Microbiome in shrimp *Litopenaeus vannamei* aquaculture: dynamic changes and bacterial lifestyles

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EXECUTIVE SUMMARY

The Pacific white leg shrimp *Litopenaeus vannamei* is the most frequently cultured shrimp species, comprising more than 70% of the world shrimp commodities since 2010. Shrimp indoor and outdoor aquacultures are affected by water quality deteriorations and bacterial diseases. These cultivation problems lead to a substantial annual loss of shrimp harvest. Traditionally, water quality is quantified by plankton density, total suspended solids/particulate matter, pH, salinity and inorganic nutrients, particularly ammonium, nitrite and phosphate. Especially, the quantitative assessment of the carbon cycle in shrimp aquaculture is required to determine oxygen demand and depletion. However, optimal and acceptable but potentially stressful conditions have not systematically been determined in pond aquaculture. Excess biomass formed by photosynthesis of phytoplankton and by heterotrophic bacteria in the microbial loop poses a particular problem in shrimp aquaculture. While particulate matter in pond water may serve as additional feed source for shrimps, it also provides niches for particle-associated bacteria, such as pathogenic *Vibrio* species.

In this study, water quality parameters as well as bacterial community compositions were observed in Indonesian shrimp aquacultures over the complete rearing process. Semi-intensive and intensive aquacultures differed in suspended particulate matter content, chlorophyll a, pH, and dissolved oxygen, while inorganic nutrient concentrations and the population size of cultivable heterotrophic bacteria were comparable. *Halomonas*, *Salegentibacter*, and *Sulfitobacter* were the most abundant free-living bacteria, whereas particle-associated bacteria were dominated by *Halomonas* and *Psychrobacter*. *Vibrio* were more frequently found in the intensive aquaculture system, particularly in the particle-associated fraction and at low pH conditions.

White feces disease (WFD) event, which is a disease repeatedly occurring in shrimp aquaculture, was further documented in this thesis. To better understand pond water conditions as well as bacterial community dynamics during the disease event, the quantification of physico-chemical water parameters was combined with molecular analyses of the microbiome of shrimps, feces, and aquaculture water including the particles. The WFD event coincided with a low water pH and a high proportion of *Alteromonas* in the feces. Virulence genes of *Vibrio*, i.e toxin regulator (*toxR*) and termolabile hemolysin (*tlh*) were detected in the particle fraction (> 3 μm), in the intestine of healthy shrimps and feces of WFD-infected shrimps. An increase of pH above 8 via limestone addition enabled a recovery from WFD. In addition, the bacterial community composition also changed with the rise in

pH. This observation led to a recommendation, that aquacultures of *L. vannamei* should maintain a pH above 8.

Lastly, bacterial dynamic on aggregates, which form in large numbers in shrimp aquaculture, was investigated to monitor the growth of potential pathogenic *Vibrio* species. In the rolling tank experiments, the addition of carbon-rich molasses was shown to rapidly reduce toxic ammonium and nitrite pools. Furthermore, it enabled the fast growth of halophilic heterotrophic bacteria, such as *Halomonas*, *Psychrobacter*, and *Salegentibacter*. Conversely, in the presence of *Chlorella vulgaris* aggregates *V. parahaemolyticus* population remained constant without decay. The algal biomass seemed to maintain the *Vibrio* population and density.

I conclude that water parameters such as salinity and pH shape bacterial communities in shrimp pond aquaculture, and that a deterioration of water quality may cause detrimental shifts in bacterial community composition. However, bacterial communities will recover to the initial composition if water parameters are adjusted to former condition. To improve shrimp farming practices, I propose to perform regular pond water assessment, not only for the physicochemical parameters, but also for bacterial community composition. For this purpose, I recommend to analyze the bacterial communities in the particle fraction, including virulence genes of pathogenic bacteria. In addition, sludge discharge and regular addition of lime stones are necessary to improve and maintain shrimp production. As consequence, sustainable shrimp pond farming systems should include sludge as well as clean water reservoirs.

ZUSSAMMENFASSUNG

Die Pazifische Weissbein-Garnele *Litopenaeus vannamei* ist die am häufigsten in Aquakultur gezüchtete Garnelenart. Seit 2010 trägt sie mehr als 70% zur weltweiten Garnelenproduktion bei. Sowohl Innenraum- als auch Freiluftaquakultur für die Garnelenzucht leiden unter schlechter Wasserqualität und bakteriellen Krankheiten. Diese Faktoren führen jährlich zu einem erheblichen Ernteverlust. Traditionell wird Wasserqualität basierend auf Planktondichte, der Menge suspendierter Partikel, pH Wert, Salinität, und der Konzentration anorganischer Nährstoffe, insbesondere Ammonium, Nitrit und Phosphat, quantifiziert. Von besonderer Bedeutung ist die quantitative Erfassung des Kohlenstoffkreislaufs, die benötigt wird, um den Sauerstoffverbrauch des Aquakultursystems zu bestimmen. In Teichaquakulturanlagen wurden optimale bzw. akzeptable aber potentiell belastende Bedingungen bis her jedoch nicht systematisch ermittelt. Ein Überschuss an Biomasse produziert durch photosynthetisches Phytoplankton und heterotrophe Bakterien als Teil des so genannten 'Microbial Loop' stellt ein besonderes Problem für die Garnelenzucht in Teichaquakultur dar. Während organische Partikel im Wasser der Aquakulturteiche als zusätzliche Futterquelle für die Garnelen dienen können, bieten sie ebenfalls eine Nische für partikel-assoziierte Bakterien, u.a. pathogene *Vibrio* Arten.

In dieser Arbeit wurden Wasserqualität und Bakteriengemeinschaften während des vollständtigen Zuchtzyklus dokumentiert. Semi-intensive und intensive Aquakultursysteme unterschieden sich in der Menge suspendierter Partikel, Chlorophyll a Konzentration, pH Wert, und dem Gehalt gelösten Sauerstoffs, während beide System vergleichbare Konzentrationen an anorganischen Nährstoffen und der Anzahl kultivierbarer heterotropher Bakterien auswiesen. *Halomonas*, *Salegentibacter*, und *Sulfitobacter* zählten zu den häufigsten freilebenden Bakterien, während partikel-assoziierte Bakterien von *Halomonas* und *Psychrobacter* dominiert wurden. *Vibrio* trat vermehrt in intensive Aquakultur auf, vor allem in der partikulären Fraktion unter verringerten pH Bedingungen.

Desweiteren untersuchten ich in dieser Arbeit einen Ausbruch der Weißen Fäzes Krankheit (White Feces Disease: WFD), die regelmäßig in der Garnelenzucht auftritt. Um ein besseres Verständnis von den Wasserbedingungen in den Aquakulturteichen und den Veränderungen der Bakteriengemeinschaften während der Krankheitsphase zu erhalten, wurde die Analyse physikalischer und chemischer Wasserparameter mit molekularen Untersuchungen des Mikrobioms der Garnelen, der Fäzes, und des Tichwassers einschließlich der Partikel kombiniert. Der WFD-Ausbruch wurde von veringerten pH Werten und einem erhöhten

Anteil an *Altermonas* in den Fäzes begleitet. Virulenzfaktoren von *Vibrio*, d.h. der Toxinregulator *toxR* und das thermolabile Hämolysin *tlh*, wurden sowohl in der partikulären Fraktion (> 3 µm), im Darm gesunder Garnelen und den Fäzes erkrankter Tiere detektiert. Eine Eröhung des pH Werts auf über 8 mittels Kalkzugabe führte zu einem Rückgang der Krankheit und einer Änderung in der Zusammensetzung der Bakteriengemeinschaften. Eine der Hauptaussagen dieser Arbeit ist, dass *L. vannamei* Aquakultur bestrebt sein sollte, pH Werte von mehr als 8 aufrecht zu erhalten.

Abschließend wurde die Entwicklung von Bakteriengemeinschaften auf Aggregaten, die sich in großer Anzahl in Aquakultursystem bilden, untersucht, um das Wachstum pathogener *Vibrio* Arten zu beobachten. Rolltank-Experimente zeigten, dass die Zugabe kohlenhydratreicher Molasse einen rapiden Abfall toxischer Ammonium- und Nitritkonzentrationen herbei führten. Zusätzlich wurde das schnelle Wachstum halophiler heterotropher Bakterien wie *Halomonas*, *Psychrobacter* und *Salegentibacter* gefördert. Im Gegensatz dazu blieb die Population von *V. parahaemolyticus* in der Anwesenheit von *Chlorella vulgaris* Aggregaten konstant und nahm nicht ab. Algenbiomasse scheint demnach *Vibrio* Populationen und Dichte zu erhalten.

Ich schlussfolgere, dass Wasserparameter wie Salinität und pH Wert Bakteriengemeinschaften in Teichaquakultur für die Garnelenzucht beeinflussen, und dass eine Veschlechterung der Wasserqualität nachteilige Veränderungen der Bakteriengemeinschaft verursachen könnte. Die Bakteriengemeinschaften scheinen jedoch ihre ursprüngliche Zsuammensetzung wieder anzunehmen, wenn die vorherigen Wasserparameter erneut eingestellt werden können. Um existierende Aquakulturpraktiken zu verbessern, empfehlen wir regelmäßige Wasserüberwachungen, die nicht nur physikalische und chemische Wasserparameter sondern auch die Zusammensetzung der Bakteriengemeinschaften einschließen. Zu diesem Zweck sollten vor allem die Bakterien der partikulären Fraktion analysiert werden, einschließlich der Virulenzfaktoren pathogener Bakterien. Desweiteren ist die Entfernung von Schlamm aus den Teichen und die regelmäßige Zugabe von Kalk angebracht, um die Garnelenproduktion aufrecht zu erhalten bzw. zu verbessern. Demnach sollten nachhaltige Teichaquakultursysteme sowohl Sammelbecken für Schlamm als auch Vorratsbehälter mit sauberem Wasser enthalten.

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No branch can bear fruit by itself; it must remain in the vine.

CONTENTS/INHALTSVERZEICHNIS

ABBREVIATIONS

ANOSIM   Analysis of similarity

FL   free-living

I   intensive aquaculture system

IMTA   integrated multi-trophic aquaculture

MFS   microbiome of fecal string

MI   microbiome of intestine

NMDS   non-metric multidimensional scaling

OTU   operational taxonomic unit

PA   particle-associated

pir   photorhabdus insect-related protein

PCA   principal component analysis

PL   post-larvae shrimp

RDA   redundancy analysis

SI   semi-intensive aquaculture system

TEP   transparent exopolymer particle

tdh   thermostable direct hemolysin

tlh   thermolabile hemolysin

toxR   toxin regulator

WFD   white feces disease

WM   water microbiome

Chapter 1. General introduction

The importance of aquaculture activities to meet global food needs and security is increasing as natural resources are becoming more and more depleted. Apart from various fish species, also other aquatic animals have proven useful for the rearing in farming structures, including for example shrimps.

1.1 Shrimp aquaculture and shrimp farming in Indonesia

Shrimp pond farming is a promising industry in Latin America and Asia, especially South-East Asia, making countries in these areas the main shrimp producers in the world (Lebel *et al*., 2016; Wurmann *et al*., 2004). Supporting factors such as government and private sector initiatives, low production cost compare to operational cost for indoor farming, sufficient area for new shrimp farms, and a supporting annual climate for shrimp farming, have accelerated the establishment of the centers of shrimp industries (Kautsky *et al*., 2000; Lebel *et al*., 2016).

Shrimp pond farming consists of several phases, forming a standard operating procedure (SOP) for shrimp rearing. The steps encompass i) rearing preparations, which can be divided into pond preparation and shrimp fry selection, ii) shrimp rearing management, and iii) post-harvest management (Figure 1). Modern shrimp farming improves the SOP to gain optimum harvest and efficiently spend operational costs for examples by nurture **s**pecific **p**athogen **f**ree **p**ost-**l**arvae (SPF-PL) from hatcheries, intensification of culture (increase stocking density), supply of formulated feed in the proper amounts, at the right times and locations. Additionally, shrimp growth and weight gain are carefully observed, the feed conversion ratio (FCR) is respected, water quality is monitored and probiotics can be added to enhance inorganic nutrient cycles as well as to improve shrimp growth performance (Arias-Moscoso *et al*., 2018; Miao *et al*., 2017; Moriarty, 1999; Patnaik and Samocha, 2009; Smith, 2002; Tacon *et al*., 2002, 2013).

Optimum nutrient requirements as well as feed management have been thoroughly investigated in shrimp farming (Patnaik and Samocha, 2009; Tacon et al., 2013). In addition, exploration of alternative feeds has led to an invention of plant proteins or microbial protein as feed which can substitute fishmeal and fish oil in shrimp diet (Amaya *et al*., 2007; Avnimelech, 2015; Dersjant-li, 2002). These findings secure feed supply, reduce production costs, and increase shrimp growth. Furthermore, pond management related studies have been carried out aiming at maintaining pond water and soil quality, and minimizing the negative impact of shrimp farming on adjacent ecosystems. Additionally, estimations on feeding efficiencies, explorations of optimum stocking densities, determinations of required nutrients and micro-elements, and

calculations on rates of nutrient incorporation into shrimp bodies as well as quantification of inorganic nutrients in shrimp farm effluents and solid waste had been performed (Burford and Lorenzen, 2004; Funge-Smith and Briggs, 1998; Herbeck *et al*., 2013; Martin *et al*., 1998; Sahu *et al*., 2013).
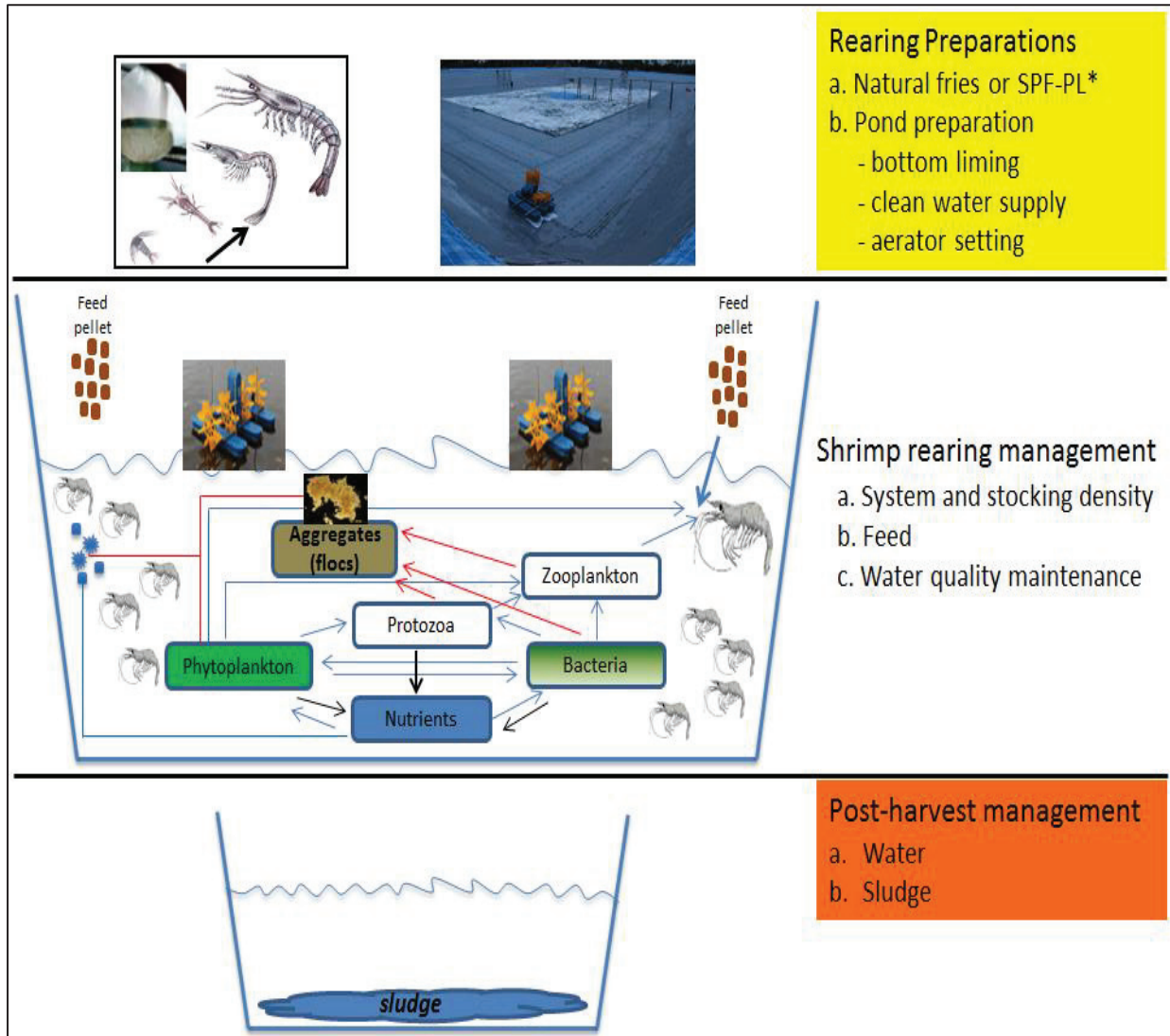
Figure 1. Illustration of shrimp rearing processes and ecological interaction in shrimp pond. The rearing processes are divided into three steps, i) rearing preparations, ii) shrimp rearing management, iii) post-harvest management. SPF-PL: specific pathogen free of post larvae *L. vannamei* where the fries are free for pathogenic virus. Above-left: arrow indicates metamorphosis of the *L. vannamei*: nauplii, protozoea, mysis, and post-larvae. Above-right: empty shrimp pond. During the rearing preparations, SPF-PL are acquired for the rearing process. Red lines indicate aggregate formations which may be composed by different particles, while blue and black lines indicate microbial loop in shrimp pond. Source of shrimp metamorphosis pictures: https://www.pinterest.com/pin/270990102550835714/, other pictures by Yustian Rovi Alfiansah

In Indonesia, shrimp farming has been conducted for more than three decades (KKP, 2018). Within this period, shrimp farming has adopted several farming systems ranging from traditional over extensive to super-intensive (Kautsky *et al*., 2000; Thong, 2014). Almost all

semi-intensive and intensive farms have shifted to the production of *Litopenaeus vannamei*, while farms with traditional, extensive monoculture and polyculture still culture black tiger shrimps, *Penaeus monodon*. Based on the world shrimp production in 2017, Indonesia was the fifth main aquaculture shrimp producer in the world, with a production of 676,000 tons from about 1.3 million hectare ponds. The main shrimp producing areas are Lampung, West Java, Central Java, East Java and South Sulawesi (Ministry of Marine Affairs and Fisheries (KKP), 2015; Shrimp News International, 2018).

