.0	21.2	121.8	14.9	39.6	22.3				
11/11/2018 10:00	76	8.5	0.3	28.1	0.0	7.72	0.01	130.0	0.0	117.5	1.6	43.9	11.7				
11/13/2018 10:00	78	9.0	0.1	27.9	0.1	7.70	0.01	155.0	7.1	127.4	24.5	42.2	0.2	143.50	0.00		
11/15/2018 10:00	80	8.9	0.3	28.7	0.1	7.62	0.01	145.0	7.1	104.1	6.8	44.3	15.4				
11/17/2018 10:00	82	8.5	0.0	28.9	0.2	7.57	0.07	135.0	7.1	120.0	24.9	34.2	8.0				
11/19/2018 10:00	84	8.4	0.1	27.9	0.1	7.57	0.06	145.0	7.1	128.9	19.9	48.3	8.6	156.75	6.72		
11/21/2018 10:00	86	8.9	0.3	28.1	0.0	7.48	0.07	125.0	7.1	118.3	7.3	44.3	4.0				
11/23/2018 10:00	88	8.5	0.2	28.0	0.1	7.51	0.07	150.0	0.0	124.4	20.9	35.1	4.5				
11/25/2018 10:00	90	8.5	0.4	27.5	0.0	7.50	0.02	130.0	0.0	125.7	14.1	32.7	4.1				
11/27/2018 10:00	92	8.1	0.1	27.0	0.1	7.53	0.06	125.0	7.1	118.1	28.7	32.4	2.9	150.00	9.19		

Date/Time	Day	DO (mg L^{-1})		Temp (°C)		pH		Alkalinity (mg-$CaCO_3$ L^{-1})		ORP-water (mV)		ORP-biofilter (mV)		SS (mg L^{-1})		COD (mg L^{-1})	
		Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
11/29/2018 10:00	94	8.9	0.3	26.2	0.1	7.60	0.02	150.0	0.0	112.4	12.7	41.0	1.8				
12/1/2018 10:00	96	8.5	0.0	26.9	0.1	7.56	0.04	125.0	7.1	118.3	0.9	46.9	7.6				
12/3/2018 10:00	98	8.6	0.4	28.2	0.0	7.57	0.01	150.0	0.0	122.6	28.8	44.6	13.2	135.00	53.03		
12/5/2018 10:00	100	8.7	0.1	28.9	0.0	7.59	0.01	120.0	14.1	122.3	14.8	33.0	7.4				
12/7/2018 10:00	102	8.8	0.2	29.1	0.3	7.59	0.06	150.0	0.0	126.3	0.5	29.2	8.1				
12/9/2018 10:00	104	8.7	0.3	26.6	0.1	7.68	0.06	180.0	0.0	122.2	19.4	41.8	0.1				
12/11/2018 10:00	106	8.5	0.6	26.9	0.0	7.66	0.04	170.0	0.0	116.0	5.7	42.9	1.0	141.00	3.54		
12/13/2018 10:00	108	8.2	0.0	26.6	0.0	7.66	0.04	165.0	7.1	109.2	8.9	25.1	19.0				
12/15/2018 10:00	110	8.4	0.4	26.1	0.1	7.71	0.01	155.0	7.1	127.4	2.3	35.5	9.7				
12/17/2018 10:00	112	8.3	0.0	26.3	0.1	7.73	0.00	140.0	14.1	131.9	8.4	62.3	1.3	161.25	14.50		
12/19/2018 10:00	114	8.4	0.4	26.6	0.6	7.68	0.09	150.0	0.0	133.5	11.0	60.3	11.5				
12/21/2018 10:00	116	8.6	0.1	27.6	0.0	7.59	0.03	155.0	7.1	128.9	3.2	40.7	3.7				
12/23/2018 10:00	118	8.6	0.1	27.5	0.1	7.62	0.10	155.0	7.1	139.0	5.0	39.4	3.1				
12/25/2018 10:00	120	2.3	0.1	27.5	0.3	7.51	0.09	160.0	0.0	76.5	16.5	10.0	2.1	81.75	37.83	355.56	31.43
12/27/2018 10:00	122	0.2	0.0	28.0	0.1	7.36	0.01	200.0	0.0	-18.0	5.4	-56.5	1.3			422.22	0.00
12/29/2018 10:00	124	0.2	0.0	28.5	0.2	7.51	0.02	310.0	0.0	-81.8	22.2	-151.0	3.7			322.22	78.57
12/31/2018 10:00	126	0.2	0.0	27.4	0.1	7.83	0.03	325.0	7.1	-67.0	27.5	-166.6	19.7	100.35	2.76	255.56	47.14
Shrimp cultivation #2																	
1/2/2019 10:00	128	8.7	0.5	25.2	0.1	7.86	0.00	320.0	14.1	127.9	9.0	33.4	13.9			133.33	62.86
1/4/2019 10:00	130	8.4	0.0	24.3	0.1	7.88	0.04	310.0	0.0	142.7	18.6	40.0	2.5			166.67	78.57
1/6/2019 10:00	132	8.1	0.0	25.7	0.0	7.77	0.08	300.0	0.0	133.5	29.1	55.9	3.7			44.44	31.43
1/8/2019 10:00	134	8.5	0.6	27.1	0.1	7.67	0.16	290.0	0.0	162.9	0.7	37.2	17.5	117.00	24.04	55.56	15.71
1/10/2019 10:00	136	8.5	0.1	27.3	0.1	7.67	0.05	280.0	0.0	158.1	7.3	33.9	1.1			22.22	0.00
1/12/2019 10:00	138	8.6	0.2	27.4	0.1	7.69	0.06	270.0	0.0	127.9	34.5	35.5	10.1				
1/14/2019 10:00	140	8.5	0.2	27.5	0.0	7.77	0.02	260.0	0.0	118.7	28.3	46.6	5.3	120.75	57.63		
1/16/2019 10:00	142	8.9	0.1	27.6	0.1	7.74	0.04	255.0	7.1	142.3	9.6	57.1	7.4				

Date/Time	Day	DO (mg L^{-1})		Temp (°C)		pH		Alkalinity (mg-$CaCO_3$ L^{-1})		ORP-water (mV)		ORP-biofilter (mV)		SS (mg L^{-1})		COD (mg L^{-1})	
		Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
1/18/2019 10:00	144	8.5	0.1	26.3	0.0	7.68	0.09	245.0	7.1	130.2	23.1	48.5	10.1				
1/20/2019 10:00	146	8.4	0.2	26.3	0.1	7.65	0.11	240.0	14.1	140.6	1.6	47.3	4.1				
1/22/2019 10:00	148	8.4	0.0	26.8	0.0	7.63	0.12	230.0	14.1	129.8	7.4	29.2	15.0	97.25	8.84		
1/24/2019 10:00	150	8.7	0.2	25.8	0.1	7.57	0.08	225.0	21.2	153.2	6.2	46.7	0.3				
1/26/2019 10:00	152	8.7	0.0	25.9	0.1	7.60	0.07	230.0	0.0	132.0	17.7	41.8	16.0				
1/28/2019 10:00	154	8.2	0.1	25.0	0.0	7.65	0.04	220.0	0.0	142.7	0.4	38.3	23.5	97.00	19.09		
1/30/2019 10:00	156	9.0	0.1	27.0	0.0	7.49	0.09	225.0	21.2	118.4	9.2	37.2	2.1				
2/1/2019 10:00	158	8.8	0.1	27.3	0.0	7.46	0.07	210.0	28.3	118.9	22.6	38.0	4.9				
2/3/2019 10:00	160	8.7	0.4	27.8	0.0	7.45	0.09	200.0	28.3	122.1	10.7	42.3	3.0				
2/5/2019 10:00	162	8.2	0.1	27.9	0.2	7.43	0.08	200.0	28.3	121.0	16.6	47.8	8.3	155.25	6.72		
2/7/2019 10:00	164	8.3	0.4	28.1	0.1	7.39	0.04	200.0	28.3	102.7	1.3	48.0	6.1				
2/9/2019 10:00	166	8.6	0.2	28.2	0.1	7.41	0.01	210.0	28.3	127.4	3.4	36.7	18.2				
2/11/2019 10:00	168	9.0	0.2	28.4	0.1	7.39	0.02	220.0	28.3	121.2	3.5	32.8	4.2	107.00	57.28		
2/13/2019 10:00	170	8.2	0.5	28.0	0.1	7.43	0.04	195.0	21.2	114.7	14.2	36.4	7.1				
2/15/2019 10:00	172	8.3	0.1	28.1	0.1	7.40	0.03	175.0	21.2	114.6	12.8	43.3	0.5				
2/17/2019 10:00	174	8.8	0.1	27.3	0.1	7.32	0.15	170.0	28.3	113.8	11.1	34.2	2.1	129.25	51.27		
2/19/2019 10:00	176	8.4	0.1	27.2	0.0	7.34	0.08	155.0	35.4	119.3	4.2	32.4	0.4				
2/21/2019 10:00	178	8.6	0.0	27.7	0.0	7.44	0.01	145.0	49.5	107.1	6.6	29.8	7.9				
2/23/2019 10:00	180	8.6	0.4	27.9	0.1	7.43	0.01	160.0	14.1	114.7	18.1	37.8	8.5				
2/25/2019 10:00	182	8.8	0.5	27.8	0.1	7.44	0.04	150.0	14.1	128.9	5.3	35.1	3.6	176.50	10.61		
2/27/2019 10:00	184	3.9	1.6	28.0	0.1	6.41	0.17	195.0	7.1	18.2	7.5	-11.1	2.2			352.78	3.93
3/1/2019 10:00	186	0.5	0.1	29.1	0.0	7.12	0.14	270.0	42.4	-78.2	25.4	-111.4	12.0			233.33	47.14
3/3/2019 10:00	188	0.5	0.0	29.7	0.0	7.87	0.04	375.0	21.2	-137.9	3.4	-200.5	12.2			155.56	94.28
Shrimp cultivation #3																	
3/5/2019 10:00	190	8.3	0.5	28.5	0.1	7.85	0.18	400.0	56.6	68.6	17.0	21.6	5.3			100.00	109.99
3/7/2019 10:00	192	8.5	0.1	28.0	0.1	7.91	0.11	395.0	49.5	94.7	12.4	35.1	8.7			33.33	15.71