1.2 Characteristics of Pacific white leg shrimp *Litopenaeus vannamei*

Litopenaeus vannamei (Figure 2)*,* a decapod crustacean*,* is one of the most important species in shrimp farming. It was originally described as an edible shrimp species in Panama (Boone, 1931) and as a native species which inhabits along the tropical Pacific coast of Latin America (Wyban *et al*., 2009). The Pacific white leg shrimp *L. vannamei* is a euryhaline shrimp, which lives in both coastal and oceanic area up to 70 m deep (Boone, 1931). It utilizes coastal lagoons during the juvenile phase and migrates offshore for subsequent maturation and reproduction (Garcia & Le Reste, 1981). Mating female have a carapace length (CL) in range from 30 to 50 mm, while for male is between 30 to 40 mm CL (Calderon-Perez *et al*., 2007). Once the females spawn shortly after mating, the larvae hatch and during a few weeks they undergo pelagic stages and experience a metamorphosis from nauplius up to juvenile (post-larvae) which consists of six nauplii stages, followed by three protozoea stages, and three mysis stages (Kitani, 1986). Subsequently, it becomes a post-larva and adopts a benthic lifestyle with the CL of post-larvae ranges from 0.88 to 3.00 mm (Kitani, 1993).
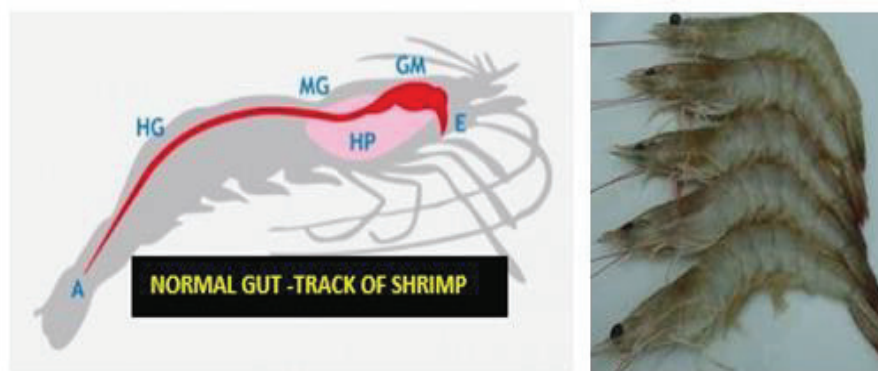
Figure 2. *Litopenaeus vannamei* and its digestive tract compartments. E: esophagus, GM: gastric mill (stomach), HP: hepatopancreas, MG: midgut, HG: hindgut, A: anus. Source of pictures: left picture: www.thefishsite.com/articles/2166/prevention-of-white-feces-syndrome-white-gut-disease-and-white-muscle-disease-in-shrimp, right picture by Yustian Rovi Alfiansah.

L. vannamei has advantageous characteristics over other shrimp species, which make this species very suitable for aquaculture (Table 1). It grows rapidly, has decent survival rates even at high density populations, possesses a wide range of tolerance for salinity, temperature, pH, and light intensity, and can be cultivated in indoor and outdoor facilities (Cuzon *et al*., 2004, Zhang P. *et al*., 2006). Moreover, fish-meal protein in their diet can be substituted by plant protein, such as soybean, wheat, and canola meal (Suarez *et al*., 2009) or bacterial protein (Ekasari *et al*., 2014) resulting in a decrease of rearing operational costs (Avnimelech, 2015).

Table 1. Comparison of size of fries, culture condition requirement (nutrient and water condition) and expected harvesting size of the Pacific white leg shrimp *L. vannamei*, the black tiger shrimp *P. monodon*, and the fresh water prawn *Macrobrachium rosenbergii*.

Parameters	*L. vannamei*	*P. monodon*	*M. rosenbergii*
PL-culture (days)	10-15	10-20	7-15
Dietary nutrient (per kg feed)			
Fiber (% kg^{-1})	1-5	< 4	5-9
Protein (% kg^{-1})	30-40	40-46	33-40
Lipid (% kg^{-1})	7-10	8-12	2-8
Ash (% kg^{-1})	7-12	16.5	10-20
Carbohydrates (% kg^{-1})	10-40	10-40	25-35
Vitamin C (mg kg^{-1} diet)	9-12	20	10
Density (PL m^{-2})	up to 150	15-40	17-25
Harvest time (days)	90-120	120-150	120-150
Water requirement			
Dissolved oxygen (mg L^{-1})	0.7-13.3	>5	> 2
pH	6-8	7.5-8.5	7.0-8.5
Salinity (ppt)	0-85	5-45	0-10
Temperature (°C)	21-37	18- 34.5	26-30
TAN (mg L^{-1})	< 42	< 3.7	< 1.5
Nitrite (mg L^{-1})	< 153	< 4.5	< 0.3
Body weight (g)			
Male	28.2-45.6	33	35-50
Female	33.5-46.8	82-97	21-27

Notes: PL: post-larvae, TAN: total ammonium-nitrogen. Data were retrieved from Cuzon *et al*., 2004, Schuler, 2008, and Volstorf & Roque, 2019.

Since 1990, this species has been widely reared in Asia and Latin America due to successful species domestication and continuous supply of specific pathogen free post-larvae/SPF-PL (Wyban, 2009). In recent decade, the Pacific white leg shrimp *L. vannamei* has been dominating shrimp productions, representing 75% of world shrimp commodities with a peak yield 3.7 million tons in 2014 (Anderson, 2016). They have undertaken other shrimp

supplies such as *P. monodon*, *P. stylirostris*, and fresh water prawn *Macrobrachium rosenbergii* since year 2004 (FAO, 2018).

1.3 Bacterial studies related to shrimp farming

Microorganisms, especially bacteria and phytoplankton, play vital roles in pond aquaculture. They live in various habitats, such as soil/sediment, water column, as well as symbiont in and on shrimp. They even structure particular ecological niches (Moriarty, 1997). Bacteria along with algae govern the oxygen content in pond through photosynthesis and respiration (Avnimelech, 2015). They also contribute significantly to the food web, the energy and nutrient flux in shrimp ponds (Azam *et al.*, 2002), as they may be directly consumed by the shrimp, or by zooplankton or protozoa on which the shrimp feed.

Aquatic bacteria have different life-styles. They occur free-living (FL) in the water columns, particle-associated (PA) or alternating between these two states (Grossart, 2010). The different life-styles entail different biogeochemical or ecological functions, which is why microbiologists commonly separate the two fractions via sequential filtration (e.g. Lyons and Dobbs, 2012). In intensive shrimp pond farming, pond water contains bacterial cells in concentration of 10^7-10^9 cell mL^{-1} (Burford *et al.*, 2003; De Schryver *et al.*, 2008).

1.3.1 Organic matter remineralization by heterotrophic bacteria

Bacteria are involved in the decomposition of organic matter and the inorganic nutrient cycles in pond systems (Avnimelech and Ritvo, 2003). Heterotrophic bacteria degrade particulate organic matter (POM) and release dissolved organic matter (DOM) and inorganic nutrients. The degradation of organic matter can occur aerobically (i.e. requiring O_2) or anaerobically (i.e. using other electron acceptors, such as nitrate or sulfate) in the water column or the sediments. During the degradation of organic matter, ammonium is released, which is further converted to nitrate by bacteria. During the nitrification process, ammonium oxidizing bacteria (AOB) such as *Halomonas* sp., *Psychrobacter* sp., *Bacillus sp.*, *Nitrosomonas* sp., *Nitrosococcus* sp., *Nitrosospira* sp., *Nitrosovibrio* sp., and *Nitrosolobus* sp., convert ammonia to nitrite (Chankaew *et al.*, 2016; Sangnoi *et al.*, 2016). Nitrite oxidizing bacteria (NOB), including *Nitrobacter* sp., *Nitrospira* sp., *Nitrospina* sp., *Nitrococcus* sp., and *Nitrocystis* sp., then convert nitrite to nitrate (Chankaew *et al.*, 2017). All mentioned bacterial processes will influence the water quality such as dissolved oxygen concentration, pH and alkalinity which then affect the rearing process in pond farming (Boyd and Tucker, 2002). Furthermore, the occurrence of pathogenic bacteria may severely threaten the survival of shrimps.

1.3.2 Shrimp diseases

Shrimp may suffer from diseases, which are caused by virus, bacteria, protozoa and metazoan parasites (Hasson *et al*., 2009; Lightner *et al*., 2012; Mastan, 2015; Sriurairatana *et al*., 2014; Walker and Winton, 2010; Zhou *et al*., 2012). Among the various shrimp diseases, viral and bacterial diseases usually lead to mass mortality events and cause great economic losses (Brun *et al*., 2009; Kautsky *et al*., 2000). While viral disease outbreaks can be prevented with the supply of SPF-PL and proper water treatment (Lakshmi *et al*., 2013; Taslihan *et al*., 2013; Wyban, 2009), lethal bacterial disease outbreaks still frequently occur and even devastate shrimp farming in some countries such as China, Malaysia, Mexico, Philippines, Thailand, and Vietnam (Dong *et al*., 2017; Hasson *et al*., 2009; Hong *et al*., 2016).

The various bacterial diseases have different signs and infection sites. The red-leg disease infects the pleopods, periopods and gills (Chen, 1992), while the white feces disease (WFD) provokes the lysis of gut tissue and induces the excretion of white fecal string (Mastan, 2015; Sriurairatana *et al*., 2014). The early mortality syndrome (EMS)/ acute hepatopancreatic necrosis syndrome (AHPND) attacks the hepatopancreas, causing necrosis of the hepatopancreatic tissue in *L. vannamei*. This disease can lead to mass mortalities of infected shrimps at the beginning of rearing (Hong *et al*., 2016; Joshi *et al*., 2014). Streptococcosis, another shrimp disease caused by bacteria, attacks the lymphoid organ (Hasson *et al*., 2009). For these diseases, pathogenic bacteria such as *Vibrio alginolyticus*, *V. campbellii*, *V. fischeri*, *V. harveyi*, *V. mimicus*, *V. vulnificus*, *V. parahaemolyticus* and *Streptococcus* sp. have been reported to be the main causative agent of bacterial diseases (Austin and Zhang, 2006; Hasson *et al*., 2009; Hong *et al*., 2016; Joshi *et al*., 2014; Mastan, 2015; Sung *et al*., 2001).

Several efforts have been proposed to minimize and to cure infected shrimps from bacterial diseases, for example through water preparation via disinfection and chlorination (Kautsky *et al*., 2000; Vaseeharan and Ramasamy, 2003). Furthermore, the application of galangal extract to treat WFD-infected or AHPND-infected shrimps has been proposed (Chaweepack *et al*., 2015). The addition of green tea extract in hatcheries can inhibit *V. parahaemolyticus* growth (Kongchum *et al*., 2016). The incorporation of microalga *Isochrysis galbana* in shrimp diet can be performed to inhibit *V. campbellii*, *V. alginolyticus*, and *V. harveyi* (Molina-Cárdenas *et al*., 2014), and in general the usage of probiotics, which prevent quorum sensing mechanism among *Vibrio*, has proven useful (Defoirdt *et al*., 2011; Farzanfar, 2006; Liu *et al*., 2010; Zheng *et al*., 2016). However, bacterial diseases remain frequently occur in various lethal infection spectrums and still lead to mass mortalities among reared shrimps (Cornejo-

6

Granados *et al.*, 2017; Han *et al.*, 2015; Hong *et al.*, 2016; Xiao *et al.*, 2017). Recently, the use of bio-flocs has shown promising advancements in the prevention and treatment of diseases in shrimp ponds (Arias-Moscoso *et al.*, 2018; Ekasari *et al.*, 2014; Hargreaves, 2013).

1.3.3 Bio-flocs in aquacultures

Particulate matter in shrimp pond water tend to congregate to form aggregates with diameter in the range of 0.1 mm to several mm (Avnimelech, 2015). The aggregates or flocs (later on called bio-flocs) are made up of a mixture of live and dead cells, including bacteria, detritus, phytoplankton, and other eukaryotic microorganisms such as fungi and protozoa (De Schryver *et al.*, 2008). The different types of particles forming bio-flocs are glued together with slimy polymers made of polysaccharides, proteins, or humic compounds (Avnimelech, 2015; Mecozzi *et al.*, 2001; Ploug, 2001) which are either produced by bacteria or algae (Gärdes *et al.*, 2011; Grossart and Simon, 2007; Mohamed, 2008).

For aquaculture, the bio-flocs provide nutritious feed for cultured animals such as shrimp or fish. They contain dry-weight protein in range from 25 to 50 % (with most estimates between 30 and 45 %), fat ranges from 0.5 to 15 percent % (with most estimates between 1 and 5 %), amino acids like methionine and lysine, vitamins and minerals especially phosphorus (Hargreaves, 2013). Nonetheless, it is unlikely that dried bio-flocs could replace animal or plant protein sources used in commercial-scale aquafeed manufacturing due to limited availabilities/ quantities (Hargreaves, 2013), but they may still be used in addition to other feed.

Bio-flocs are densely colonized by PA bacteria because they provide several benefits. The permeability of the flocs allows advective flow to pass through the pores since the water tends to follow the path of least resistance (Li and Ganczarczyk, 1992). As a result the amount of nutrient supplied to the bacteria in the flocs by mixed flow is considered to be higher as compared to the amount supplied by water flow to an individual cell. Thus, the substrate availability can increase up to a factor two (Avnimelech, 2015), which is especially relevant in oligotrophic waters. In eutrophic waters, bio-flocs may also provide important services to bacteria. They protect bacteria from detrimental impact of light, reduce grazing by zooplankton or protozoa and provide more nutrient from the water column (Avnimelech, 2015). Therefore, some beneficial bacterial which are usually added for aquaculture, for example *Bacillus* spp, *Halomonas aquamarina*, *Lactobacillus plantarum*, *Lactobacillus* spp., *Saccharomyces* spp. are added into bio-flocs to facilitate bacterial proliferation and increase the probiotic function of bio-flocs (Arias-Moscoso *et al.*, 2018; Suantika, 2013). These bio-flocs have been shown to be one

of the possible solution for feed supply as well as improvement of biosecurity in shrimp farming (Avnimelech, 2015).

1.4 Addition of carbon sources for shrimp farming

Carbohydrates in the form wheat or rice bran, tapioca, corn starch, and sugar such as molasses are usually added during shrimp rearing. They serve as an additional carbon-rich feed to satiate shrimp. The addition of carbohydrates into shrimp diet has been reported to decrease required dietary protein levels without causing weight loss in shrimps, improve the feed efficiency ratio and increase the survival rates (Shiau and Peng, 1992). However, the addition of wheat-rice bran, tapioca and starch into shrimp pond may lead to an increase of suspended particulate matter (SPM), which might lead to a decrease in water quality. Therefore, readily dissolvable carbohydrates, such as glucose, sucrose, mannose, dextrose, and molasses are preferentially chosen (Miao *et al.*, 2017; Shiau and Peng, 1992). Among these substrates, shrimps, for example *P. monodon* and *L. vannamei* incorporate molasses better than other carbohydrates such as glucose and sucrose (Shiau and Peng, 1992). Molasses, which is a side product of sugar production, contains saccharose, nitrogenous materials, and inorganic constituents (Table 2.)

Table 2. Average composition per 100 gram of molasses (wet weight)

Constituent	Beet molasses (%)	Cane molasses (%)
Water	16.5	20
Organic constituents		
Sugar: Saccharose	51.0	32.0
Glucose	-	14.0
Fructose	-	16.0
or Invert sugar	1.0	-
Raffinose	1.0	-
Non-sugars: nitrogenous materials, free and bound acids, soluble gummy substances	19.0	10
Inorganic constituents (ash)		
SiO_2	0.1	0.5
K_2O	3.9	3.5
CaO	0.26	1.5
MgO	0.16	0.1
P_2O_5	0.06	0.2
Na_2O	1.3	-
Fe_2O_3	0.02	0.2
Al_2O_3	0.07	-
Soda and carbonate residue (as CO_2)	3.5	-
Sulfate residue (as SO_3)	0.55	1.6
Chlorides	1.6	0.4

Data were retrieved from Olbrich, 1963.

1.5 Gaps of knowledge and aims of thesis

Shrimp pond aquaculture has to deal with great quantities of water, which are regularly enriched by nutrients from left-over feeds, feces and other metabolite excretions of the cultured shrimps. In addition, it contains microorganisms, such as bacteria and algae, in greater concentrations than those found in other aquatic ecosystems (Avnimelech, 2015). The measurement and identification of bacteria in shrimp farming are usually performed by cultivation based method. However, this method may fail to culture viable but non-culturable bacteria and elucidate bacterial communities (Amann *et al.*, 1995; Amel *et al.*, 2008), which may affect in shrimp rearing processes, particularly for those which provoke bacterial diseases.

Microbial processes, which transform the metabolite excretions and unconsumed feed, may affect the pond water quality. Therefore, the assessment of bacterial communities and nutrient dynamics are important to avoid rearing failure. Moreover, investigation of the causative agent of bacterial diseases has to consider whole bacterial community profile instead of targeting common pathogenic bacteria via cultivation method. For this purpose, the study on bacterial diversity, including the detection of bacterial virulence genes, seems to be appropriate to complement the pathogenic bacterial identification through cultivation method. Likewise, combined analyses of bacterial community composition and record of biogeochemical parameters during a complete shrimp rearing may enhance an understanding and optimization of shrimp farming. Still, these studies have so far been neglected.

The overarching scope of my doctoral thesis was to investigate the bacterial community and water quality dynamics in shrimp pond aquaculture systems. I compared the bacterial community composition (BCC) in water samples of different pond systems over the rearing period. I particularly identified bacterial community profiles of free-living (FL) and particle-associated (PA) bacteria using amplicon sequencing of the 16S rRNA gene. So far in shrimp aquacultures, the separation of FL and PA bacteria has been neglected. However, since bio-flocs can harbour very different bacterial communities than those found in the FL fraction (Ortega-Retuerta *et al.*, 2013; Rieck *et al.*, 2015), I aimed at highlighting the dynamic of shrimp pond water quality and the importance of studying aggregates (bio-flocs), including their distinct bacterial communities and environmental factors which shape the bacterial communities. In addition, I detected and quantified the virulence genes *toxR*, *tlh*, and *tdh*, which belong to *Vibrio parahaemolyticus*. Furthermore, I investigated bacterial dynamics and quality of aggregates in shrimp pond water after molasses addition. In parallel, I observed *V. parahaemolyticus* populations which were grown in a limited nutrient medium with the addition of aggregates of microalgae *Chlorella*

vulgaris. I hypothesized that water quality parameters fluctuate over rearing process where different shrimp farming systems affect the water quality. The differences on water quality will affect microbial dynamics, especially the abundance of potential pathogenic *Vibrio*. Moreover, because shrimp pond waters contain great amount of particulate matter (aggregates), they accumulate bacteria including pathogenic bacteria than the aggregate-free waters. I also hypothesized that the addition of molasses enhances bacterial growth and may improve aggregate quality. Lastly, *V. parahaemolyticus* may survive in nutrient limited condition if there is a supply of nutrient from microalgae aggregates.

My thesis consists of six chapters. In chapter 2, "**Aggregates in aquaculture: recent condition and challenge for Indonesian marine culture**", I provide a review of aggregates and its bacterial community composition (BCC) as well as research on the BCC in Indonesian aquatic ecosystems, particularly in shrimp aquaculture and conclude that the aggregate study in aquaculture needs to be intensified. In shrimp culture, separation of the bacterial community into the FL and PA fraction is neglected. In addition, I propose to include research on aggregates for a relatively new aquaculture technic called 'integration multi-trophic aquacultures (IMTA). In chapter 3, "**Bacterial dynamics in aggregates: a preliminary study of bacterial attachment and proliferation on aggregates in rolling tanks experiments with different nutrient availability**", I investigated the bacterial dynamics once different carbon source such as molasses was added into shrimp pond water. Moreover, I investigated the concentration of pure-single culture of *V. parahaemolyticus* which was grown in limited nutrient medium with the aggregates of *C. vulgaris* over 48 hours. In chapter 4, "**Bacterial abundance and community composition in pond water from shrimp aquaculture systems with different stocking densities**", I investigated water quality fluctuations and water microbiomes from different farming systems. In chapter 5,"**Structure and co-occurrence patterns of bacterial communities associated with white faeces disease outbreaks in the Pacific shrimp *Litopenaeus vannamei***", I explored bacterial dynamics during a shrimp disease event called "white feces disease (WFD)". Finally, in chapter 6, I discussed shrimp pond aquaculture systems, important factors which affect shrimp rearing processes, such as water quality, bacterial dynamics, and aggregates, and proposed some ideas for sustainable shrimp aquacultures.

Chapter 2. Aggregates in aquaculture: recent condition and challenge for Indonesian marine culture

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Contribution to the manuscript:

Literature review concept and design	**100%**
Acquisition of experimental data	**100%**
Data analysis and interpretation	**100%**
Preparation of figures and tables	**100%**
Drafting manuscript	**100%**

Chapter 2. Aggregates in aquaculture: recent condition and challenge for Indonesian marine culture

Abstract

Agglomerations of suspended particulate matter, known as aggregates or bio-flocs, play a major role in aquatic ecosystems. They participate in nutrient and energy fluxes and are involved in important food web processes. While comprehensive studies on aggregates are available from natural freshwater and marine ecosystems, little is known about the role of aggregates in aquacultures, particularly in shrimp pond farming. As a particle-rich system, shrimp ponds constitute interesting objects for aggregate studies, particularly with regard to the bacterial community composition, including pathogenic bacteria. Therefore, the aims of this review are i) to compile the current knowledge on the role of aggregates in aquacultures, ii) to discuss advantages and negative side effects of aggregates in aquacultures, ii) to explore the role of aggregates in disease ecology. Since South-east Asia, especially Indonesia, are among the most important countries for aquaculture activities, the focus of this review lies on Indonesian aquacultures. I conclude that aggregates, including its associated bacterial communities, are rarely investigated in Indonesian aquacultures, particularly in shrimp pond farming. Most of studies focused on bacterial cultivation and utilization of isolates for aquaculture. The understanding of the ecology of aggregates in aquacultures may support the improvement aquaculture managements and yields.

Keywords: particulate organic matter, food web, microbiome, bacteria, shrimp

Introduction

Aggregates formation is a common phenomenon in aquatic ecosystem, in lakes (fresh-water), estuaries (brackish water) and in marine ecosystems (seawater). The aggregate formation is influenced by physical, chemical and biological factors, which often affect size and type of aggregates (Alldredge and Silver, 1988). Increase of aggregate density causes an increase of the sinking sedimentation rate. Therefore, the aggregates play a major role in vertical material transport and biogeochemical processes in aquatic ecosystems (Jackson and Burd, 1998). In addition, the presence of aggregates in natural ecosystems represents a food source for benthic organisms such as mussels, oysters and clams via capture of organic particles (Ward and Kach, 2009). As a result, they supply natural feed for the animals (Crab et al., 2007). In aquatic ecosystems, aggregates can be found from surface layers to the deep sea waters (Passow et al., 2012; Simon et al., 2002).

As organic-rich particles, aggregates provide a suitable habitat for microorganisms. They exploit aggregates not only for nutrient supply, but also for protection from microbial predators such as zooplankton and protozoa, as well as from destructing physical factors such as pH, UV light and salinity (Kramer et al., 2013; Lyons et al., 2010). Aggregates have a larger bacterial abundance and higher diversity than the adjacent water column (Alldredge and Silver, 1988). Kramer *et al*. (2013) mentioned that bacterial colonization and extinction rates depend on bacterial density and diversity as well as aggregate size. They proved that the colonization rates increased linearly with the bacterial background and the aggregate size.

Aggregates represent microbial hotspots and are often densely colonized by bacteria. They may even become an effective agent for the survival and spread of pathogens (Froelich et al., 2013; Lyons et al., 2005). They may thus serve as a specific target for pathogen monitoring. Despite their role in disease ecology, aggregates may also be used to improve aquaculture practices. They influence inorganic nutrient cycles, reduce toxic substances, enhance immune systems, and improve breeding periods of cultured animal (Hargreaves, 2013).

This review compiles and critically discusses the role of aggregates in aquacultures. It consists of four sections: i) an overview about aggregates and their ecological roles, ii) methods for bacterial community identification in aggregates, iii) microbial community composition in Indonesian marine ecosystems: a case study, iv) future perspective for research on aggregates in Indonesian marine aquacultures.

I. Overview of aggregates

I.1. Formation and life span of aggregates

Aggregates are the conglomeration of living and non-living particles, which occurs in the water column of fresh, brackish and sea water. The term aggregates covers marine snow, micro- and macro-sinking particles, detritus (including amorphous detritus and phyto-detritus), flocs, and mucilage (Alldredge and Silver, 1988; Danovaro *et al*., 2009; Grossart *et al*., 2006; Kiørboe *et al*., 2003). These aggregates typically consist of inorganic particles, detrital organic particles, and biological particles (e.g. mucus feeding webs, molts, transparent exopolymer particles (TEP), polysaccharides, lignin, humic materials), phyto- and zooplankton, and microbes (Alldredge *et al*., 1998; Alldredge and Silver, 1988; Danovaro *et al*., 2009; Grossart *et al*., 2006).

The formation of aggregates is influenced by a number of physical, chemical and biological interactions, which often impact the size of aggregates (Biddanda, 1988; Kiørboe, 2001). Physical coagulation by turbulent shear causes dissolved particles to collide and to attach

(Alldredge and Silver, 1988). Floating zooplankton feces or chitinous shells collide and individual or several species of phytoplankton cells adhere (Jackson and Burd, 1998; Kiørboe, 2001). Specific interactions between phytoplankton such as *Skeletonema* and *Thalassiosira* with bacteria as for example *Marinobacter* sp. support aggregate formation (Gärdes *et al*., 2011; Grossart and Simon, 2007).

In the ocean, marine snow varies in size from millimeters to centimeters (Alldredge and Silver, 1988). They episodically exist in a large formation called "mucilage" (i.e. large mucus-rich marine snow known e.g. from the Adriatic and Tyrrhenian Sea), which can reach up to 3 meters in diameter (Precali *et al*., 2005). Specific physical conditions (i.e. water column stratification and low mixing) and biological patterns in the water column including nutrient dynamics, production of TEP by microbes and phytoplankton, and other extracellular polymeric substance (exopolysaccharide (EPS)) can lead to such huge marine snow formation which was observed in the Mediterranean sea (Precali *et al*., 2005). Interactions among bacteria, phytoplankton, and zooplankton initiate the formation of smaller aggregates (Alldredge and Silver, 1988; Simon *et al*., 2002).

The life span of aggregates depends on their sizes and environmental (water column) condition. Marine mucilage floating at the surface or within the water column can have a life span of up to 3 months, while smaller aggregates (up to 4 mm spherical diameter) may have the life span of around 1 week (Danovaro *et al*., 2009; Kramer *et al*., 2013). High density may accelerate the sinking of aggregates resulting in a decrease of the numbers of aggregates in the water column. Once aggregates settle on the sea floor, they coat the sediments. Depending on the amount of export, subsequent bacterial processes involved in the degradation of the organic matter may cause hypoxic or even anoxic conditions. Moreover, pathogens, which may be thriving in aggregates, tend to persist, reside or even proliferate on the sea floor (Kramer *et al*., 2013).

I.2. Roles of aggregates in aquatic ecosystems

Aggregates serve a variety of processes in aquatic ecosystems. For examples, they are responsible for the majority of the downward transport of organic materials as well as microorganisms by gravitational settling in aquatic ecosystems (Alldredge *et al*., 1998; Alldredge and Silver, 1988; Grossart *et al*., 2006). The aggregates provide a suitable substrate for the growth of a diverse range of prokaryotic microbial communities, whose attachments and detachments influence the microbial communities in the water column (Kiørboe, 2001). In the

water column, they can also represent a major food source for meso- and macrozooplankton grazers (Kiørboe, 2001), and increase uptake of nutrients in form of particulate matter for sessile-benthic organisms, such as oysters and mussels (Ward and Kach, 2009). During algal blooms, congregation of algae cells, either among algae cells only or between particles such as detritus or clay with algae cells may decrease the algae cell density in surface waters (Anderson, 2009). This may increase light penetration and avoid oxygen depletion in surface water layers. For several invertebrate larvae, aggregates serve a sinking vehicle, so they can settle and then undergo metamorphosis (Shanks and Del Carmen, 1997). Lastly, aggregates remove pollutants from aqueous solutions through assimilation, adsorption, biodegradation and other conjoint processes (Wu *et al*., 2012).

I.3. Aggregates in aquatic ecosystem

I.3.1. Types of aggregates in aquatic ecosystems

Aggregates can be categorized based on component materials which build them up. These materials can be living or dead cells or organic matter in the form of detritus (Alldredge and Silver, 1988). Several publications reported different major components of aggregates, which are phytoplankton-bacteria based aggregates (Gardes *et al*., 2011, Grossart *et al*., 2006), dinoflagellates based aggregates (Alldredge *et al*., 1998), diatoms-fecal pellet based aggregates (Kiorboe, 2001) and heterotrophic bacteria with oil droplet aggregates (Passow *et al*., 2012).

a. Phytoplankton-bacteria based aggregates

Aggregation of phytoplankton and bacterial cells is an important process in marine ecosystems leading to the sinking of particulate organic matter in the form of marine snow (Grossart *et al*., 2007; Grossart and Simon, 1998). The phytoplankton-bacteria aggregation is facilitated by phytoplankton exudates. Transparent exopolymer particles (TEPs) act as glue for particle aggregation. Heterotrophic bacteria may attach to phytoplankton cells. *Skeletonema costatum*, *Thalasiosira weissflogii*, and *Chaetoceros debilis* form phytoplankton-bacteria aggregates with different colonization rates (Grossart *et al*., 2004). Gärdes *et al*. (2011) proposed a bilateral model system for an interaction of the diatom *T. weissflogii* with the bacterial strain *Marinobacter adhaerens*. The aggregation mechanism appeared to be highly dependent on the bacteria meaning without *M. adhaerens*, the aggregation of *T. weissflogii* never occurred. A photosynthetically active status of *T. weissflogii* seemed to be required to produce large amounts of TEP precursor material (Gärdes *et al*., 2011). Thus, marine bacteria which actively interact with the algae may influence TEP production and increase particle aggregations (Gärdes *et al*.,

2011). In addition, Amin *et al*. (2012) described that some bacteria consistently associate with growing diatoms, while other bacteria colonize sinking diatom particles and decompose organic matter therein. Hence, heterotrophic bacteria increase aggregation of microalgae and other particles (Decho, 1990). However, striking differences in aggregation dynamics and TEP abundances were observed when diatom cultures were inoculated with either diatom-attaching or free living bacteria. Free-living bacteria might not influence aggregation whereas bacteria attaching to diatom cells may increase aggregate formation (Gärdes *et al.,* 2011).

b. Dinoflagellates based aggregates

The aggregates of dinoflagellates are very large (centimeters long), brown, sticky, and unusually strong and cohesive. They are held together with thick, viscous, mucus-like material and do not readily break apart when disturbed by a diver under water (Alldredge *et al*., 1998). The thecate dinoflagellates including *Gonyaulax polyedra*, *G. polygramma*, *G. grindleyi* and *G. koeffoidi* make up over 90% of aggregates. However, other organisms are usually available on this type of aggregates, for examples diatoms, *Ceratium*, *Dynophysis*, *Prorocentrum*, *Protoperidinium*, empty frustules of dinoflagellates, and some zooplankton fecal matters and detritus (Alldredge *et al*., 1998). Dinoflagellate marine snow differs from other types of marine snow in three major ways. First, it contains conspicuous abundances of large thecate dinoflagellates. Second, it yields more particulate organic carbons (POC), particulate organic nitrogen (PON), and chlorophyll a per unit volume than other types of marine snow. Third, it is held together by copious and viscous mucus (Alldredge *et al*., 1998).

Even though it has high mucus content, the transparent exopolymers (TEP) concentration in dinoflagellate type of aggregates is very low. Alldredge *et al*. (1998) analyzed the TEP content of *G. polyedra* in a laboratory experiment and found almost no TEP. They proposed that the mucus-like material in dinoflagellate aggregates is probably not TEP (acidic polysaccharides containing sulfated half-ester groups), but some other type of mucus. This mucus appears to be of considerably higher cohesiveness, density and stickiness than TEP. Hence, they might attach component particles together more tightly and generate more compact particles with higher mass content per unit size of aggregates.

c. Aggregates derived from larvacean houses or fecal pellet

Zooplankton such as pteropods, larvaceans and salps produce gelatinous feeding nets and houses which, together with their fecal pellets, also occasionally form aggregates (Alldredge and Silver, 1988). These spherical or elliptical houses consist of an outer mucopolysaccharide wall

16

and fine mucus nets. Then, various particles from surrounding sea water (*i.e.* phytoplankton cells, bacteria, flagellates, ciliates, fecal pellets and mineral grains) attach to the houses (Hansen *et al.*, 1996). Due to the accumulation of living organisms these houses may become sites of elevated microbial activity (Davoll and Silver, 1986), and their high particle content makes them suitable as food for zooplankton (Steinberg *et al.*, 1998). Hansen *et al.* (1996) reported that the aggregates from larvacean houses sized larger than 0.8 equivalent spherical diameter (ESD) or larger than 0.3 μm, and were composed by one abandoned house of the larvacean *Oikopleura dioica* with numerous diatom, fecal pellets, ciliates and amorphous detritus.
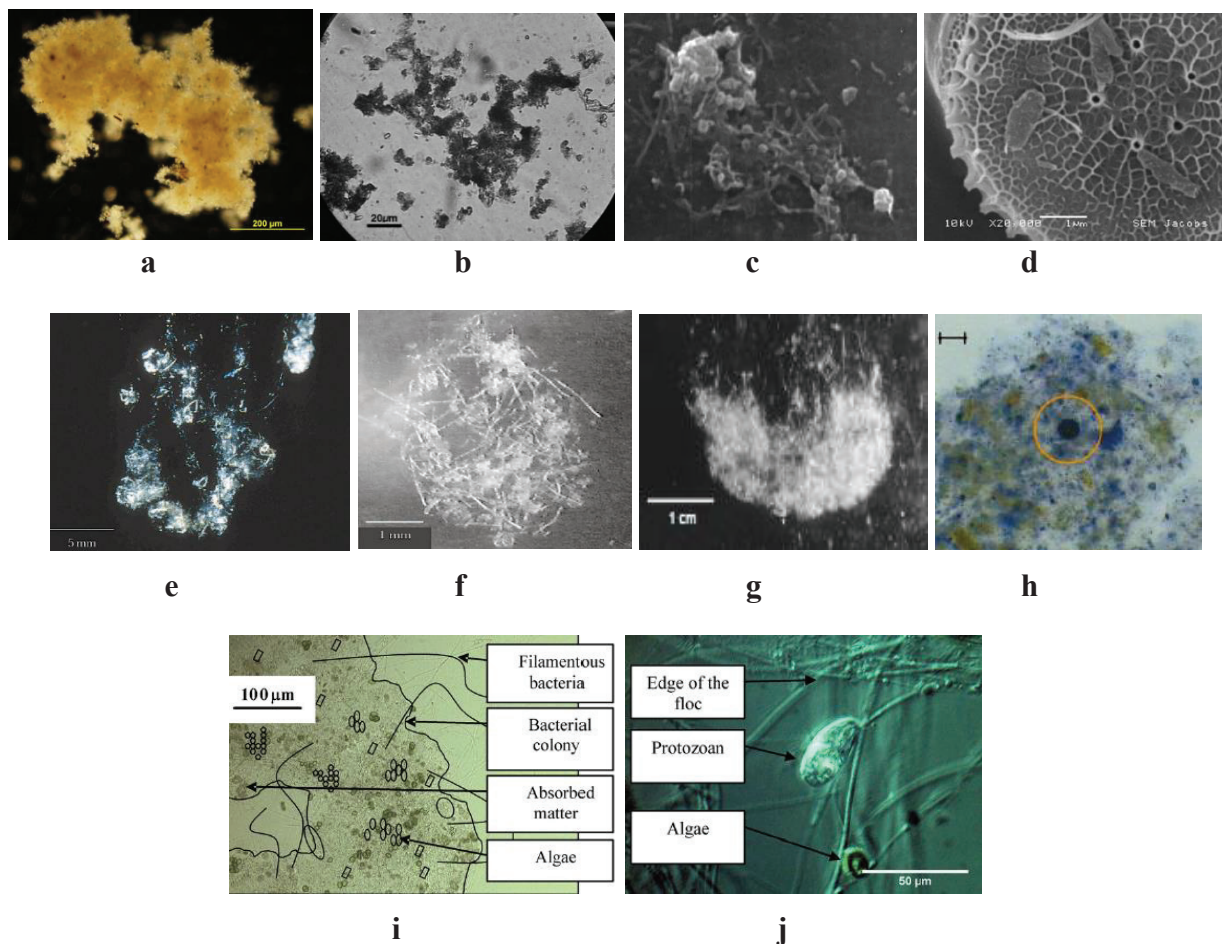
Figure 1. Different types of aggregates. a) organic marine aggregates formed in the laboratory (photo by M.M. Lyons in Lyons *et al.*, 2007), b) Micrograph of disrupted aggregates (photo by B. Froelich in Froelich *et al.*, 2013), c) Synechococcus-based aggregates (Photo by BA Biddanda in Biddanda and Pomeroy, 1988), d) Scanning electron microscopy of a *T. weissflogii* cell with four cell of HP15 strain (Photo by A. Gärdes in Gärdes *et al.*, 2011), In situ photograph of marine snow of mixed composition (e), diatom *Chaetoceros* sp (f), diatom aggregates (g) (Photo e,f,g by A. Alldredge, in Kiorboe 2001), h) photo of quahoq pathogen unknown (in dot-circle, photo by M.M. Lyons in Lyons *et al.*, 2005), i) and j) schema of bio-flocs (by P. De Schryver in De Schryver *et al.*, 2008).