Date/Time	Day	DO (mg L^{-1})		Temp (°C)		pH		Alkalinity (mg-$CaCO_3$ L^{-1})		ORP-water (mV)		ORP-biofilter (mV)		SS (mg L^{-1})		COD (mg L^{-1})	
		Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
3/9/2019 10:00	194	8.3	0.3	27.8	0.1	7.94	0.09	370.0	42.4	90.5	6.8	32.6	10.2			22.22	0.00
3/11/2019 10:00	196	8.1	0.2	27.8	0.1	7.95	0.10	345.0	21.2	84.1	3.1	33.9	2.5			33.33	15.71
3/13/2019 10:00	198	8.2	0.1	28.1	0.0	7.78	0.01	325.0	7.1	88.8	4.7	33.9	14.1				
3/15/2019 10:00	200	8.4	0.0	28.0	0.0	7.86	0.11	310.0	0.0	80.9	2.1	39.2	2.3				
3/17/2019 10:00	202	8.6	0.1	28.3	0.1	7.81	0.18	290.0	14.1	83.0	0.9	37.6	11.2				
3/19/2019 10:00	204	8.1	0.4	28.2	0.1	7.75	0.25	290.0	14.1	85.4	1.6	40.3	1.6				
3/21/2019 10:00	206	8.4	0.8	28.6	0.1	7.79	0.10	290.0	14.1	62.9	0.8	17.9	2.8				
3/23/2019 10:00	208	8.2	0.1	28.3	0.1	7.73	0.05	270.0	14.1	90.8	4.8	41.4	25.3				
3/25/2019 10:00	210	8.0	0.3	28.2	0.2	7.72	0.01	255.0	7.1	84.4	13.9	35.2	4.2				

Table C-3. Growth of shrimp cultured in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days.

Biofilter acclimation								
Start experiment			1 month of experiment			2 month of experiment		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	1.23	7.0	1	4.53	9.0	1	9.31	11.5
2	4.13	8.5	2	4.29	8.5	2	4.94	10.0
3	3.19	8.0	3	5.50	9.5	3	14.97	13.0
4	2.77	7.5	4	4.03	8.5	4	6.56	10.5
5	3.83	8.5	5	4.45	9.0	5	9.32	11.5
6	5.04	9.5	6	4.06	8.5	6	6.62	10.5
7	3.38	8.0	7	4.20	9.0	7	7.42	11.0
8	2.42	7.5	8	4.43	9.0	8	8.90	11.5
9	4.67	10.0	9	7.98	10.5	9	5.42	11.0
10	3.75	9.0	10	5.91	10.0	10	12.31	13.0
11	4.78	9.5	11	4.21	9.0	11	8.03	11.0
12	5.00	9.5	12	5.24	9.0	12	5.64	10.0
13	3.96	8.5	13	5.63	9.5	13	4.83	9.5
14	3.98	8.0	14	6.21	10.0	14	10.85	12.0
15	2.86	7.5	15	6.38	10.0	15	5.57	10.0
16	3.28	7.5	16	4.95	9.0	16	5.87	10.0
17	3.83	8.5	17	5.78	10.0	17	6.28	10.5
18	2.94	8.0	18	3.10	8.0	18	5.60	9.5
19	3.85	9.0	19	5.43	9.0	19	5.26	9.5
20	3.57	8.0	20	6.40	9.5	20	8.01	10.5
21	4.37	8.5	21	3.75	8.5	21	6.19	10.0
22	2.97	7.5	22	4.59	9.0	22	9.82	12.0
23	3.50	8.0	23	4.15	9.0	23	4.74	9.5
24	3.49	8.5	24	3.75	8.5	24	5.32	10.0
25	4.13	8.5	25	4.08	8.5	25	4.71	9.5
26	4.87	10.0	26	4.14	9.0	26	7.88	11.0
27	2.43	7.5	27	5.20	9.0	27	8.33	11.0
28	5.32	10.0	28	4.43	9.0	28	5.24	9.5
29	2.78	7.5	29	3.91	8.5	29	4.78	10.0
30	4.46	9.0	30	4.98	9.5	30	9.41	11.5
31	2.90	8.5	31	6.43	10.5			
32	3.12	8.5	32	5.02	9.0			
33	3.53	8.0	33	3.95	8.5			
34	2.72	8.0	34	5.55	10.0			
35	3.04	8.0	35	4.58	9.0			
36	4.84	9.0	36	4.52	9.0			
37	4.85	9.5	37	5.48	9.5			
38	3.50	8.5	38	3.41	8.5			
39	4.04	9.0	39	5.37	10.0			
40	3.08	8.0	40	2.67	7.5			
41	3.12	8.0	41	6.21	10.5			
42	1.74	7.0	42	4.94	9.5			
43	4.20	9.0	43	3.46	8.5			
44	3.05	8.0	44	5.06	9.0			
45	3.43	9.0	45	3.73	8.5			
46	2.64	7.0	46	4.74	9.5			
47	2.72	8.0	47	6.16	10.5			
48	3.18	7.5	48	5.03	9.0			

No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
49	3.00	7.5	49	4.31	9.0			
50	2.47	7.5	50	6.61	10.0			
51	3.44	8.5	51	5.83	10.0			
52	2.91	8.0	52	2.93	8.0			
53	4.11	9.0	53	8.10	11.5			
54	3.46	8.5	54	2.71	8.0			
55	2.88	7.5	55	7.93	10.5			
56	2.69	7.5	56	4.26	8.5			
57	2.72	7.5	57	5.80	10.5			
58	3.69	8.0	58	4.95	9.5			
59	3.02	8.0	59	4.09	9.0			
60	3.09	7.5	60	3.73	9.0			
61	2.29	7.5	61	6.13	10.0			
62	3.40	8.0	62	3.58	8.0			
63	2.98	8.0	63	4.43	9.5			
64	2.83	7.5	64	3.54	8.5			
65	4.58	9.0	65	3.51	8.5			
66	3.40	8.0	66	2.46	7.5			
67	3.68	8.0	67	3.95	8.5			
68	3.82	8.5	68	4.20	9.0			
69	2.54	7.5	69	3.52	8.5			
70	3.55	9.0	70	4.20	9.0			
71	2.93	8.0	71	3.72	8.5			
72	3.70	9.0	72	4.01	8.5			
73	3.62	8.5	73	3.56	8.5			
74	4.58	8.5	74	3.20	8.0			
75	2.02	6.5	75	4.02	9.0			