17

I.3.2. Aggregates as islands for microorganisms

Bacteria in aquatic environments can be distinguished into free-living (FL), i.e. floating in the water column, and particle-associated (PA), i.e. associated with a substrate, bacteria. Colonization of aggregates by the so called "PA" bacteria is mainly determined by bacterial attachment and detachment probabilities (Kiorboe *et al.*, 2002). Bacterial aggregate dynamics are affected by physical factors, such as water movement and light penetration. Additionally, bacterial interactions, such as antagonistic activities, govern bacterial microscale dynamics on aggregates (Grossart *et al.*, 2004; Grossart and Simon, 2007). Inhibitory activity greatly influences inter-specific interactions and may impact microbial degradation and remineralization of particulate organic matter in aquatic environments (Grossart *et al.*, 2004).

a. Bacterial settlement and abundances in aggregates

In aquatic ecosystems, the adhesion of bacteria to both biotic (*e.g.* phytoplankton, macrophytes, zooplankton, benthic invertebrates, pelagic vertebrates) and abiotic particles (*e.g.* clay and sediment grains) surfaces is considered a protective mechanism to survive (Colwell *et al.*, 2003; Davies *et al.*, 1995). Thereby, bacterial attachment may reduce effects of environmental stressors (*e.g.* sunlight, changes in temperature, salinity, pH, competition and lack of nutrients) and predation which may suppress bacterial growth.

The abundance of bacteria associated with an aggregate depends on the size of the aggregate and the abundance and diversity of microbes in the ambient water (Kramer *et al.*, 2013). The concentration of heterotrophic bacteria on phytoplankton based aggregates is about 10^5-10^7 cells per aggregate (Kiørboe, 2001). In addition, Lyons *et al.* (2007) reported that the concentration of total heterotrophic bacteria (THB) in aggregates is also influenced by seasonal change, with concentration ranges of 10^3-10^7 cells mL^{-1}, while THB concentration in the water column is about 10^3-10^4 cells mL^{-1}. Once aggregates formed, free-living bacteria will attach and colonize the aggregate surface.

Aggregates are often considered to represent microscale islands in a sea of potential colonists, which can be understood with reference to the theory of island biogeography (MacArthur and Wilson, 1967 <u>in</u> Lyons *et al.* 2010). The theory suggests that bacterial colonization and extinction rates largely depend on the size of the aggregate and its distance from the source of dispersing organisms/ water (Lyons *et al.*, 2010). Originally, the island biogeography theory was developed to explain the composition of biological communities found on oceanic islands. The theory predicts a dynamic equilibrium between colonization of new

species and extinction of resident species in which the total number of species (*i.e.* species richness) is an increasing function of island size and a decreasing function of the distance to a source of potential colonizers (*e.g.* continental mainland) (Lyons *et al.*, 2010). They observed bacterial population in some individual organic aggregates in size from 1.9 to 3.7 mm with surface areas from 2.6 to 8.6 mm² and volume of 0.024 to 0.137 ml. They found that a dynamic equilibrium within the aggregates-associated microbial community was reached within 1 day and was maintained for at least 1 week. During this period, the species composition in an aggregate might change, but the bacterial numbers remained constant.

b. Aggregate associated bacterial communities vary in different ecosystems

Bacterial communities on aggregates from marine environments, lakes, fresh and estuaries are strikingly different even on low taxonomic resolution. The *Cytophaga*, *Flavobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* are the dominant bacterial groups in marine aggregates (Simon *et al.* 2002). Aggregates of diatom detritus, for example, are mostly colonized by *Gammaproteobacteria*, *Sphingobacteria*, *Flavobacteria* and *Alphaproteobacteria* (Bidle and Azam, 2001). In lake aggregates, *Betaproteobacteria* (such as *Duganella zoogloeoides, Acidovorax facilis* and *Hydrogenophaga palleroni*) and *Alphaproteobacteria* (such as *Sphingomonas* spp. and *Brevundimonas diminuta*) are the most dominant bacteria (Schweitzer *et al.*, 2001). On river aggregates, *Betaproteobacteria* constitute up to 50% of total bacterial cell numbers, followed by *Alphaproteobacteria* and *Gammaproteobacteria*, which constitute around 5 to 25% and 15 to 30%, respectively (Böckelmann et al., 2000). However, this is a generalization and other factors, e.g. seasonal changes, may cause major shifts in dominant aggregate-associated bacteria. For example, *Cytophaga* and *Flavobacteria* clusters made up less than 15% of total bacterial cells from summer to winter, but they increased up to 40% in spring (Simon *et al.*, 2002). Despite the major taxonomic differences, heterotrophic aggregate-associated bacteria are mainly involved in the degradation of the organic matter in aggregates (Grossart *et al.*, 2007; Mecozzi *et al.*, 2005).

I.4. The role of aggregates in aquaculture: are they curse or blessing?

An intense aquaculture setting coincides with considerable amounts of particulate and soluble wastes due to an excess of organic materials and nutrients from excreted metabolites (*i.e.* feces and urine) and left-over feed. These materials may cause an acute toxic effect and long term environmental risks (Piedrahita, 2003), not only for the aquaculture areas, but also for the ecosystems nearby aquaculture activities (Herbeck *et al.*, 2013). If the nitrogen and phosphorous

contents of farming effluents are continuously high, they may lead to eutrophication and changes in the ecosystem. These include for example algal blooms or the mass mortality of cultured organisms due to the depletion of dissolved oxygen (Avnimelech, 2015; Crab *et al*., 2007). Thereby, aggregates can be involved in processes, which have negative effects on the ecosystem and reared species.

Heterotrophic (aggregate-associated) bacterial activity can lead to major draw-downs of oxygen in the water column and on the sediments (Holmer *et al*., 2003). This can result in the generation of anaerobic conditions, which is often observed in sediments below farming structures. In pond farming, the anaerobic conditions may occur due to the outgassing of hydrogen sulfide, ammonia and methane (Avnimelech and Ritvo, 2003). Hence the over-fertilization of aquaculture systems with organic matter, and subsequent aggregate formation can have detrimental effects on the environment and aquaculture process. Furthermore, since they represent a suitable habitat for many pathogenic bacteria, they may be enriched on aggregates, which are fed on by the reared species.

The risk of disease for cultured animals in aquaculture often increases with low water quality, intense culture and stocking density, and particularly when polyculture is replaced by monoculture (Kautsky *et al.*, 2000). Furthermore, the presence of potentially pathogenic bacteria as well as antibiotic-resistant bacteria raises particular concern in aquaculture, where a large use of antibiotics has been common in recent years (Seyfried *et al.* 2010). Aquaculture is believed to contribute to the spread and persistence of antibiotic resistant bacteria in the environment and indeed antibiotic-resistant bacteria have frequently been detected at aquaculture sites (Tamminen *et al*., 2011, Di Cesare *et al*., 2013). Even though no antibiotic treatment was applied in aquaculture, a potential threat of antibiotic-resistant bacteria in aquaculture, especially coastal aquaculture, remains viable (Di Cesare *et al*., 2013). Antibiotic-resistant bacteria can reach aquaculture sites also via agricultural and urban wastewaters, which contain intestinal flora and pathogens of animals and humans, which are usually resistant to antibiotics (Cabello, 2006). These emerging contaminants may accumulate in the underlying sediments of aquaculture. Even in the absence of continuous antimicrobial application, antibiotic-resistant microorganisms, as well as pathogenic bacteria, can persist in protected reservoirs such as sediments or cultured animal gut. Thus, certain groups of microbes may potentially be pathogens or provoke diseases in cultured animals (Reichardt *et al*., 2013; Zhang *et al*., 2014).

Pathogenic or parasite bacteria have been found on aggregates from aquatic ecosystems and shellfish aquaculture. Quahoq hard clam (*Mercenaria mercenaria*) unknown parasites

(QPX), mesophilic pathogenic bacteria (*Vibrio cholera*, *V. vulnificus*, *V. alginolyticus*, *Aeromonas hydrophyla* and *Pseudomonas aeruginosa*) and *E. coli* were found on the aggregates from aquaculture of hard clams and oysters (Lyons *et al*., 2005, Lyons *et al*., 2007). Other studies reported that human pathogenic bacteria such as *V. parahaemolyticus* and *V. cholera* were detected on aggregates from a Bangladesh river (Colwell *et al*., 2003; Reichardt *et al*., 2013). Therefore, it is essential to analyze the presence of potential pathogenic bacteria on aggregates. The presence of pathogen-laden aggregates in areas subjected to disease outbreaks would suggest a vector for the spread and survival of pathogens between epidemics. Aggregates may then provide a specific target for environmental monitoring of those pathogens.

Aggregates have also proven to be part of the solution for some of the aquaculture related challenges. Recent aquaculture practices use aggregates, commonly known as "bio-flocs" in this context, during rearing. They provide two critical services for aquaculture. Firstly, they provide nutrition for the cultured organisms and secondly, they can treat wastes from left-over pellets (Hargreaves, 2013). Once bio-flocs form, the supply of food source in an aquaculture system naturally increases, which may provide additional sources of proteins (around 25 to 50%), fats (around 0.5 to 15 %), vitamins and minerals, and especially phosphorus (Hargreaves, 2013). An addition of extra carbons (an external carbon source or elevated carbon content of the feed) may enhance bio-floc formation (Crab *et al*., 2012). The increase of carbon contents in aquaculture systems will promote nitrogen uptake by heterotrophic bacteria (Avnimelech, 2015). Amongst those carbon sources, molasses, glucose and starch give the best bio-flocs formation (Avnimelech, 2015). The addition of molasses in zero water exchange aquaculture of shrimp *Penaeus monodon* can ameliorate shrimp culture by reducing the ammonium and nitrite concentrations, increasing the heterotrophic bacterial numbers, and improving the shrimp survival rates, the percentage of weight gain and the feed conversion ratio at optimum levels (Panjaitan, 2010). Furthermore, the application of bio-flocs in aquacultures can reduce water exchange rates in pond aquacultures. In fact, subsequent rearing can be undertaken using the same water. Therefore, this system is applied in areas where water is scarce (Crab *et al*., 2012).

In shrimp aquaculture, bio-flocs can be a novel strategy for disease management in opposite to conventional approaches such as antibiotic, antifungal, probiotic and prebiotic applications. The natural probiotic effect in bio-flocs could act internally and externally against, *i.e.*, to *Vibrio* sp., and ectoparasites, respectively. Sinha *et al*. (2008) reported that bacteria like *Bacillus sp., Alcaligenes sp.,* and *Pseudomonas sp.* may synthesize and accumulate polyhydroxyalkanoates (PHA). These polymers may comprise 16% of the bio-flocs dry weight,

21

and when they are degraded in an animal gut, they will provide antibacterial activity similar to short chain fatty acids (Liu *et al*., 2010). Studies in shrimp farming *Litopenaeus stylirostris* and *Farfantepenaeus duorarum* showed that bacteria in bio-flocs could enhance spawning performance which might be caused by better control of water quality parameters and continuous availability of food in a form of fatty acids rather than in conventional systems (Emerenciano *et al*., 2013). Crab *et al.* (2012) inoculated bio-flocs with a probiotic *Bacillus* mixture in an attempt to produce probiotic bio-flocs for improving shrimp growth performance, survival, immunity, and disease resistance. They showed that the water of shrimp tanks fed bio-flocs inoculated with *Bacillus* had an on average 5 times lower *Vibrio* population when compared to the shrimp tanks fed an artificial feed. It is possible that bacterial conglomeration mechanisms in bio-flocs involve cell-to-cell interactions (quorum sensing). However, the understanding of this mechanism is far from complete. Consequently, more studies of the microbial dynamics in the bio-flocs are required (De Schryver *et al*., 2008).

II. Methods for bacterial community identification in aggregates

Because of the limitations of bacterial cultivation (Amann et al., 1995), culture independent-based methods are necessary to be conducted to elucidate the broader microbial communities on aggregates. In addition, potential pathogenic bacterial groups such as *Vibrio* and other pathogens may exist on the viable but not cultivable phase (Colwell *et al*., 1985), causing cultivation problems. Moreover, conventional phenotypic methods for the accurate identification of different potential pathogenic bacterial species (*i.e. Vibrio, Salmonella* etc.) are problematic, especially when requiring the discrimination of closely related species (Kwok *et al*., 2002), because of the greater variability in biochemical characteristics within bacterial strains, either isolated from clinical samples or from the environment (Thompson *et al*., 2004). Therefore, bacterial identification and enumeration techniques via cultivation independent methods are as essential as bacterial cultivation.

Microbial identification and enumeration via culture independent methods have been well developed resulting in robust methods such as Polymerase Chain Reaction (PCR) and Fluorescence *In Situ* Hybridization (FISH). These methods allow tracing bacterial communities. Invention of PCR based methods such as sequencing of the 16S rRNA gene is very useful for bacterial identification and classification. However, identical sequences of 16S rRNA genes among bacterial strains causes problems on differentiating these strains into the same or different species (Nhung *et al*., 2007). Some bacterial species, for example *Vibrio mimicus* with *Vibrio cholera*, *Vibrio scophthalmi* with *Vibrio ichthyoenteri* or within *Flavobacterium* genera have

identical 16S rRNA sequences with 100% sequence similarity (Thompson *et al*., 2004). To avoid this difficulty, the use of housekeeping sequences such as protein-encoding genes has been proposed for species delineation (Stackebrandt *et al*., 2002). Modification of PCR methods into quantitative PCR allows quantifying of nucleic acids and observing a dynamic range of the nucleic acids concentration (Raso and Biassoni, 2014).

III. Bacterial community composition in Indonesian marine ecosystems: a case study

Having about 17,000 islands surrounded by shallow waters as well as deep sea areas, Indonesia provides ideal condition for a variety of tropical marine ecosystems from estuarine, mangroves, sea grass, and coral reefs to open ocean and deep-sea ecosystem. In addition, aquaculture areas are stretched out over the coastline of Indonesian waters. However, microbial studies do not yet cover these various ecosystems. They have commonly been conducted for particular ecosystems such as mangrove and coral reef ecosystems or for biotechnological purposes such as screening of metabolite compounds (Kurniawan *et al*., 2018; Murniasih *et al*., 2013; Radjasa *et al*., 2005; Sabdono *et al*., 2015).

III.1. Bacterial communities in coastal ecosystems

In the last five years, some studies used culture-independent methods to elucidate microbial communities in Indonesian coral reef ecosystems or sponge symbiont microbes (Cleary *et al*., 2018; de Voogd *et al*., 2015; Kegler *et al*., 2017a, 2017b). Kegler *et al*. (2017a) investigated the bacterial community compositions (BCC) in the water column of three coral reef habitats, which were affected by changes in environmental and water quality parameters along the eutrophication gradient. Authors investigated the BCC separately on free-living (FL) and particle-associated (PA) fractions. They found that human pathogen bacteria, for example *Gammaproteobacteria* such as *Escherichia*, *Shigella*, and *Stenotrophomonas*, *Betaproteobacteria* such as *Ralstonia*, and *Alphaproteobacteria* such as *Phenylobacterium* were dominant bacteria in the FL and PA fraction. Moreover, Kegler *et al*. (2017b) reported that bacteria such as *Gammaproteobacteria, Alphaproteobacteria*, and *Cyanobacteria* dominated the BCC in eutrophic water. They may also inhibit coral larvae recruitment on ceramic tile substrates. In addition, they also suggested that some bacterial may potentially induce coral larvae settlement in the ceramic tiles.

III.2 Bacterial communities in Indonesian marine aquaculture

Despite diverse marine aquaculture areas in Indonesia, studies on bacterial ecology including bacterial community composition in Indonesian aquaculture systems are still limited.

Most of published studies related to aquaculture were focused on the isolation, identification, and utilization on strain level. Screening of bacteria through cultivation method was done to determine pathogenic bacteria as well as to isolate bacteria, which may inhibit the pathogenic ones (Azizah *et al*., 2017; Hatmanti *et al*., 2008; Herfiani *et al*., 2010). Furthermore, some studies attempted to use bacterial isolates to improve water quality, specifically to reduce the ammonium concentration (Azizah *et al*., 2017; Suantika *et al*., 2013).

Identification of the microbial symbiont composition of fish and shrimp via 16S rRNA amplicon sequencing has been recently reported. Hennersdorf *et al*. (2016a,b) and Oetama *et al*., (2016) analyzed the microbial symbionts in cultured and wild fish *Epinephelus fuscoguttatus* and shrimp *Penaeus monodon* to compare the microbial symbionts in both types of organisms, respectively. *Hennersdorf et al. (2016b)* reported that feces samples from cultured fish *E. fuscoguttatus* revealed a highly stable distribution of several orders of bacteria such as *Vibrionales*, *Pseudomonales*, *Rhizobiales* and non-classifiable *Alphaproteobacteria*, while Oetama *et al*. (2016) described that wild *P. monodon* contained more pathogenic bacteria such as *Pseudoaltermonadales* and *Vibrionales*, e.g. *Vibrio alginolyticus* and *Photobacterium damselae*, as well as viruses, which provoke white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), and yellow head virus (YHV).

IV. Future perspective for research on aggregates in Indonesian marine aquacultures

Active marine and pond aquaculture areas in Indonesia are about 200.000 and 650.000 hectares, respectively, with seaweed, shrimp and fish as the main commodities amounting to a harvest of 6.5, 0.5, and 0.6 million tons, respectively (Kementerian PPN/Bappenas, 2014). Marine and pond aquaculture have to deal with ecological aspects such as degradation of environmental quality and disease outbreaks (KKP, 2015). Even though water monitoring as well as preventive and curative disease procedures have been established, water deterioration and diseases, especially viral and bacterial diseases have frequently occurred in Indonesian aquaculture (Kementerian PPN/Bappenas, 2014).

Aggregates might also be part of the new aquaculture system called "integrated multi-trophic aquaculture (IMTA)". In this system, several species of different trophic levels are cultivated together to optimize the use of nutrients (Buschmann *et al*., 2009). The idea of the system is to re-use the resources, especially with regard to space and feed to get more than one harvest. To achieve the objective, farmers have to cultivate main biota such as fish or shrimp, detritivore such sea cucumbers, and nutrient absorbers such as seaweed or mussels. This

promising aquaculture system was tested in an IMTA in Nusa Tenggara Barat, Indonesia, which included oyster (*Crassostrea* sp.), seaweed (*Gracilaria* sp.) and black tiger shrimps (*Penaeus monodon*) (Astriana, 2012). Unfortunately, microbial parameters have not been observed yet. Research on the aggregates can be performed in this aquaculture system, especially on the particle flux, aggregate capture and sedimentation rates, bio-fouling and pathogenic bacteria which may be available in the waters.

Aquaculture management, which includes a microbial perspective, is needed to sustain Indonesian aquaculture. The assessment of pathogenic bacteria via cultivation based method as well as single bacterial utilization in outdoor facilities seems to be ineffective to improve rearing practices. Besides the needs of rapid and precise assessments of the causative diseases, other investigations which involve microbial monitoring, particle fluxes in water column as well as carrying capacity for aquaculture activities are highly necessary to be taken. In addition, regular records of water quality parameters over rearing periods are still needed to improve rearing processes. It is expected that having holistic information about the bacterial community compositions in water column as well as biogeochemical parameters of aquaculture systems with particular emphasis on shrimp pond aquaculture will minimize rearing failures and improve both quality and quantity of production.

Conclusions and outlook

Aquatic ecosystems, particularly aquaculture sites, contain aggregates which are loaded with bacteria. They play vital roles in ecosystems, especially on the vertical flux, biogeochemical cycles, and nutrient supply. It is evident that certain pathogenic bacteria live in aggregates. Their presence may endanger aquaculture. Therefore, identification of the microbial community composition, especially pathogenic bacteria on aggregates is necessary to gain better knowledge on the abundance and diversity of potential pathogenic microorganisms. As an integral part of aquatic ecosystems, particularly in aquaculture ecosystem, aggregates may play significant roles in the sustainability of shrimp aquaculture and IMTA. Regular observation of aggregates in term of its quantity and quality, sinking rates, and microbial communities with an emphasis on pathogenic bacteria surveillance, may improve aquaculture via enhancement of cultured animal performance and reduction of operational costs and avoid rearing failure due to disease, eutrophication and water quality deterioration.

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Chapter 3. Bacterial dynamics in aggregates: a preliminary study of bacterial attachment and proliferation on aggregates in rolling tanks experiments with different nutrient availability

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Contribution to the manuscript:

Experimental concept and design	**90%**
Acquisition of experimental data	**100%**
Data analysis and interpretation	**90%**
Preparation of figures and tables	**100%**
Drafting manuscript	**90%**

Chapter 3. Bacterial dynamics in aggregates: a preliminary study of bacterial attachment and proliferation on aggregates in rolling tanks experiments with different nutrient availability

Abstract

Shrimp pond water contains high concentrations of suspended particulate matter (SPM), phytoplankton, and microorganisms. These components can be utilized to improve shrimp farming by the formation of larger aggregates which also provides a feed source and govern inorganic nutrient content in pond water. The aggregates harbor associated bacteria, including pathogenic bacteria. We conducted rolling tank experiments to generate large aggregates and analyzed bacterial attachment and proliferation of the pathogenic bacteria, *Vibrio parahaemolyticus*. In the first experiment, we added molasses, an easy dissolved sugar which may accelerate particulate matter aggregation, in tanks containing shrimp pond water and rolled them for 48 hours. We recorded water quality parameters, analyzed the water microbiome in the free-living (FL) fraction and associated to aggregates via 16S rRNA amplicon sequencing and measured macromolecules (carbohydrates, lipids and proteins) of the aggregates. Our results showed that molasses increased bacterial cells numbers, specifically in the aggregate fraction with $2.5 \times 10^7 \pm 1.0 \times 10^6$ cells mL^{-1}. Moreover, potential beneficial bacteria, so called probiotics were detected such as *Halomonas*, *Psychrobacter*, *Mesonia*, and *Chromohalobacter*, which dominated the aggregate fraction after 24 hours of aggregation. Furthermore, carbohydrate contents of aggregates increased up to 4-fold higher (43.98 ± 5.35 mJ mg^{-1}) compared to those of aggregates generated without molasses. Since potential pathogens such as *Vibrio* and *Alteromonas*, were found in the aggregates without molasses addition, we specifically conducted the second experiment using pathogenic *Vibrio parahaemolyticus* and analyzed its growth on generated aggregates with the microalgae *Chlorella vulgaris* which served as sole carbon source. *V. parahaemolyticus* with *C. vulgaris* aggregates were incubated for 48 hours. Bacterial cells in free-living, gel-like associated and aggregate associated fractions were measured using the DAPI method. Cell numbers of *V. parahaemolyticus* attached to aggregates in size greater than 3.0 μm reached 1056 cells mL^{-1} when they grew in saline Walne medium at pH 8.5 after 48 hours, while *V. parahaemolyticus* in Walne medium without *C. vulgaris* aggregates decreased constantly to 11 cell mL^{-1} at the same incubation time. We conclude that *V. parahaemolyticus* can grow on *C. vulgaris* aggregates.

Keywords: bio-flocs, particulate organic matter, bacterial colonization, water fractionation

Introduction

Shrimp ponds contain organic matter as dissolved and particulates, autotrophic and heterotrophic microorganisms, and shrimps which engage in various ecological interactions (Azam *et al*., 2002). Regular feed input results not only in shrimp growth, but also in rise of suspended particulate matter and in release of inorganic nutrients which may initiate blooms of microbes and phytoplankton (De Schryver *et al*., 2008; Funge-Smith and Briggs, 1998). Since shrimp ponds contain elevated densities of microalgae and bacteria (Alonso-Rodrıguez and Paez-Osuna, 2003; Moriarty, 1997), these microorganisms are target to improve shrimp cultivation (Avnimelech, 2015). Thus, shrimp farming may consider phytoplankton composition and the processes of the microbial loop which can support the rearing process.

Aggregation of particulate matter and bacterial dynamics on aggregates including colonization, replacement, and extinction, have been well studied (Kramer *et al*., 2013; Lyons *et al*., 2005). They depend on random motion of microorganisms, the bacterial density and diversity, physical processes, such as water movement, particle size, and sinking rate, biological process like predation from higher tropic levels (Kiørboe, 2001; Kiørboe *et al*., 2003) and production of particular substance such as exopolysaccharides which facilitate bacteria to attach onto substrates (Simon *et al*., 2002). Gärdes *et al*. (2011) demonstrated the formation of diatom-bacteria based aggregate in which photosynthetically active diatom cells, *Thalassiosira weissflogii*, and specific diatom-attaching bacteria were needed to form aggregates. Avnimelech (2015) reported that addition of carbohydrate sources such as molasses, wheat or rice bran and tapioca accelerated growth of heterotrophic bacteria as well as aggregate formation. Aggregates may attract bacteria which live freely in surrounding water column, so-called free-living (FL) bacteria, to attach onto aggregates (Kiørboe *et al*., 2003; Kramer *et al*., 2013). The FL bacteria then turn to be particle-associated (or aggregate-associated) bacterial (Grossart, 2010). Thus, aggregates provide a habitat for particle-associated bacteria, including pathogenic bacteria.

Potentially pathogenic bacteria such as *Aeromonas hydrophyla*, *Mycobacteria* sp., *Pseudomonas aeruginosa*, *Vibrio cholerae*, *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* seem to prefer living as particle-associated bacterium in contrast to a free-living physiology (Froelich *et al.*, 2013; Lyons *et al*., 2005, 2010). In addition, shrimp pond waters may contain opportunistic pathogenic bacteria (Heenatigala and Fernando, 2016; Xiong *et al*., 2015; Zhang *et al*., 2014a; Zheng *et al*., 2016) which may cause shrimp diseases (Hasson *et al*., 2009; Joshi *et al*., 2014). Among pathogens, *Vibrio* are commonly reported as the causative agent (Mastan *et al*., 2015). Furthermore, *V. parahaemolyticus* provokes lethal diseases in shrimp (Munro *et al*., 2003; Soto-

Rodriguez *et al*., 2015) and causes sea food contamination which harmed human (Makino *et al*., 2003). If aggregates harbor pathogenic bacteria, it may menace shrimp aquaculture because the shrimps may suffer disease when they consume pathogen laden aggregates.

Studies on aggregates, so called bio-flocs, for shrimp aquaculture have been usually focused on the addition of known-beneficial bacterial strains into substrates (Miao et al., 2017; Suantika et al., 2013). However, augmentation of non-halophilic probiotic bacteria may not be effective in salty pond water (Chankaew *et al*., 2017; Sangnoi *et al*., 2016). Furthermore, the indigenous bacterial community composition (BCC) is one of the important factors which affects pond aquacultures (Moriarty, 1997). Bacteria, such as *Halomonas*, *Nitrosomonas*, *Nitrobacter*, *Exiguobacterium*, *Pseudomonas*, and *Sulfitobacter*, which are involved in nitrification, organic matter decomposition and sulphur oxidation (Ray *et al*., 2010; Sangnoi *et al*., 2016; Sombatjinda *et al*., 2011; Xiao *et al*., 2017) are commonly found in shrimp pond water and sediment. They may be incorporated into suspended particulate matter (SPM) forming aggregates and serve as additional or even substitutive feed in shrimp aquaculture (Hargreaves, 2013; Ray *et al*., 2010; Suantika *et al*., 2013). These particles, commonly called bio-flocs, can be naturally generated due to water movement (Avnimelech, 2015; Hargreaves, 2013). In the present of suspended particles at size between 0.5 to 5 μm, shrimp growth rates increased 53% over growth rates attained in clear well water, while particle greater than 5.0 μm improved growth by an additional 36% (Moss, 2002). Today little is known about bacterial community changes in pond water upon aggregate formation. Therefore, we attempted to examine the indigenous bacterial community dynamics in the aggregates and free-living form from shrimp pond water after molasses addition. We hypothesize that the molasses increase bacterial numbers and affect the BCC in pond water.

Shrimp pond waters also contain algae, especially microalgae. They serve as nutrients, provide oxygen through photosynthesis and also assimilate inorganic nutrients (Kumar and Babu, 2015; Shaari *et al*., 2011; Yaakob *et al*., 2014). In hatcheries, microalgae, along with zooplankton such as rotifers, have been widely used to nourish fish or shrimp larvae (Kumar and Babu, 2015; Shaari *et al*., 2011). Among microalgae, *Chlorella vulgaris* was reported as a diet supplement in shrimp culture (Palm *et al*., 2015; Yaakob *et al*., 2014). *C. vulgaris* is rich in proteins, vitamins (B complex and ascorbic acid), minerals (potassium, sodium, magnesium, iron and calcium), β carotene, chlorophyll, and other beneficial substances (Tokusoglu and Ünal, 2006). These cellular compounds make the microalga a prominent meal replacement in the diet of fishes and shrimps. Moreover, partial replacement of fish meal by *C. vulgaris* can enhance shrimp growth,

for example in freshwater prawn, *Macrobrachium rosenbergii* (Radhakrishnan *et al*., 2015) and *Litopenaeus vannamei* (Pakravan *et al*., 2018).

This study aims (i) to generate aggregates after addition of molasses in shrimp pond waters and therein changes in bacterial communities towards beneficial bacteria, and (ii) to investigate the attachment and growth of the pathogen *V. parahaemolyticus* on generated *C. vulgaris* aggregates.

Materials and Methods

Experimental design

Two rolling-tank experiments were conducted in Diponegoro University, Semarang, Indonesia and the Leibniz-Zentrum für Marine Tropenforschung (ZMT) Bremen, Germany, on November 2016 and March-May 2017, respectively.

Experiment 1. Bacterial dynamics in aggregates from shrimp pond water with molasses addition

12 liters of pond water at 60th day of shrimp rearing were collected from a shrimp pond in Rembang Regency, Central Java, Indonesia (-6°42`11.66" S 111°21`54" E) on November 2016 and placed into cylindrical tanks (1 L per tank). 6 tanks served as control and other 6 tanks were treated with the addition of 1 mL (equivalent to 1.4 g dry weight mL^{-1}) molasses per tank. All tanks were rolled in roller tables (Gärdes *et al*., 2011) to generate aggregates at 12 rpm per minute for 48 hours in room temperature (29°C) and darkness. Samplings were conducted at 24 and 48 hours by sacrificing 3 tanks per treatment.

Aggregate collection

Clumps of aggregates in each tank were collected using disposable pipettes, placed in 15 mL centrifuge tubes, and settled for 15 minutes. Water in upper layer of collected aggregates was discarded. Total aggregates were weighted using an analytical balance (ME 36S, Sartorius, Göttingen, Germany). From these aggregates, 100 µL were used for bacterial counting for aggregate fraction using a DAPI method as mentioned above, while 500 µL were used for bacterial community analysis on particle-attached (aggregates). Both samples were filtered on 3 µm, ø 47 mm polycarbonate filters. Remaining aggregates and filters were stored in -20°C until macromolecule content (such as C/N, carbohydrate, lipid and protein content) and bacterial analyses (genomic DNA extraction and cell counting), respectively, while water in each tank

(macro aggregate-free water) was used for water quality and bacterial community analyses for the free-living (FL) fraction which were explained below.

Water quality measurements

Water parameters (inorganic nutrients, physical parameters, suspended particulate matter (SPM), total carbon/nitrogen (C/N) , C-organic carbon (Corg) content, and transparent exopolymer particles (TEP),) were measured as follow, the inorganic nutrient measurements were done in triplicates according to the colorimetry method by Strickland and Parsons (1972), with a Shimadzu UV-1800 spectrophotometer. Physical water parameters such as salinity, temperature, and pH were measured *ex-situ*, using calibrated Manta Eureka 2 multi-probes (Eureka Environmental Engineering, Texas, USA). SPM was measured as dry mass on pre-combusted GF/F filters (porosity 0.7 μm, ø 47 mm, VWR, France) in triplicates after filtration of 500 mL water sample. Weight of the filters (SPM) was determined using a precision balance (ME 36S, Sartorius, Göttingen, Germany) after drying the filters for 24 h at 40°C. Half of the filter were cut and used for total carbon (TC) and total nitrogen (TN) measurement, while another half was for particulate organic carbon (C-org) measurement. The C-org was measured after acidification of the filter with 1N HCl to remove the inorganic carbon. CN ratios were calculated from C-org and TN. Total carbon (TC), total nitrogen (TN), and C-org concentrations were measured on an Elemental Analyzer (EA-3000, EuroVector, Italy). TEP analysis was performed for 100 mL macro aggregate-free water filtered onto 0.4 μm pore size polycarbonate filters (Whatman, Dassel, Germany), using a spectrophotometric method introduced by Alldredge *et al*. (1998), with an updated protocol by Engel (2009). Briefly, this method relates the adsorption of an Alcian blue dye to the weight of polysaccharides filtered on 0.4 μm pore size polycarbonate filters (Whatman, Dassel, Germany). A calibration curve was prepared using the reference polysaccharide gum xanthan from *Xanthomonas campestris* cultures.

Organic macromolecule (C/N total, carbohydrates, lipid and protein) content of aggregates

Samples for the organic macromolecule analyses were prepared from harvested aggregates. Nutrient analyses were done in triplicates, except for C/N measurement. 2 mL of aggregates were centrifuged at 1000x *g* for 5 minutes to form a pellet. Wet weight of the pellet were measured using a precision balance (ME 36S, Sartorius, Göttingen, Germany). Due to insufficient amount of pelleted material for C/N total measurement, samples from replicates were pooled and then analyzed as mentioned before. Carbohydrate and lipid measurements were performed according to Van Handel (1965), while protein analyses were done according to

Bradford (1976). Samples were prepared as follow: pellets of aggregates were homogenated in phosphate buffer (pH 7.4) in dilution factor 1:5 to 1:6. Homogenates were then sonicated for 2 minutes using a sonicator (Sonophis Bendelin HD 3100, Germany). From these, 150 µL of homogenate was collected for carbohydrates and protein measurements, while 150-300 µL of homogenate was collected for lipids.

Samples for carbohydrates and proteins were precipitated using 50 µL of 15%-trichloro-acetic acid (TCA), incubated in -20°C for 10 minutes and centrifuged at 1000x g at 4°C for 10 min. Supernatant was collected for carbohydrate measurement, while the pellet was dissolved in 1 M NaOH for protein measurement. Serial dilution of D-glucose and bovine serum albumin (BSA) served as standard curve for carbohydrates and proteins, respectively (Supplementary information 1). For carbohydrates, 50 µL of the 5% phenol and 200 µL of concentrated sulphuric acid (98% H_2SO_4) were added into 50 µL supernatant, followed by incubation at room temperature (27°C) for 30 minutes, while for proteins 270 µL Bradford reagent was added into 30 µL of adjusted sample (pH 6-8) and then incubated as previous condition. Optical density of the samples was measured using a spectrophotometer (Infinite M200 Pro, Tecan, Germany) at 492 nm and 592 nm for carbohydrates and proteins, respectively.

Lipid fraction from 150 µL homogenates was extracted with 250 µL chloroform and 250 µL methanol and centrifuged at 1000x g for 5 minutes. The extracts were transferred into glass tubes, followed by addition of 500 µL 98% H_2SO_4. Samples were incubated in an oven at 200°C for 20 minutes, and added with 1.5 mL distillate water after samples reached room temperature. 300 µL samples were measured spectrophotometrically using the spectrophotometer at 375 nm.

Bacterial cell measurement

For both experiments, bacterial cell numbers were counted using a 4',6-diamidino-2-phenylindole (DAPI) staining method according to Kepner and Pratt (1994). Briefly, filters containing bacterial cells were stained with 1 µg mL^{-1} of DAPI solution for 5 minutes, then washed in 80% ethanol and rinsed with sterile distillated water. Stained filters were air dried in the dark for 30 minutes, and then mounted with 10 µL of mounting solution consisting of 3:1 Citifluor AF mounting medium (Citifluor Ltd, London, UK) and Vecta shield (Vector Laboratories Inc., Burlingame, USA). Bacterial cells observation was performed with a fluorescence microscope *Axio Imager.D2* (Zeiss, Jena, Germany) at 1000x magnification. Bacterial cell abundance was calculated from 30 photos per filter, using the free software *ImageJ* (https://imagej.nih.gov/ij/index.html, Ducret *et al*., 2016).

Samples for bacterial community analyses

Bacterial community compositions from the particle-associated (PA) fraction were collected from aggregates as mentioned above. Free-living bacteria (FL) fraction was collected by filtering filtrate of TEP samples on 0.2 μm polycarbonate filter with a diameter 47 mm and 25 mm in amount 100 mL and 200 μL for bacterial community analysis and cell counting, respectively. The filters were then stored in -20°C until analyses.