Shrimp cultivation #1

Start experiment			1 month of experiment			2 month of experiment		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	3.25	7.5	1	3.66	9.0	1	11.70	12.0
2	4.87	10.0	2	4.88	10.0	2	7.23	11.0
3	3.77	8.5	3	3.35	8.0	3	12.42	13.0
4	3.62	9.0	4	3.63	9.0	4	13.00	13.0
5	4.13	9.0	5	3.98	9.0	5	9.23	11.0
6	4.14	9.0	6	4.42	9.5	6	7.42	10.0
7	2.68	8.0	7	4.92	9.5	7	7.03	10.0
8	4.40	9.5	8	5.24	10.0	8	12.63	12.5
9	3.38	8.5	9	4.06	9.0	9	8.58	10.5
10	3.74	9.0	10	5.74	10.0	10	9.87	11.0
11	4.01	9.0	11	4.74	9.5	11	9.14	11.0
12	3.77	8.5	12	5.17	9.5	12	7.50	10.5
13	3.55	8.0	13	5.60	10.0	13	11.11	12.0
14	3.32	8.5	14	5.04	10.0	14	14.12	12.5
15	2.93	8.0	15	4.76	9.5	15	15.09	13.5
16	2.90	7.5	16	4.65	10.0	16	10.42	12.0
17	3.75	8.0	17	4.15	10.0	17	9.81	12.5
18	2.67	7.5	18	5.78	11.0	18	9.46	11.5
19	2.43	7.5	19	3.70	9.0	19	16.71	14.0
20	4.10	9.0	20	3.82	9.0	20	10.61	12.0
21	3.10	8.0	21	3.84	9.0	21	9.55	12.0
22	2.98	7.5	22	4.38	9.0	22	7.68	10.5
23	3.86	9.0	23	3.73	9.0	23	6.14	10.0

No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
24	3.68	8.5	24	4.77	9.5	24	5.14	10.0
25	4.21	9.0	25	4.60	9.0	25	14.00	13.5
26	3.15	8.0	26	4.66	9.5	26	6.40	10.0
27	3.88	8.5	27	5.54	9.5	27	12.67	13.0
28	3.77	8.5	28	4.88	10.0	28	10.54	12.0
29	3.70	9.0	29	2.73	7.0	29	6.25	10.0
30	3.17	8.5	30	3.86	8.5	30	5.82	10.0
31	3.72	8.5	31	5.84	10.0	31	5.92	9.5
32	3.16	8.5	32	4.88	9.0	32	5.78	9.5
33	3.41	9.0	33	5.07	9.5	33	7.31	11.0
34	4.18	9.0	34	8.07	11.0	34	6.60	10.0
35	4.13	9.5	35	6.64	10.0	35	5.76	10.0
36	3.45	8.5	36	4.35	9.0	36	11.00	11.5
37	3.77	9.0	37	4.82	9.5	37	6.53	10.5
38	3.10	8.5	38	4.28	8.5	38	4.50	9.5
39	2.06	7.5	39	4.44	8.5	39	4.97	9.5
40	3.30	8.5	40	3.78	8.5	40	5.34	9.5
41	3.27	8.5	41	4.48	9.0	41	6.85	11.0
42	3.88	8.5	42	4.88	9.5	42	10.64	13.0
43	4.61	9.5	43	4.57	9.0	43	4.86	10.0
44	2.99	8.5	44	4.31	8.5	44	10.44	12.5
45	2.58	7.5	45	5.69	9.5	45	4.85	9.0
46	3.18	8.0	46	6.48	11.0	46	7.76	11.0
47	3.25	8.0	47	2.83	8.0	47	9.53	11.5
48	3.64	9.0	48	3.86	8.5	48	6.27	10.5
49	3.79	9.0	49	3.75	9.0	49	8.65	11.0
50	3.67	8.5	50	3.86	8.5	50	9.73	11.5
51	3.21	8.5	51	4.34	9.0	51	11.57	13.0
52	5.33	10.0	52	4.78	9.0	52	5.83	10.5
53	3.92	9.0	53	4.75	9.5	53	8.95	12.0
54	3.48	8.0	54	5.02	9.5	54	6.50	10.0
55	3.68	8.5	55	3.94	9.0	55	5.86	10.0
56	3.10	8.0	56	4.39	9.5	56	4.89	10.0
57	3.42	8.5	57	4.82	9.5	57	5.44	10.0
58	4.16	8.5	58	3.82	9.0	58	6.23	10.5
59	3.92	9.0	59	4.77	9.5	59	4.97	10.0
60	3.38	8.5	60	4.57	9.0	60	6.80	10.0
61	4.24	9.0	61	5.08	9.0	61	4.89	9.5
62	4.74	10.0	62	4.17	9.0	62	5.55	10.0
63	3.57	8.5	63	5.86	10.0	63	6.42	10.0
64	2.78	8.0	64	5.07	10.0	64	6.50	10.0
65	3.01	8.5	65	4.02	9.0	65	5.48	10.0
66	2.80	8.0	66	3.93	8.5	66	6.20	10.0
67	2.42	7.5	67	5.77	10.0	67	6.55	10.5
68	4.27	9.5	68	4.96	9.5	68	4.94	9.5
69	3.84	9.5	69	5.07	9.0	69	12.32	11.0
70	3.75	8.5	70	4.16	9.0	70	6.25	10.5
71	3.35	8.5	71	4.72	9.5	71	9.15	11.5
72	3.41	8.5	72	5.29	10.0	72	10.82	12.0
73	3.51	8.5	73	5.55	10.0	73	5.80	10.0
74	4.14	9.5	74	7.81	11.0	74	5.50	11.0
75	2.90	8.0	75	4.83	9.5	75	8.38	11.0