Molecular analysis of bacterial communities

Genomic DNAs from aggregate and free-living fraction of water samples were extracted using the phenol-chloroform-isoamylalcohol method, according to Nercessian *et al*. (2005). DNA pellets were dissolved in 40 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5). DNA concentrations were measured photometrically and checked for purity (ratio of light absorption at 260 to 280 nm) using a nanoquant plate reader (Infinite M200 Pro, Tecan, Germany). Genomic DNAs in concentration between 0.2-100 ng μL^{-1} were then sent for 16S rRNA gene amplification and sequencing at LGC genomics (Berlin, Germany).

16S rRNA gene amplification was performed from genomic DNA extracts. DNA sequences of the V3-V4 hypervariable region of the 16S rRNA gene were obtained from amplicon sequencing with the primer set S-D-Bact-0314-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') / S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAAKCC-3') (Klindworth *et al*., 2013). Sequencing at LGC genomics (Berlin) was done on an Illumina MiSeq using the V3 Chemistry (Illumina) in a 2x300 bp paired-end run. Demultiplexing, i.e. grouping of sequences by sample, and the removal of the primer sequences from the raw paired-end reads were performed by LGC genomics (Berlin, Germany). Sequences were quality trimmed with a sliding window of 4 bases and a minimum average quality of 15 with *trimmomatic* v.033 (Bolger *et al*., 2014). Quality trimmed sequences were merged using PEAR v0.9.8 (Zhang et al., 2014b). Minimum Entropy Decomposition (MED) was used to cluster sequences (Eren *et al*., 2013; Ramette and Buttigieg, 2014). MED applies the principle of oligotyping (Eren *et al*., 2013), of which Shannon entropy was used to iteratively partition amplicons at single nucleotide resolution, which provide more accurate descriptions of closely related but distinct taxa (Utter *et al*., 2016). During MED, we used a minimum substantive abundance (-M) of 50 read numbers to filter low-abundant OTU with the decomposition of one nucleotide position at a time (-d 1). For each OTU, one representative sequence (node) was taxonomically classified with SINA (SILVA Incremental Aligner) v1.2.11 using the SILVA rRNA project reference database (SILVA v.128) at a

minimum alignment similarity and quality of 0.9 and a last common ancestor consensus of 0.7 (Pruesse *et al*., 2012). Unwanted lineages (such as archaea, chloroplasts, and mitochondria) were removed.

Experiment 2. Growth performance of *Vibrio parahaemolyticus* (*V. parahaemolyticus*) in limited nutrient media with *Chlorella vulgaris* (*C. vulgaris*) aggregates.

Pathogenic *Vibrio parahaemolyticus* DSM 11058 was obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen (*DSMZ), Braunschweig, Germany, while axenic *Chlorella vulgaris* SAG 211-12 was provided by culture collection of algae, Georg August University, Göttingen, Germany.

V. parahaemolyticus was grown in 100 mL alkaline peptone water (APW) for 24 hours. 10 ml of *V. parahaemolyticus* culture were taken and centrifuged at 1844x *g* for 10 minutes to obtain bacterial pellet. The pellet was then washed twice with 1 mL PBS to remove remaining nutrient from APW medium. Pellet was dissolved in 1 mL Walne medium (Walne, 1970) which then served as *V. parahaemolyticus* inoculum for attachment experiment. Bacterial numbers in the inoculum was determined by filtering 1 mL of *V. parahaemolyticus* inoculum. The inoculum sample was filtered on a 0.2 μm polycarbonate filter with diameter 25 mm (Whatman, Dassel, Germany) and counted using DAPI method as mentioned previously.

Axenic cultures of *C. vulgaris* was grown in 500 mL Walne medium (Supplementary Information 2) at 28°C for two weeks. After two weeks, *C. vulgaris* cells were counted using Fuchs Rosenthal counting chamber (Thermo Scientific, Braunschweig, Germany) under light microscope (Axio Zeiss, Germany). 250 ml of culture were placed in a cylindrical tank. Volume was adjusted with Walne medium until fully filled without air bubbles. Cultures were rolled for 48 hours at 12 rpm per minute to generate aggregate formations. Aggregate of *C. vulgaris* was harvested and used for attachment experiment.

Walne medium with different pH (6.5, 7.5, and 8.5) and salinity (0 and 35 ppt) levels were prepared. 100 mL of Walne medium from each level combination were placed in 250 ml Erlenmeyer. *C. vulgaris* aggregates and *V. parahaemolyticus* cells were then added in amount 500 μL and 100 μl, respectively. Co-cultures were incubated for 48 hours. Sampling was done at 6, 24, and 48 hours by sacrificing the co-cultures. For control, *V. parahaemolytiocus* and aggregate of *C. vulgaris* were placed in Walne medium separately and sampled at respective sampling points.

Determination of bacterial cells was performed in 3 fractions, aggregates (using 3 µm, ø 47 mm polycarbonate filters), gel-like material (polysaccharides particles/exudates, using 0.4 µm, ø 47 mm polycarbonate filters), and free-living fraction (using 0.2 µm, ø 47 mm polycarbonate filters). For bacterial cell numbers from exudate and free-living fraction, a subsequent filtration was done for aggregate free-culture. Bacterial cell numbers were counted using DAPI method mentioned above.

Statistical analyses

For first experiment, water quality, including physical parameters, inorganic nutrients, and bacterial cell numbers as well as organic nutrient content (carbohydrates, lipid, and protein) in aggregates was tested with one-way ANOVA, followed by Tukey-HSD post-hoc test. Bray-Curtis dissimilarity values were calculated to evaluate dissimilarity in the BCC. Bacterial cell numbers from second experiment were tested using Multivariate Anova (MANOVA) followed by ANOVA as post-hoc MANOVA test. All statistical analyses were performed in R (R version 3.4.2, R Core Team, 2017, using R Studio v.0.98.1056) with the packages vegan (Oksanen *et al.* , 2017).

Results

Experiment 1. Bacterial dynamics in aggregates from shrimp pond water with molasses addition

Water quality parameters

The addition of molasses and the herein generation of aggregates affected water quality and bacterial dynamics. In tanks with molasses addition, suspended particulate matter (SPM) decreased from 35.7 ± 1.9 mg L^{-1} to 4.5 ± 1.8 mg L^{-1}, while SPM in control tanks decreased to 16.1 ± 3.9 mg L^{-1}. Transparent exopolymer particles (TEP) declined drastically in both treatments after 24 hours from 7.7 ± 0.3 mg L^{-1} to 1.4 ± 0.3 mg L^{-1} and 0.8 ± 0.2 mg L^{-1} for molasses and control tanks, respectively. pH in molasses treatment increased up to 8.17, which was moderately higher than the initial pH (7.91), while pH in control tanks decreased to 7.55 . After 48 hours, salinity slightly decreased to 32.64 and 33.07 in molasses and control tanks, respectively. Moreover, C-total and N-total in pond water with molasses increased up to 10 fold higher after 24 hours (Table 1). Inorganic nutrient concentrations, except phosphate and silicate decreased drastically in molasses tanks after 48 hours. Concentration of toxic inorganic nutrients such as N-ammonium and N-nitrite decreased below 0.1 mg L^{-1} in molasses treatment. Salinity, SPM, C-org and ammonium were among measured quality parameters which differed significantly between tank with molasses and control (one-way ANOVA, Table 1).

Table 1. Physical parameters, carbon and nitrogen content and inorganic nutrient concentrations in water from rolling tank experiment. Differences in ANOVA were written in bold; superscript letters indicate significant differences after pairwise t-test. Data are shown as mean ± standard deviation.

Parameters	Initial pond water	Control 24 h	Control 48 h	Molasses 24 h	Molasses 48 h	Sig.
		Samples				
Temp. (°C)	30.24 ± 0.09	29.43 ± 0.09	29.32 ± 0.02	29.36 ± 0.03	29.31 ± 0.02	
Salinity (psu)	**33.64 ± 0.17^a**	**33.31 ± 0.05^{ab}**	**33.07 ± 0.18^b**	**32.75 ± 0.21^c**	**32.64 ± 0.07^c**	***
pH	**7.91 ± 0.02^a**	**7.55 ± 0.09^{ab}**	**7.2 ± 0.05^b**	**8.17 ± 0.09^c**	**7.97 ± 0.04^a**	***
TEP (mg L^{-1})	**7.7 ± 0.4^a**	**0.8 ± 0.2^b**	**1.8 ± 0.3^c**	**1.4 ± 0.3^c**	**3.4 ± 1.7^d**	***
SPM (mg L^{-1})	**35.7 ± 1.9^a**	**18.0 ± 2.4^b**	**16.1 ± 3.9^b**	**5.1 ± 1.9^c**	**4.5 ± 1.8^c**	***
C-org (% dry weight SPM)	**2.4 ± 0.2^a**	**5.0 ± 0.7^a**	**3.1 ± 0.6^a**	**7.5 ± 2.2^b**	**7.6 ± 0.5^b**	***
C total (% dry weight SPM)	2.57 ± 0.58	19.38 ± 25.09	2.74 ± 0.56	25.45 ± 9.91	30.18 ± 14.58	
N total (% dry weight SPM)	0.38 ± 0.07	3.29 ± 4.41	0.47 ± 0.12	4.48 ± 2.08	5.27 ± 2.80	
C:N ratio	1:7	NA	1:6	1:6	1:6	
Inorganic nutrients (mg/L)						
Ammonium	**0.63 ± 0.09^a**	**0.39 ± 0.13^b**	**0.17 ± 0.06^c**	**0.07 ± 0.04^d**	**0.007 ± 0.002^e**	***
Nitrate	**0.68 ± 0.27^a**	**0.03 ± 0.02^b**	**0.001 ± 0.0008^c**	**0.01 ± 0.009^b**	**0.004 ± 0.003^d**	***
Nitrite	**0.03 ± 0.003^a**	**0.12 ± 0.04^b**	**0.002 ± 0.001^c**	**0.01 ± 0.007^a**	**0.002 ± 0.0002^c**	***
Phosphate	1.46 ± 0.89	0.55 ± 0.15	0.56 ± 0.04	0.63 ± 0.03	0.52 ± 0.03	***
Silicate	**0.76 ± 0.33^a**	**0.25 ± 0.02b**	**0.27 ± 0.03^b**	**0.53 ± 0.09^a**	**0.37 ± 0.03^b**	**

Notes: Sig.: significance p-value in ANOVA; *** : <0.001, *** : <0.01. NA: Not applied due to high standard deviation of samples

Total aggregates with molasses varied in range 6.47 ± 0.50 to 6.57 ± 0.31 mL per 1 L pond water, which were equivalent to 77.17 ± 18.30 to 93.82 ± 25.39 mg of wet weight of the aggregates, while in control tank, total aggregates were 6.40 ± 0.79 to 6.93 ± 0.32 mL, which was equivalent to 86.26 ± 24.46 to 119.74 ± 26.37 mg of wet weight of the aggregates per 1 liter pond water.

Molasses contained total carbon and total nitrogen of 34.71± 0.60 and 0.21 ± 0.04 % per mg dry weight, respectively. They contained proteins and lipids in quantities 9.22 ± 1.56 and 81.68 ± 5.43 mJ mg^{-1}, correspondingly. Once the molasses were added into pond water, concentrations of lipid, total C and total N in aggregates with molasses increased to 16.0% and 2.9% per mg of

aggregates in 1 L pond water after 48 hours, respectively, which were slightly higher than those in aggregates without molasses (Table 2). In addition, molasses increased carbohydrate content in the aggregates up to 4-fold higher (44.0 ± 5.4 mJ carbohydrates mg^{-1}aggregates). In contrast, protein and lipid contents in the aggregates with molasses did not differ from those in the aggregates without molasses (Table 2).

Table 2. Volume and macromolecule contents (carbohydrates, lipids and proteins) in molasses and aggregates

Parameters		Aggregates				p-value
		Control		With Molasses		
	Molasses	24 h	48 h	24 h	48 h	
Volume (mL L^{-1})	1	6.4 ± 0.8	6.9 ± 0.3	6.6 ± 0.3	6.5 ± 0.5	
Wet weight (g)	1.2 ± 0.2	$86.3 \pm 24.5*$	$119.7 \pm 26.4*$	$77.2 \pm 18.3*$	$93.8 \pm 25.4*$	
C total (% per mg dw)	34.7 ± 0.6	10.1	13.7	12.0	16.0	NA
N total (% per mg dw)	0.21 ± 0.04	1.7	2.2	1.9	2.9	NA
Carbohydrates (mJ mg^{-1})	> 100	13.6 ± 2.7^a	9.7 ± 1.8^a	9.4 ± 8.3^a	44.0 ± 5.4^b	<0.01
Lipid (mJ mg^{-1})	81.7 ± 5.4	58.9 ± 2.8	69.0 ± 7.5	89.3 ± 36.5	56.1 ± 9.5	0.22
Protein (mJ mg^{-1})	9.2 ± 1.6	30.0 ± 5.4	50.8 ± 5.9	49.2 ± 27.3	71.7 ± 11.9	0.06

Notes: *: aggregates after centrifugation at 1000xg for 5 minutes, dw: dry weight, C and N content is from a pool of triplicates. Superscripts after values indicate a significant difference

The water movement which formed aggregates and the addition of molasses influenced bacterial numbers as well as bacterial community compositions. The addition of molasses increased the bacterial cell numbers in the particle-associated (PA) fraction, as well as in the free-living (FL) fraction. After 24 hours, bacterial concentrations in the PA and the FL were higher in pond water with molasses, in amount of $2.5 \times 10^7 \pm 1.0 \times 10^6$ and $5.5 \times 10^6 \pm 2.0 \times 10^5$ cell mL^{-1}, respectively. However, the cell numbers decreased significantly to $4.5 \times 10^6 \pm 1.0 \times 10^5$ cells mL^{-1} in the FL fraction, while the cells numbers in the PA fraction stayed steady thereafter (Table 3).

Table 3. Concentrations of bacterial cells in free-living and aggregates from rolling tank experiment with and without molasses.

Fraction	24 hours		48 hours	
	Control	Molasses	Control	Molasses
Aggregates (in 10^7 cell/mL)	1.6 ± 0.2^a	2.5 ± 0.1^b	2.2 ± 0.1^b	2.3 ± 0.2^b
Free-living (in 10^6 cell/mL)	3.9 ± 0.2^a	5.5 ± 0.2^b	3.3 ± 0.1^c	4.5 ± 0.1^d

Water microbiome

Bacterial community composition (BCC) in pond water changed with molasses addition. Dominant BCC in PA (>3 µm particles) and FL to smaller particles (0.2 - < 3 µm) fraction of initial pond water were *Halomonas* and *Salegentibacter* (Figure 1). After 24 hours incubation, the BCC in the FL and the PA fraction differed from the initial BCC. *Alteromonas* and *Vibrio* became dominant bacteria in the PA fraction (aggregates) of pond water without molasses addition. In contrast, the FL fraction shared relatively similar bacterial OTUs proportions without any dominant OTUs genera at the same sampling time. In water with molasses addition, *Halomonas* and *Sulfitobacter* were dominant bacteria in the PA fraction, while *Chromohalobacter*, *Mesonia*, *Halomonas*, and *Vibrio* dominated the FL fraction at 24 hours. After 48 hours, bacterial shifts occurred in the PA and FL fraction in both water samples. In water without molasses, *Halomonas* became the dominant bacteria in the PA fraction, while *Alteromonas*, *Vibrio* and OTUs of unclassified *Rhodobacteriaceae* dominated the FL fraction. In water with molasses, *Halomonas* and *Salegentibacter* were the dominant bacteria in the PA fraction, while *Mesoflavibacter*, *Sulfitobacter* and OTUs of unclassified *Sphingomonadaceae* dominated the FL fraction (Figure 1).
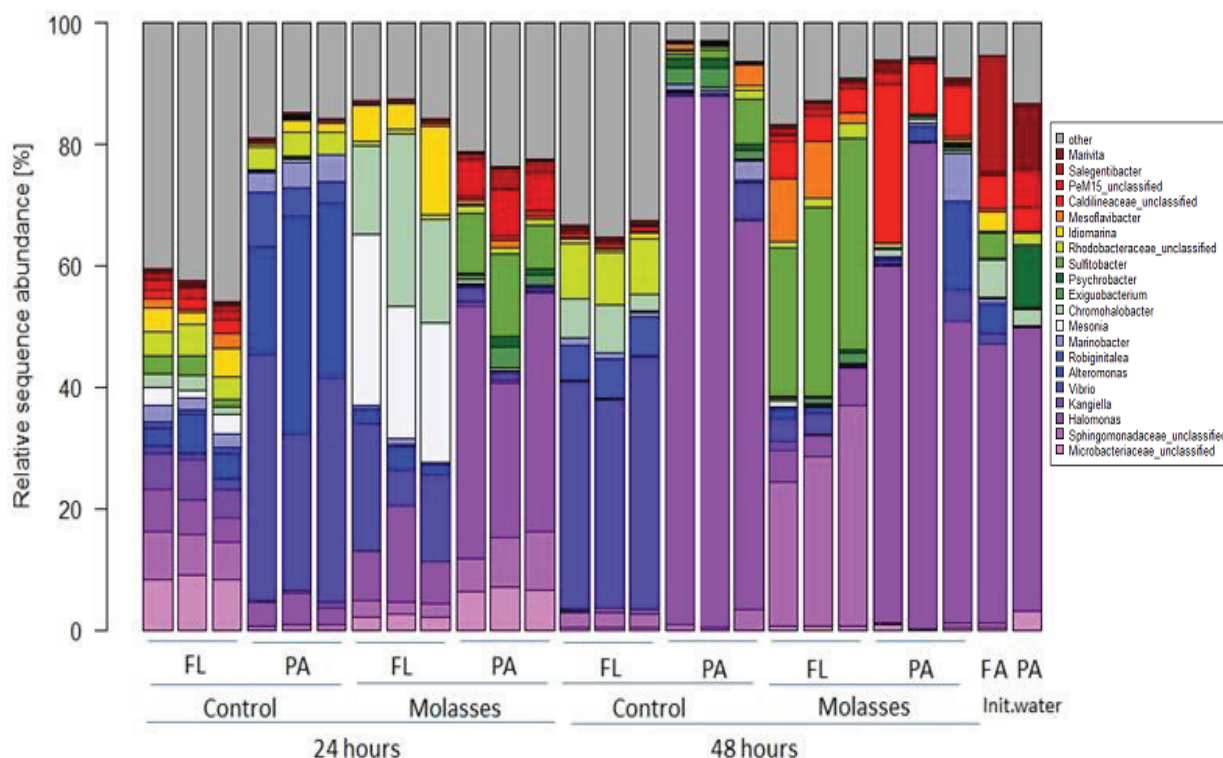
Figure 1. Contribution of the most abundant bacterial Operational Taxonomic Units (OTUs) in free-living (FL) and particle-attached/aggregate (PA) fractions in pond water without molasses (control) and with molasses within 48 hours. Taxonomic affiliation for OTUs is provided for genus level. The bacterial community composition in FA of initial water (Init.water) was bacteria which were available in free-living and particle in range > 0.2 to < 3 μm, while in the FL of the control and the molasses was bacteria which were available in range 0.2 to 0.4 μm. The PA fraction included bacteria which attached to particles larger than > 3μm

Bacterial community compositions (BCC) in FL and PA fraction for both water samples differed over the time with the dissimilarity values higher than 0.6 (Table 4). Moreover, the BCC of the same fraction and treatment were dissimilar between the BCC at 24 and 48 with the Bray-Curtis dissimilarity values higher than 0.8 (Table 4).

Table 4. Bray-Curtis dissimilarity values of bacterial community composition in free-living (FL) and particle-attached (PA) fraction of water without molasses (control/C) and with molasses (M) at 24 (1d) and 48 hours (2d)

Samples	C_FL-1d	C_PA-1d	C_FL-2d	Samples	M_FL-1d	M_PA-1d	M_FL-2d
C_FL-1d				M_FL-1d			
C_PA-1d	0.80			M_PA-1d	0.88		
C_FL-2d	0.79	0.68		M_FL-2d	0.89	0.63	
C_PA-2d	0.86	0.90	0.94	M_PA-2d	0.87	0.86	0.86

Experiment 2. Growth performance of *Vibrio parahaemolyticus* in limited nutrient media with *Chlorella vulgaris* aggregates.

Vibrio parahaemolyticus numbers on *Chlorella vulgaris* aggregates fraction increased in all treatments (pH of 6.5, 7.5 and 8.5, and salinity 34 and 0). Due to limited equipment, experiments were done in two rounds with three combined levels of Walne medium (LW) per experiment. First run covered three conditions which were salinity 34-pH 7.5, salinity 34-pH 8.5 and salinity 0-pH 7.5, while second run were salinity 34-pH 6.5, salinity 0- pH 6.5, and salinity 0-pH 8.5. Bacterial inoculates for first and second run contained 726 ± 20 cells mL^{-1} and 678 ± 43 cells mL^{-1}, respectively. After 48 hours *V. parahaemolyticus* cells in control (FL fraction) decreased to 11 ± 2 cells mL^{-1} (Figure 2) and 88 ± 31 cells mL^{-1} (Figure 3). *V. parahaemolyticus* decreased over the time in every level of Walne medium without aggregates of *C.vulgaris* (control). In general, most abundant *V. parahaemolyticus* cells were obtained in aggregate fraction, followed by polysaccharide and free-living fraction (Figure 2 and 3). Furthermore, saline Walne medium with pH 8.5 yielded highest *V. parahaemolyticus* numbers, in concentration 1056 cells mL^{-1}.

Due to only one bacterial fraction in control, analyses of multivariate Anova (MANOVA) were performed by excluding values in the control. In the first and second run, bacterial numbers in three fractions differed significantly in each variable. Anova which served as posthoc test MANOVA indicated that bacterial abundances were different in all fractions in first run, while in second run, bacterial numbers were different only in polysaccharides-attached and aggregates fraction (Table 5).

Table 5. Significance of *Vibrio* abundance at tested variables (levels of Walne medium (LW), incubation time (IT), and interaction of LW and IT) in MANOVA test (Pillai) followed by ANOVA test as a post-hoc MANOVA for bacterial abundances in 3 different fractions.

Experiments	Fractions (filter sizes in μm)	Variables	Df (numdf, dendf)	*F*-value	*P*- value
Run1 (MANOVA)		LW	2 (6,34)	0.70	0.02
		IT	2 (6,34)	0.95	<0.01
		Int. LW-IT	4 (12,54)	1.32	<0.01
Run 1 (ANOVA)	Free-living (0.2)	LW	2	0.69	0.51
		IT	**2**	**1.78**	**0.20**
		Int. LW-IT	4	5.41	<0.01
	Polysaccharides (0.4)	LW	2	2.05	0.16
		IT	2	4.79	0.02
		Int. LW-IT	4	10.55	<0.01
	Aggregates (3.0)	LW	2	19.56	<0.01
		IT	2	9.18	<0.01
		Int. LW-IT	4	5.15	<0.01
Run 2 (MANOVA)		LW	2 (6,34)	0.82	<0.01
		IT	2 (6,34)	1.24	<0.01
		Int. LW-IT	4 (12,54)	1.13	<0.01
Run 2 (ANOVA)	Free-living (0.2)	LW	2	2.39	0.12
		IT	2	1.51	0.25
		Int. LW-IT	4	1.93	0.15
	Polysaccharides (0.4)	LW	2	4.98	0.02
		IT	2	13.58	<0.01
		Int. LW-IT	4	4.14	0.01
	Aggregates (3.0)	LW	2	3.15	0.07
		IT	2	14.53	<0.01
		Int. LW-IT	4	3.96	0.02

Notes: LW: level of Walne Medium (3 pH and 2 salinity levels), IT: incubation time, Int.LW-IT: interaction level of Walne medium and incubation time, numdf: numerator degree of freedom, dendf: denumerator degree of freedom.

Figure 2. Total *V. parahaemolyticus* cell numbers in three different fractions; free living bacteria (red), polysaccharide attached bacteria (blue), and aggregate associated bacteria (green) in control (CO) and Walne medium with aggregates (AG). Walne media had two pH (8.5 and 7.5) and salinity (seawater/SW and freshwater/FW) levels.
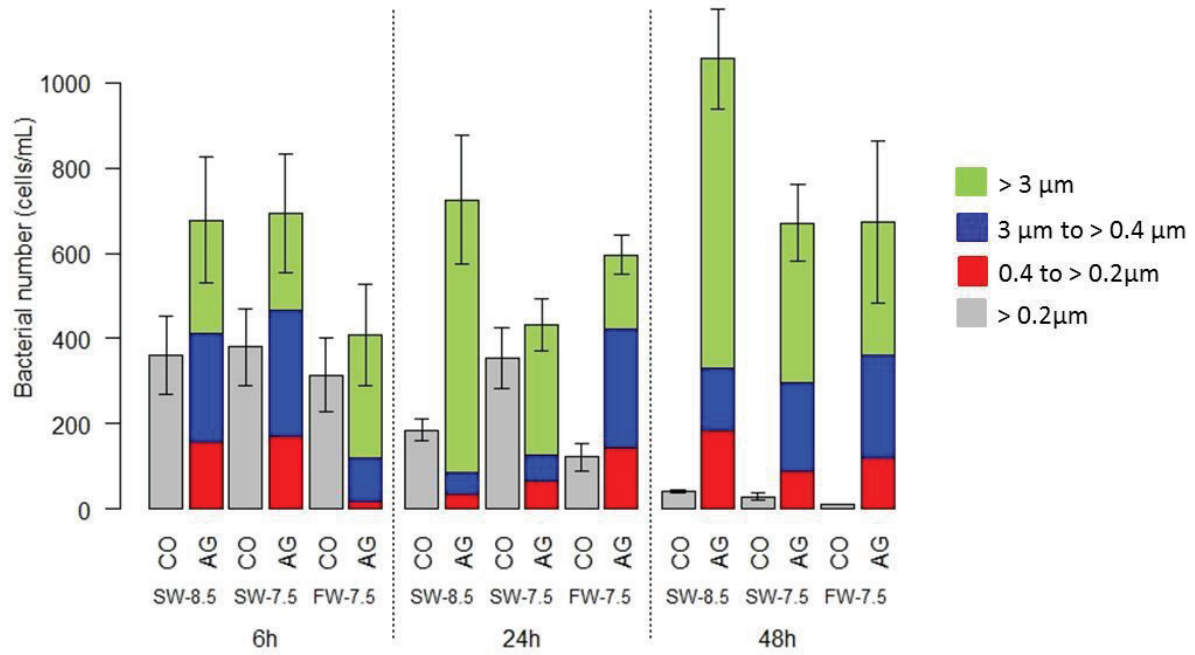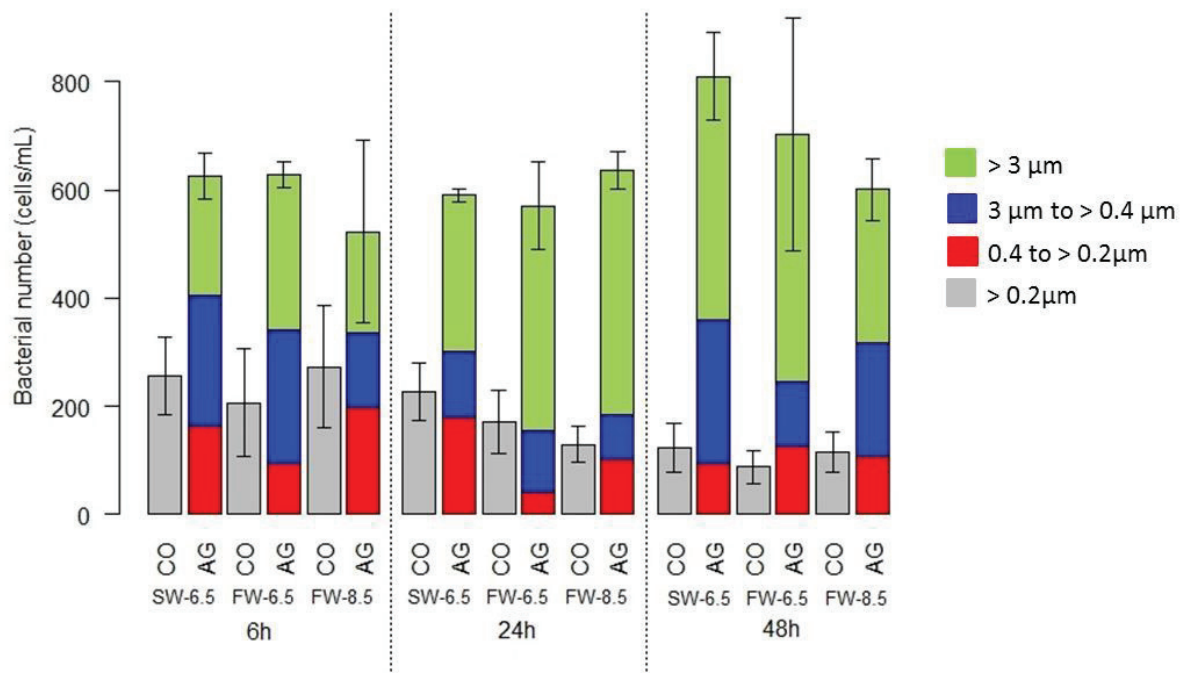

Figure 3. Total *V. parahaemolyticus* cell numbers in three different fractions; free living bacteria (red), polysaccharide attached bacteria (blue), and aggregate associated bacteria (green) in control (CO) and Walne medium with aggregates (AG). Walne media had two pH (8.5 and 6.5) and salinity (seawater/SW and freshwater/FW) levels.

Discussion

Coagulation of particulate matter in aquatic environment can change water quality parameters. The aggregate formation reduced transparent exopolymer particles (TEP) and suspended particulate matter (SPM) concentrations in pond water. They also attracted bacteria to attach resulting in concentrated bacterial cells in the aggregates. Thus, the aggregates facilitate particle removal from water columns (Jackson and Burd, 1998).

Molasses has been reported to ameliorate food, aquaculture, and municipal waste process (Gökşen and Ekiz, 2018; Miao *et al.*, 2017; Waligórska *et al.*, 2000). In shrimp aquaculture, molasses has been used to increase C/N ratio to form nutritive aggregates, so-called bio-flocs (Avnimelech, 2015; Miao *et al.*, 2017). In our study, water quality changed when molasses were added into pond water. However, they did not affect the total quantity of aggregates. In contrast, organic macromolecules such as carbohydrates rise significantly than those in aggregates without molasses. Our findings showed that molasses are able to maintain water pH and inorganic nutrient such as N-ammonium and N-nitrite into favorable conditions/levels for shrimp. These findings concur with previous study which reported that molasses increase pH and maintain N-ammonium and N-nitrite steady over 90 days farming (Miao *et al.*, 2017).

In this study, the addition of molasses ameliorated the growth of beneficial bacteria. The beneficial bacteria, such as *Halomonas*, *Salegentibacter*, *Psychrobacter*, *Mesonia* and *Chromohalobacter* dominated BCC in water. In contrary, potential pathogenic bacteria such as *Alteromonas* and *Vibrio* dominated the BCC of pond water without molasses addition. Therefore, the addition of molasses is useful for shrimp aquaculture, especially for maintaining water quality, improving beneficial bacterial growth, and providing nutritive bio-flocs. This can be achieved if the quantity of molasses with suitable C:N ratio is precisely determined. Our study failed to fulfill the recommended C:N ratio for bio-floc formation due to insufficient quantity of added molasses. However, we observed that the addition of molasses increased macromolecules such as carbohydrates, proteins, and lipids in aggregates. These nutritious aggregates may improve shrimp diets, especially for the need of carbohydrates. The presence of high carbohydrate level (30%) associated with crude protein (35% CP) tend to improve energy retention up to 19% in shrimps (Cuzon *et al.*, 2000). Nutritious bio-flocs can be formed by adjusting C:N ratio at 1:15 till 1:20 (Avnimelech, 2015; Miao *et al.*, 2017). Moreover, if we want to obtain better quality of aggregates, especially for probiotic content, additional beneficial strains such as *Halomonas*, *Bacillus*, *Nitrococcus*, *Nitrospira*, and *Lactococcus* into aggregates is also suggested (Miao *et al.*, 2017; Suantika *et al.*, 2013; Zhang *et al.*, 2009).

Aggregates provide a suitable habitat for microorganisms (Grossart and Simon, 1998; Kiørboe, 2001; Simon *et al*., 2002). The experiments shows that the highest bacterial abundances were reached when bacteria attached to aggregates, which were 10-fold and 5-fold higher than bacterial abundances in FL fraction after 24 and 48 hours, respectively. Bacterial abundances in aggregates may decrease due to detachment, predation, bacteria-bacteria inhibition or mortality due to phage (Grossart *et al*., 2004; Kramer *et al*., 2013; Riemann and Grossart, 2008). In pond water with molasses, bacterial abundances in aggregates as well as in FL fraction tended to decrease after 48 hours. Our results is in opposite to those of Kramer *et al*. (2013) and Lyons *et al*. (2010) who mentioned that bacterial numbers on aggregates remain constant over the time. We suppose that dissolved oxygen (DO) depletion due to microbial processes, such as respiration and sulphur oxidation as well as the presence of hydrogen sulfide (H_2S), influence bacterial abundances in both fractions. We observed H_2S in shrimp pond water due to their characteristic odor. Unfortunately, we could not measure H_2S as well as DO concentration directly in our treatments. Our results correspond to previous studies (Kiørboe *et al*., 2003; Kramer *et al*., 2013; Lyons *et al*., 2005, 2010), which reported that aggregates content higher bacterial abundance and diversity, including pathogenic bacteria than those in adjacent water column. Bacteria may use aggregates as sources of nutrient and shelter from destructing physical factors such as pH and salinity (Lyons *et al*., 2010, Kramer *et al*., 2013), however the presence of toxic substance, such as H_2S or depletion of oxygen in surrounding water may limit heterotrophic bacterial growth.

Bacteria can use exudates of algae or even break-down algae cells for their growth (Grossart and Simon, 2007). We observed this phenomenon in second experiment of which *V. parahaemolyticus* grew better in the presence of aggregates of *C. vulgaris*. Because Walne medium contain limited inorganic nutrient, we suppose that *V. parahaemolyticus* used exudates from *C. vulgaris* aggregates or even break-down *C. vulgaris* cell to absorb nutrients for their growth. Knowing that *V. parahaemolyticus*, a pathogenic bacteria in shrimp, can attach to *C. vulgaris* aggregates and may utilize the exudates and aggregates of *C. vulgaris* for their growth, and that intensive shrimp farming might contain more than 1×10^6 phytoplankton cell L^{-1} (Alonso-Rodrıguez and Paez-Osuna, 2003), it is necessary to monitor and maintain bacterial abundances, especially pathogenic bacteria as well as phytoplankton density in shrimp pond water. In addition in our study, *V. parahaemolyticus* has their maximum numbers once they grew in Walne medium with aggregates of *C. vulgaris* at pH 8.5 which is a typical pH in shrimp pond waters. Detection of *V. parahaemolyticus* in the aggregates fraction of shrimp pond water is necessary to monitor such pathogenic bacteria which may harm shrimps.

Conclusions

In shrimp aquaculture, aggregates can be generated from suspended particulate matter, transparent exopolymer particles and microorganisms which are available in pond water. Addition of external carbon such as molasses, as well as aggregates of microalgae or its exudates can ameliorate bacterial growth including heterotrophic halophilic bacteria and pathogenic *V. parahaemolyticus*. To obtain nutritious aggregates which also contain beneficial indigenous bacteria which serve as natural feed source in shrimp aquaculture, C:N ratio in pond water as well as the presence of pathogenic bacteria has to be determined and measured carefully. Application of molasses may increase C content, provide nutrient for bacterial growth, and at the same time maintain pH to above 8. Feeding experiment using different size of aggregates would be promising to understand the impact of aggregates on shrimp performance.

Acknowledgement

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Supplementary Information 1. Dilution of standard for carbohydrates, lipid, and protein and wavelength for spectrophotometer

Standards	Concentration (mg/mL)	Wavelengths (nm)
Carbohydrates (D-Glucose)	0.25	492
	0.125	
	0.0625	
	0.032	
	0.016	
	0.0078	
	0	
Protein (Bovine Serum Albumin)	0.5	592
	0.25	
	0.125	
	0.0625	
	0.0313	
	0.0155	
	0	
Lipid (Glyceryl Tripalmitate)	2.4	375
	1.6	
	0.8	
	0.4	
	0.2	
	0.1	
	0.05	
	0	

Supplementary Information 2. Composition of Walne medium (Walne, 1970)

Stocks per 100 mL
(1) Trace metal solution (TMS)

$ZnCl_2$	2.1 g
$CoCl_2.6H_2O$	2.0 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.9 g
$CuSO_4.5H_2O$	2.0 g

Make up to 100 mL with distilled water.
Acidify with a few drops of concentrated HCl to give a clear solution.