Shrimp cultivation #2								
Start experiment			1 month of experiment			2 month of experiment		
No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
1	7.00	10.5	1	9.60	11.5	1	10.10	12.0
2	7.30	10.0	2	10.00	12.0	2	16.80	14.0
3	6.90	10.0	3	9.70	11.5	3	19.60	12.0
4	3.90	8.5	4	13.10	12.5	4	11.00	11.5
5	7.80	10.5	5	10.40	12.0	5	13.20	13.0
6	8.00	10.5	6	10.40	12.5	6	11.10	12.0
7	3.40	8.0	7	9.00	11.0	7	13.10	12.0
8	4.30	8.5	8	9.70	12.0	8	12.20	12.0
9	4.90	9.0	9	9.10	11.0	9	13.80	13.0
10	4.80	9.0	10	8.40	10.5	10	12.40	12.0
11	8.10	11.0	11	11.20	12.0	11	12.40	12.0
12	4.00	8.5	12	9.90	11.0	12	16.20	13.0
13	4.40	7.5	13	10.80	12.0	13	12.90	12.5
14	5.10	9.0	14	8.30	11.0	14	13.20	12.5
15	4.60	8.5	15	10.00	11.0	15	10.30	11.0
16	5.10	9.0	16	8.10	10.5	16	13.50	12.5
17	7.20	10.0	17	9.80	12.0	17	12.00	12.0
18	9.00	10.5	18	11.10	12.0	18	13.20	12.5
19	7.40	9.5	19	9.40	12.0	19	20.00	14.0
20	7.60	9.5	20	8.20	11.0	20	13.90	12.5
21	8.10	11.0	21	10.90	12.0	21	12.20	12.5
22	7.20	10.0	22	10.50	12.0	22	12.10	12.0
23	7.40	10.0	23	10.50	11.5	23	8.90	11.0
24	6.10	10.0	24	10.20	12.0	24	11.10	12.0
25	6.20	9.5	25	11.20	12.0	25	12.10	12.5
26	5.80	10.0	26	9.40	10.5	26	12.30	12.0
27	9.10	10.0	27	10.70	12.0	27	17.30	13.0
28	6.60	10.0	28	8.90	11.0	28	15.60	13.0
29	7.00	10.5	29	10.50	11.5	29	14.60	13.0
30	7.20	0.0	30	14.10	13.0	30	14.50	12.5
31	7.80	10.0	31	11.50	12.0	31	13.50	18.1
32	7.70	10.0	32	9.90	11.0	32	8.30	18.1
33	7.90	10.5	33	9.00	10.5	33	19.90	14.0
34	7.30	10.5	34	11.00	12.0	34	11.20	12.5
35	6.60	9.5	35	12.30	12.5	35	9.50	11.0
36	7.10	10.0	36	8.10	11.0	36	12.60	12.0
37	7.30	10.0	37	8.60	11.0	37	13.30	12.0
38	7.00	10.0	38	11.90	12.0	38	9.90	11.5
39	7.10	10.0	39	7.10	10.0	39	9.70	11.5
40	6.30	9.5	40	12.30	12.5	40	11.30	11.5
41	7.10	10.0	41	10.00	11.0	41	11.70	11.5
42	7.60	10.0	42	10.30	11.5	42	11.90	12.0
43	6.40	10.0	43	10.20	11.5	43	13.00	12.0
44	7.70	10.5	44	12.10	13.0	44	14.80	13.0
45	7.20	10.0	45	8.10	10.5	45	13.80	13.0
46	6.10	9.5	46	9.50	12.0	46	12.50	12.0
47	7.50	9.5	47	8.60	11.0	47	12.70	12.0
48	8.90	10.5	48	9.90	11.0	48	12.20	12.0
49	6.60	9.5	49	9.80	11.0	49	8.90	11.0
50	7.40	10.0	50	10.60	12.5	50	16.50	13.0
51	5.80	9.5	51	9.50	11.0	51	15.80	13.0
52	8.00	10.5	52	9.70	12.0	52	10.20	11.5

No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)	No.	Weight (g)	Length (cm)
53	7.10	10.0	53	7.60	10.5	53	11.40	12.0
54	7.30	10.0	54	10.30	11.5	54	12.00	12.0
55	6.00	9.0	55	10.70	11.5	55	10.60	11.5
56	6.90	10.0	56	7.30	10.5	56	10.90	11.5
57	6.80	10.0	57	10.60	11.5	57	11.50	11.5
58	6.30	9.5	58	10.60	12.0	58	9.70	11.5
59	8.10	10.5	59	11.70	12.0	59	10.30	12.5
60	4.70	6.0	60	9.30	11.5	60	10.80	12.0
61	7.20	10.0	61	10.90	12.5	61	7.40	11.0
62	7.20	9.5	62	10.00	12.0	62	10.20	12.0
63	6.40	9.5	63	7.50	10.5	63	15.90	13.0
64	7.40	10.5	64	14.70	13.0	64	11.50	11.5
65	7.50	10.5	65	10.50	12.0	65	18.60	13.5
66	7.00	10.0	66	6.20	10.0	66	9.30	11.5
67	6.20	9.5	67	10.50	12.0	67	8.10	10.5
68	7.70	10.5	68	13.00	12.5	68	13.80	12.5
69	6.40	10.0	69	10.10	11.0	69	11.10	11.5
70	7.10	10.0	70	11.70	12.0	70	11.40	12.0
71	6.00	9.5	71	9.60	11.0	71	8.40	10.0
72	5.80	9.5	72	9.00	11.0	72	10.70	11.5
73	6.40	10.0	73	10.00	11.5	73	14.00	12.5
74	8.50	11.0	74	9.30	11.0	74	11.70	11.5
75	7.10	10.5	75	12.30	12.5	75	11.20	12.0

Table C-4. Nitrification and denitrification rates of fibrous Biocord^{TM} biofilter in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

Week	Nitrification rate (mg-N m^{-2} d^{-1})	SD	Denitrification rate (mg-N m^{-2} d^{-1})	SD
0	0.00	0.00	0.00	0.00
2	40.84	19.37	6.88	2.82
4	123.00	37.63	14.32	1.98
6	115.04	17.38	19.25	2.97
8	23.12	10.09	21.23	4.35
9	17.05	12.44	12.59	4.84
11	42.76	4.72	11.96	2.82
13	41.61	6.85	20.25	4.55
15	39.49	17.02	18.06	1.10
17	63.10	8.47	9.80	2.23
18	59.87	7.44	66.80	15.45
20	91.64	16.79	21.14	5.71
22	95.26	26.31	20.63	3.78
24	112.30	50.22	21.60	2.75
26	93.79	12.80	19.24	2.39
27	67.59	6.04	67.68	4.74
29	74.33	12.51	16.79	2.60

Table C-5. Relative abundances (%) of bacterial phyla observed on fibrous Biocord™ biofilter in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

Phylum	Week 2		Week 4		Week 8		Week 13		Week 17		Week 18		Week 22		Week 26		Week 27		Week 29	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Other	0.76	0.50	0.92	0.73	0.31	0.52	0.91	4.61	1.80	1.57	2.05	1.06	1.03	0.92	1.37	1.21	1.62	1.36	2.02	1.39
Actinobacteria	3.21	4.48	0.79	1.41	6.83	2.31	3.65	5.87	2.49	2.99	0.98	0.57	3.39	2.59	2.28	2.29	0.80	1.99	4.11	22.68
Bacteroidetes	18.78	7.14	29.57	20.60	10.63	11.84	17.45	14.54	30.29	21.77	13.49	7.74	10.37	18.54	22.85	21.19	12.40	16.02	22.12	21.84
Caldithrix	0.00	0.00	0.00	0.00	0.02	0.02	0.88	2.40	0.52	0.99	0.27	0.47	0.02	0.25	0.68	0.25	0.20	0.31	0.11	0.26
Chlamydiae	0.11	2.24	0.28	2.29	1.84	0.22	0.76	0.36	1.78	0.41	0.61	0.08	0.61	0.63	0.53	0.44	0.20	0.32	0.77	0.47
Chlorobi	1.43	0.06	0.37	0.02	0.84	0.05	0.24	0.96	0.32	0.77	1.10	0.81	1.27	0.57	1.17	0.34	0.36	0.41	1.17	0.37
Chloroflexi	0.36	0.48	1.18	0.86	16.67	45.99	38.27	7.13	4.36	21.63	1.54	2.34	28.23	32.06	18.76	22.04	9.51	10.92	24.42	10.77
Cyanobacteria	0.02	0.04	1.25	0.41	0.20	0.05	0.17	0.30	0.15	0.63	2.02	0.90	0.13	0.24	0.57	0.15	0.53	0.12	0.35	0.03
Dependentiae	0.08	0.11	0.21	0.50	1.64	0.20	0.67	0.25	0.57	0.28	0.22	0.07	0.15	0.13	0.18	0.10	0.03	0.04	0.29	0.39
Firmicutes	0.32	0.12	9.57	1.20	0.41	0.09	0.13	0.20	0.22	0.11	2.42	1.23	0.23	0.17	0.20	0.35	2.87	1.33	0.34	0.53
Gemmatimonadetes	0.47	0.18	1.46	0.83	0.09	0.07	0.49	1.28	2.00	1.22	0.96	0.39	0.56	0.96	2.64	2.82	1.15	1.79	2.43	0.64
Gracilibacteria	0.04	0.24	0.02	1.00	0.02	0.01	0.18	0.82	0.13	0.40	1.39	3.44	0.37	0.32	0.11	0.14	0.48	0.12	0.33	0.22
Latescibacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.09	0.16	0.09	0.19	0.12	0.02	0.18	0.19	0.80	0.15	1.51	0.10	0.31
Nitrospirae	0.00	0.00	0.00	0.00	0.02	0.05	0.33	0.31	0.27	0.57	0.06	0.10	1.87	0.38	0.17	0.20	0.13	0.18	0.10	0.03
Planctomycetes	9.53	6.35	3.81	3.02	10.30	4.78	7.58	12.87	5.69	6.77	2.48	0.89	9.40	5.31	8.86	9.62	3.51	5.64	7.93	4.12
Proteobacteria	60.96	72.35	49.31	65.43	47.74	32.78	25.84	44.09	47.72	37.33	69.91	79.62	40.58	35.54	38.38	35.39	65.76	57.25	32.33	34.87
Tenericutes	0.94	1.14	0.02	0.03	0.12	0.14	0.06	0.08	0.32	0.04	0.01	0.02	0.09	0.00	0.02	0.05	0.00	0.00	0.03	0.11
Verrucomicrobia	2.98	4.56	1.25	1.67	2.32	0.87	2.36	3.85	1.22	2.43	0.30	0.15	1.69	1.21	1.05	2.62	0.29	0.70	1.04	0.97
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table C-6. Relative abundances (%) of bacterial class observed on fibrous Biocord™ biofilter in long-term marine RAS during 60 days of biofilter acclimation, followed by two rounds replication of aerobic shrimp cultivation for 60 days and 7 days of denitrification (anoxic, no shrimps).

Class	Week 2		Week 4		Week 8		Week 13		Week 17		Week 18		Week 22		Week 26		Week 27		Week 29	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Other	1.10	0.92	3.30	1.64	2.15	1.03	1.82	6.67	3.72	3.21	3.98	2.15	3.29	2.52	5.97	5.30	4.44	4.40	4.26	3.16
Acidimicrobiia	1.94	2.00	0.51	0.96	5.22	1.75	2.19	3.30	1.25	1.43	0.51	0.25	1.77	1.17	1.64	1.41	0.43	1.13	2.16	3.16
Actinobacteria	1.22	2.43	0.26	0.42	1.44	0.49	1.31	2.48	1.12	1.28	0.43	0.30	1.26	1.06	0.42	0.64	0.30	0.65	1.65	19.25
Cytophagia	1.23	0.18	0.19	0.14	0.42	0.41	1.31	2.27	1.92	1.11	0.78	0.35	0.85	1.18	2.03	0.64	0.92	0.78	2.52	1.02
Flavobacteriia	15.25	6.25	18.89	10.99	6.83	3.89	4.32	8.30	19.06	11.37	10.66	5.52	6.71	12.87	13.34	13.62	8.07	10.20	12.43	14.93
[Rhodothermi]	0.01	0.01	0.05	0.15	0.69	0.15	1.88	1.34	1.43	2.10	0.71	0.72	0.69	1.65	3.01	2.60	1.72	2.35	2.43	3.05
[Saprospirae]	2.16	0.52	10.30	9.11	2.22	7.22	9.86	2.46	7.65	7.10	1.26	1.02	2.10	2.78	4.30	4.21	1.44	2.32	4.65	2.55
Caldithrixae	0.00	0.00	0.00	0.00	0.02	0.02	0.88	2.40	0.52	0.99	0.27	0.47	0.02	0.25	0.68	0.25	0.20	0.31	0.11	0.26
Chlamydiia	0.11	2.24	0.28	2.29	1.84	0.22	0.76	0.36	1.78	0.41	0.61	0.08	0.61	0.63	0.53	0.44	0.20	0.32	0.77	0.47
OPB56	1.43	0.06	0.37	0.02	0.84	0.05	0.24	0.96	0.32	0.77	1.10	0.81	1.27	0.57	1.17	0.34	0.36	0.40	1.15	0.37
Anaerolineae	0.30	0.44	1.08	0.82	16.36	45.86	37.95	6.71	4.04	21.37	1.41	2.29	27.11	30.89	17.17	20.33	8.85	9.92	22.66	8.09
Thermomicrobia	0.06	0.01	0.06	0.03	0.21	0.05	0.23	0.32	0.24	0.17	0.08	0.03	0.95	1.03	1.39	1.66	0.64	0.94	1.60	2.52
ML635J-21	0.00	0.00	0.00	0.00	0.15	0.04	0.00	0.14	0.03	0.24	1.96	0.87	0.03	0.01	0.27	0.00	0.35	0.01	0.14	0.01
Synechococcophycideae	0.02	0.00	1.24	0.34	0.02	0.01	0.01	0.01	0.03	0.22	0.00	0.02	0.01	0.03	0.01	0.01	0.00	0.00	0.01	0.01
Clostridia	0.29	0.03	9.56	1.19	0.32	0.07	0.09	0.16	0.10	0.09	2.39	1.22	0.06	0.15	0.07	0.33	2.84	1.28	0.23	0.38
BB34	0.03	0.00	0.01	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BD1-5	0.00	0.24	0.00	0.00	0.01	0.00	0.14	0.00	0.01	0.00	0.50	3.17	0.00	0.01	0.01	0.00	0.24	0.06	0.03	0.00
Gemm-2	0.47	0.18	1.46	0.83	0.09	0.07	0.49	1.28	2.00	1.22	0.95	0.38	0.56	0.96	2.59	2.81	1.13	1.78	2.39	0.64
Nitrospira	0.00	0.00	0.00	0.00	0.02	0.05	0.33	0.31	0.27	0.57	0.06	0.10	1.87	0.38	0.17	0.20	0.13	0.18	0.10	0.03
OM190	1.98	0.10	0.16	0.05	0.02	0.00	0.20	0.13	0.17	0.12	0.06	0.04	0.06	0.16	0.14	0.25	0.05	0.19	0.10	0.12
Phycisphaerae	0.20	0.03	0.84	0.10	0.93	1.96	2.18	8.92	1.83	2.04	1.04	0.41	0.82	1.22	2.83	3.08	1.27	2.87	3.66	1.46
Planctomycetia	7.30	6.21	1.41	2.39	8.44	2.68	4.94	3.45	2.66	4.16	0.80	0.32	7.66	3.49	2.72	3.22	0.94	1.08	3.22	2.04
Alphaproteobacteria	43.83	56.09	24.08	55.25	29.04	20.69	15.52	22.81	15.93	20.67	16.38	15.78	32.40	19.04	16.30	18.44	10.95	13.91	15.40	19.02
Betaproteobacteria	1.44	0.08	0.94	0.35	0.49	0.30	0.30	1.26	0.89	0.48	2.10	4.40	0.76	0.38	0.39	0.45	1.93	2.34	0.50	0.21
Deltaproteobacteria	12.39	7.50	8.89	2.27	2.68	0.76	2.61	4.76	13.57	3.43	2.54	0.72	1.88	7.05	7.73	7.38	3.22	4.12	4.46	3.16
Gammaproteobacteria	3.27	8.68	14.51	7.50	15.03	10.91	6.97	14.33	16.47	12.09	48.58	58.16	5.37	8.46	12.43	7.95	48.46	35.66	10.92	11.42
TA18	0.01	0.00	0.13	0.00	0.50	0.13	0.40	0.70	0.76	0.56	0.18	0.07	0.06	0.59	1.34	0.90	0.45	0.61	1.04	0.91
SJA-4	0.01	0.09	0.21	0.47	1.63	0.19	0.67	0.25	0.57	0.28	0.22	0.07	0.15	0.13	0.18	0.10	0.02	0.04	0.29	0.39

Class	Week 2		Week 4		Week 8		Week 13		Week 17		Week 18		Week 22		Week 26		Week 27		Week 29	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Mollicutes	0.94	1.14	0.02	0.03	0.11	0.13	0.06	0.08	0.32	0.04	0.00	0.02	0.09	0.00	0.02	0.05	0.00	0.00	0.03	0.11
Verrucomicrobiae	2.97	4.56	1.25	1.67	2.28	0.86	2.31	3.74	1.21	2.40	0.22	0.15	1.61	1.15	0.98	2.57	0.28	0.64	1.00	0.94
PRR-12	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.09	0.16	0.09	0.19	0.12	0.02	0.18	0.19	0.80	0.15	1.51	0.10	0.31
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Appendix D

Calculations

Example calculations in the study 1: effects of salinity, stocking density, and acclimation period on nitrogen removal efficiency and microbial community.

D.1 Surface of biofilter

SSA of Biocord^{TM} biofilter	=	2.8 m^2 m^{-1}
Length used in study 1	=	0.1 m
Total surface	=	(2.8 m^2 m^{-1}) x (0.1 m)
	=	**0.28 m^2**
SSA of Japanese filter mat	=	300 m^2 m^{-3}
Volume used in study 1	=	0.1×0.23×0.04 m^3
Total surface	=	(300 m^2 m^{-3}) x (0.1×0.23×0.04 m^3)
	=	**0.28 m^2**

D.2 Salinity preparation

Dilute 100 PSU of stock saline water with tap water and adjust the salt concentrations at 5, 15 and 25 PSU in 200 L of total volume.

	C_1V_1	=	C_2V_2
5 PSU:	(100 PSU) x (V_1)	=	(5 PSU) x (200 L)
	V_1	=	**10 L**
15 PSU:	(100 PSU) x (V_1)	=	(15 PSU) x (200 L)
	V_1	=	**30 L**
25 PSU:	(100 PSU) x (V_1)	=	(25 PSU) x (200 L)
	V_1	=	**50 L**

D.3 Methanol addition

Nitrate	=	**10 mg-N L^{-1}**
Volume	=	**2.5 L**
Total nitrate in system	=	(10 mg-N L^{-1}) x (2.5 L)
	=	25 mg-N

COD: Nitrate-N ratio = 5:1

Required COD = (25 mg-N) x (5)

= 125 mg-COD

Methanol (as COD) = 1.5 mg-COD mg-methanol^{-1}

$$\text{Required methanol} = \frac{125 \text{ mg-COD}}{1.5 \text{ mg-COD mg-methanol}^{-1}}$$

= 83.33 mg-methanol

Density of methanol = 792 mg mL^{-1}

$$\textbf{Required methanol} = \frac{83.33 \text{ mg}}{792 \text{ mg mL}^{-1}}$$

= **0.105 mL**

Example calculations in the study 2: application of biofilter in marine RAS for long-term operation.

D.4 Generation rate of nitrogen waste

Generation rate of nitrogen waste

= Estimated total shrimp weight (kg) x feed rate per day (%) x percentage of protein in feed (%) x nitrogen in protein (g-N g-protein^{-1}) x 10^6 mg kg^{-1}

= (5 kg) x (0.05) x (0.15) x (0.16 g-N g-protein^{-1}) x 10^6 mg kg^{-1}

= 6000 mg-N d^{-1}

D.5 Length of biofilter installed in RAS

Length of biofilter installed in RAS

$$= \frac{\text{Generation rate of nitrogen waste}}{\text{Avg. nitrification rate of fibrous biofilter (at intensive density; 25 PSU)}}$$

$$= \frac{6000 \text{ mg-N d}^{-1}}{53.55 \text{ mg-N m}^{-2} \text{ d}^{-1}}$$

= 112.04 m^2

The SSA of Biocord^{TM} biofilter is equal to 2.8 m^2 m^{-1}.

$$= \frac{112.04\ m^2}{2.8\ m^2\ m^{-1}}$$

= (40 m) x (1.2 of safety factor)

≈ 50 m

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