(2) Vitamin solution

Vitamin B12 (Cyanocobalamin)	10.0 mg
Vitamin B1 (Thiamine.HCl)	10.0 mg
Vitamin H (Biotin)	200.0 µg

Make up to 100 mL with distilled water per litre

(3) Nutrient solution

$FeCl_3.6H_2O$	1.3 g
$MnCl_2.4H_2O$	0.36 g
H_3BO_3	33.6 g
EDTA(Disodium salt)	45.0 g
$NaH_2PO_4.2H_2O$	20.0 g
$NaNO_3$	100.0 g
TMS (1 above)	1.0 mL

Make up to 1 Liter with distilled water.

Medium	per Liter
Nutrient solution (3)	1.0 mL
Vitamin solution (2)	0.1 mL
Sterilized seawater	1.0 Liter

Dispense nutrient and vitamin solutions separately into 10 mL and 1 mL, respectively. Autoclave at 121°C, 15 psi for 15 minutes. Add an aliquot of each aseptically to 10 liters of sterilized seawater.

Chapter 4. Bacterial abundance and community composition in pond water from shrimp aquaculture systems with different stocking densities

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Contribution to the manuscript:

Experimental concept and design	**90%**
Acquisition of experimental data	**80%**
Data analysis and interpretation	**80%**
Preparation of figures and tables	**60%**
Drafting manuscript	**70%**

Bacterial Abundance and Community Composition in Pond Water From Shrimp Aquaculture Systems With Different Stocking Densities

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In shrimp aquaculture, farming systems are carefully managed to avoid rearing failure due to stress, disease, or mass mortality, and to achieve optimum shrimp production. However, little is known about how shrimp farming systems affect biogeochemical parameters and bacterial communities in rearing water, whether high stocking densities (intensive system) will increase the abundance of pathogenic bacteria. In this study, we characterized bacterial communities in shrimp ponds with different population densities. Water quality, such as physical parameters, inorganic nutrient concentrations, and cultivable heterotrophic bacterial abundances, including potential pathogenic $Vibrio$, were determined in moderate density/semi-intensive (40 post-larvae m^{-3}) and high density/intensive shrimp ponds (90 post-larvae m^{-3}), over the shrimp cultivation time. Free-living and particle-attached bacterial communities were characterized by amplicon sequencing of the 16S rRNA gene. Suspended particulate matter (SPM), salinity, chlorophyll a, pH, and dissolved oxygen differed significantly between semi-intensive and intensive systems. These variations contrasted with the equal abundance of cultivable heterotrophic bacteria and inorganic nutrient concentrations. Bacterial communities were dominated by $Gammaproteobacteria$, $Alphaproteobacteria$, $Flavobacteriia$, $Bacilli$, and $Actinobacteria$. $Halomonas$ and $Psychrobacter$ were the most dominant genera in the particle-attached fractions, while $Salegentibacter$, $Sulfitobacter$, and $Halomonas$ were found in the free-living fractions of both systems. Redundancy analysis indicated that among the observed environmental parameters, salinity was best suited to explain patterns in the composition of both free-living and particle-attached bacterial communities (R^2: 15.32 and 12.81%, respectively), although a large fraction remained unexplained. Based on 16S rRNA gene sequences, aggregated particles from intensive ponds loaded a higher proportion of $Vibrio$ than particles from semi-intensive ponds. In individual ponds, sequence proportions of $Vibrio$ and $Halomonas$ displayed an inverse relationship that coincided with changes in pH. Our observations suggest that high pH-values may suppress $Vibrio$ populations and eventually pathogenic $Vibrio$.

61

Our study showed that high-density shrimp ponds had a higher prevalence of *Vibrio*, increased amounts of SPM, and higher phytoplankton abundances. To avoid rearing failure, these parameters have to be managed carefully, for example by providing adequate feed, maintaining pH level, and removing organic matter deposits regularly.

INTRODUCTION

Litopenaeus vannamei (Boone, 1931), white-leg shrimp, is an important species in aquaculture industry, which is widely reared in subtropical to tropical regions. They grow rapidly, have high survival rates even at high densities, possess a wide tolerance range of salinity and temperature, and can be cultivated in indoor (tanks or recirculating aquaculture systems) or outdoor facilities (ponds; Cuzon et al., 2004). Anderson et al. (2016) reported that world shrimp production in 2015 was 4.2 million tons of which *L. vannamei* contributed 75%. However, severe economic loss due to massive shrimp mortality has been occurring since 1987. For the period 1987–1994, economic loss reached USD 3 billion, representing 40% of the total production capacity of the industry (Brun et al., 2009; Walker and Winton, 2010). Moreover, a recent emerging bacterial disease, known as acute hepatopancreatic necrosis disease (AHPND), has occurred in Asian and Mexican shrimp aquaculture, causing an annual loss amounting to USD 1 billion with shrimp mortality exceeding 70% (Global Aquaculture Alliance [GAA], 2013, De Schryver et al., 2014; Soto-Rodriguez et al., 2015).

The intensification of the shrimp industries resulted in changes of farming systems and sustainability. Until the year 2000, 70% of shrimp farming in Indonesia was conducted in extensive pond systems (Kautsky et al., 2000), but recently changed to semi-intensive or intensive systems (Kementerian Kelautan dan Perikanan [KKP], 2015). Different shrimp stocking densities affect rearing processes, including a defined nutritional input and shrimp production. Robertson et al. (1993) reported that the shrimp growth rate increased progressively as feeding frequency increased from 1 to 4 times per day. However, only a portion of the nutrients in the feed is consumed, assimilated, and retained as shrimp biomass. Shrimps only incorporate 24 to 37% of nitrogen and 11 to 20% of phosphorus from the feed into their bodies (Funge-Smith and Briggs, 1998; Sahu et al., 2013). In addition, 15% of nitrogen losses occurs during the first 2 h of immersion of feed pellets into the pond water (Smith et al., 2002). These unused nutrients will lead to a change of pH and dissolved oxygen (DO) in the water column and pond sediment, eutrophication, proliferation of bacteria and plankton, and an increase of particulate organic matter (Avnimelech et al., 1994; Martin et al., 1998). Furthermore, Kautsky et al. (2000) reported that the risk of shrimp diseases often increased with culture intensity and high stocking densities. Thus, shrimp diseases, along with bad water quality management, might threaten shrimp farming sustainability.

Free-living (FL) and particle-associated (PA) bacterial communities from the same water sample can be distinct (Bidle and Fletcher, 1995; Riemann and Winding, 2001;

Jain and Krishnan, 2017; Yang et al., 2017). As organic-rich particles, aggregates provide a suitable habitat for microorganisms to take up nutrients, and shelter from predators, as well as from destructive physical factors (Lyons et al., 2010; Kramer et al., 2013). In addition, aggregates can accommodate higher bacterial abundance and diversity than the adjacent water column (Kiørboe et al., 2003). Due to water movement in ponds, organic matter agglomerates and forms large flocs or aggregates (Hargreaves, 2013; Avnimelech, 2014), which might facilitate bacterial settlement and proliferation. Previous studies about bacterial community compositions (BCCs) in shrimp pond waters have yet to analyze FL and PA fractions separately (Sombatjinda et al., 2011; Zhang D. et al., 2014; Xiong et al., 2016; Hou et al., 2017), resulting in a paucity of information on BCC in both fractions.

Although the causative agents for bacterial diseases in shrimps have been identified (Lavilla-Pitogo et al., 1998; Kharisma and Manan, 2012; Soto-Rodriguez et al., 2015; Xiao et al., 2017), preventive efforts to minimize disease outbreaks seem to still be ineffective. Studies in temperate ecosystems showed that pathogenic bacteria have been found in aggregates (Lyons et al., 2005; Froelich et al., 2013). Based on that evidence, we propose that particle abundance can be used to estimate the potential proliferation of pathogenic bacteria in shrimp farming, and that controlling aggregates may become an effective tool to manage the spread and survival of pathogens. Therefore, it is necessary to investigate water quality parameters, bacterial abundance, and BCC from different shrimp farming systems for both FL and PA fractions, over shrimp cultivation time.

The current study aims to comprehend the effect of shrimp farming systems of different intensity on water quality parameters and BCC, to elucidate factors affecting bacterial communities, and to evaluate the presence of pathogenic bacteria, including pathogenic *V. parahaemolyticus* in the FL and the PA/aggregates fraction. We hypothesize that (i) different shrimp farming systems affect pond water quality, including suspended particulate matter (SPM) loading, bacterial abundance and community composition, and (ii) SPM loads more bacterial cells, including pathogenic bacteria.

MATERIALS AND METHODS

Sample Collection and Sampling Sites

Water samples were collected between 9–10 a.m. from no water-exchange and plastic lining ponds (square shape in size 2700–3000 m^2, water depth 1.3–1.5 m) of semi-intensive (40 post-larvae m^{-3}, three ponds) and intensive (90 post-larvae m^{-3}, three ponds) systems, during a cycle of shrimp rearing at day 10,

62

20, 30, 40, 50, 60, and 70 (September to November 2016). Shrimp ponds were located in Rembang Regency, Central Java, Indonesia ($-6°37'41.13''$ S $111°30'1''$ E and $-6°42'11.66''$ S $111°21'54''$ E, for semi-intensive and intensive system, respectively). Two liters of water were taken from 5 points of each pond at 1 m depth, and then mixed. Two liters of the mixed water were prepared for bacterial abundance and community analysis; 3 liters for SPM and inorganic nutrient analysis. Samples were stored in a cold and dark container, and transported to the laboratory at Diponegoro University, Semarang, Indonesia, for immediate analysis. Remaining water was used for physical parameter measurement. Total harvest of each pond was recorded at the end of shrimp rearing.

Environmental Parameters

Salinity, temperature, pH, chlorophyll a, DO, and turbidity were measured *ex situ*, using calibrated Manta Eureka 2 multi-probes (Eureka Environmental Engineering, TX, United States).

Of each water sample, 0.3 L were filtered through a 0.45 μm syringe filter and the filtrate was poisoned with 1.2 mL of a 0.35 g L^{-1} $HgCl_2$ solution for inorganic nutrient analysis (ammonium: NH_4^+, nitrate: NO_3^-, nitrite: NO_2, phosphate: PO_4^{3-} and silicate: SiO_4^{4-}). The samples were stored at $-20°C$ until analysis. Inorganic nutrient measurements were done in triplicates at the Laboratory of Chemistry, Research Center for Oceanography (LIPI), Jakarta, Indonesia, according to the colorimetry method by Strickland and Parsons (1972), with a Shimadzu UV-1800 spectrophotometer.

Suspended particulate matter was measured as dry mass on pre-combusted GF/F filters (porosity 0.7 μm, ø 47 mm, VWR, France) in triplicates after filtration of a known volume of water sample (0.1–0.5 L). Weight of the filters was determined using a precision balance (ME 36S, Sartorius, Göttingen, Germany) after drying the filters for 24 h at 40°C.

Bacterial Abundance

To obtain cultivable heterotrophic bacterial number, 100 μL of water samples (dilution factor 10^{-2}–10^{-5}) were plated onto Marine Agar 2216 (Difco, United States) and incubated at 28°C (room temperature) for 48 h. While potentially pathogenic *Vibrio* were isolated by inoculating 100 μL of undiluted to 10^{-4} diluted water sample onto selective Thiosulfate Citrate Bile Salts Sucrose(TCBS) medium (Roth, Karlsruhe, Germany), followed by incubation at 35°C for 24 h.

Samples for total bacterial cell counts were prepared by fixing 50 mL water with 4% v/v paraformaldehyde and stored at 4°C for 24 h. The dilution factors for the samples were as follows: undiluted for day 10 samples, $5 × 10^{-1}$ for day 20 and 30 samples, 10^{-1} for day 40, 50, and 60 samples, and $5 × 10^{-2}$ for day 70 samples. Ten milliliters of diluted fixed samples were subsequently filtered through 3.0 and 0.2 μm polycarbonate filters (ø 47 mm and 25 mm, respectively, Whatman, Dassel, Germany) to determine bacterial cells in the particle-attached (PA) and the free-living (FL) fractions, respectively. For the FL fractions at day 30, 40, and 70, only 5 ml filtrates were filtered. Filters were air dried and stored at $-20°C$ for further staining. A 4′,6-diamidino-2-phenylindole (DAPI) staining was

performed according to Kepner and Pratt (1994) for selected samples (10, 40, 50, 60, and 70 days samples). Filters were stained with 1 μg mL^{-1} of DAPI solution for 5 min, then washed in 80% ethanol and rinsed with sterile distilled water. Stained filters were air dried in the dark for 30 min, and then mounted with 10 μL of mounting solution consisting of 3:1 Citifluor AF mounting medium (Citifluor Ltd., London, United Kingdom) and Vectashield (Vector Laboratories Inc., Burlingame, United States). Bacterial cells, as well as size and number of aggregates were observed under a fluorescence microscope *Axio Imager.D2* (Zeiss, Jena, Germany) at 1000× magnification. Bacterial cell abundance in FL filters was calculated from 30 photos per filter, using the free software *ImageJ*. Bacterial cells in PA filters were manually counted from 10 aggregates per filter of similar size. Sizes of aggregates were determined using a net micrometer grid (12.5 mm × 12.5 mm, divided into 10 × 10 fields, which is equal to 15,625 μm² at 1000× magnification). The cell average per filter was then divided by volume of filtered samples multiplied by dilution and factor of effective filter area (which was 31888 at magnification 1000×, for a filtration funnel with a diameter 25 mm, Millipore, Darmstadt, Germany) and number of aggregates (4 aggregate sizes per filter, **Supplementary Table 1**), for the FL and the PA fraction, respectively.

Molecular Analysis of Bacterial Communities

Five hundred milliliters of water samples were filtered subsequently through 3.0 and 0.2 μm polycarbonate filters (ø 47 mm, Whatman, Dassel, Germany) for PA and FL bacterial fractions, respectively. Genomic DNA was extracted according to Nercessian et al. (2005). DNA pellets were dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5). DNA concentrations were measured photometrically and checked for purity (ratio of light absorption at 260–280 nm) using a nanoquant plate reader (Infinite M200 Pro, Tecan, Germany). 16S rRNA gene amplification was performed from genomic DNA extracts from days 10, 40, 50, 60, and 70, considering cultivable bacterial abundance information (heterotrophic bacteria and potential pathogenic *Vibrio*) and bacterial disease evidence (white feces disease), which occurred at previous rearing cycles between 50 and 65 days of rearing (personal communication with shrimp farm owners).

DNA sequences of the V3–V4 hypervariable region of the 16S rRNA gene were obtained from amplicon sequencing with the primer set S-D-Bact-0314-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′)/S-D-Bact-0785-a-A-21 (5′-G ACTACHVGGGTATCTAAKCC-3′; Klindworth et al., 2013). Sequencing at LGC genomics (Berlin) was done on an Illumina MiSeq using the V3 Chemistry (Illumina) in a 2 × 300 bp paired-end run. Demultiplexing, i.e., grouping of sequences by sample, and the removal of the primer sequences from the raw paired-end reads were performed by LGC genomics (Berlin, Germany). Further bioinformatic analysis steps were carried out at ZMT, Bremen, Germany, according to Hassenrück et al. (2016). Sequences were quality trimmed with a sliding window of 4 bases and a minimum average quality of 15 with

63

trimmomatic v.033 (Bolger et al., 2014). Quality trimmed sequences were merged using PEAR v0.9.8 (Zhang J. et al., 2014). The swarming approach was used to cluster OTUs using *swarm* v2.1.1 (Mahé et al., 2014). For each OTU, one representative sequence (seed sequence) was taxonomically classified with SINA (SILVA Incremental Aligner) v1.2.11 using the SILVA rRNA project reference database (release 128) at a minimum alignment similarity and quality of 0.9 and a last common ancestor consensus of 0.7 (Pruesse et al., 2012). Unwanted lineages (such as archaea, chloroplasts, and mitochondria), as well as singletons and doubletons, i.e., OTUs occurring once or twice in the data set, were removed. Rarefaction curves were calculated based on OTU richness and inverse Simpson index to assess the quality of sequence data sets. Samples with fewer than 500 sequences were excluded from the dataset for the analysis.

DNA sequence datasets were deposited in the European Nucleotide Archive (ENA) with the project accession number PRJEB26390, using the data brokerage service of the German Federation for Biological Data (GFBio, Diepenbroek et al., 2014), while biogeochemical data were archived in PANGAEA[1].

Detection of Virulence Genes via PCR

Detection of toxin genes, i.e., transcriptional regulator (*toxR*), thermolabile haemolysin (*tlh*), thermostable direct haemolysin (*tdh*), *Photorhabdus* insect-related (*pirA* and *pirB*) was performed in a Mastercycler® (Eppendorf, Nexus gradient, Hamburg, Germany) using the set of primers described in **Supplementary Table 2**. As these genes are common genes in several representative of the genus *Vibrio* (i.e., *V. cholera, V. vulnificus, V. parahaemolyticus, V. alginolyticus, and V. owensii*), we designed specific primer pairs which only amplify DNA sequences belonging to *V. parahaemolyticus* as this bacterium causes most of bacterial diseases in shrimp (Soto-Rodriguez et al., 2015; Heenatigala and Fernando, 2016; Xiao et al., 2017). Ten samples, which had a high amplicon abundance of *Vibrio*, were chosen for PCR analysis (**Supplementary Table 3**). The PCR conditions were as follows: a reaction mixture consisted of 2 μL (20 ng μL^{-1}) of template, 2 μL of 10× PCR buffer B containing 15 mM MgCl$_2$, 0.5 μL of 25 mM MgCl$_2$, 0.5 μL of 0.2 mM dNTPs, 1 μL of 0.1 mM forward and reverse primer, 0.1 μL of 5U μL^{-1} Taq polymerase (all reagents provided by Roboklon EURx, Berlin, Germany) and 12.9 μL sterile distilled water. The amplification conditions were as follows: pre-denaturation at 95°C for 3 min, followed by 40 amplification cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s, and a final elongation at 72°C for 5 min. *Vibrio parahaemolyticus* DSM 11058 (DSMZ, Braunschweig, Germany) was used as positive control for those toxin genes, while *Vibrio vulnificus* DSM 10143 (DSMZ, Braunschweig, Germany) served as negative control.

Data Analysis

To examine differences in environmental parameters and bacterial abundances between culturing intensity (intensive and semi-intensive systems), and among days, as well as the

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interaction between culturing system and sampling day, general linear mixed models (GLMM) were performed with shrimp pond as random factor. Data were log-transformed to achieve normal distribution prior to statistical testing. *Tukey's post hoc* tests were applied in cases where there were significant differences among sampling days and/or an interaction between sampling day and shrimp farming system. Forward model selection based on the Akaike information criterion (AIC), considering collinearity of the variables and variance inflation values, was used to determine environmental parameters, which best explained observed counts of cultivable heterotrophic bacteria and potential pathogenic *Vibrio*. Total shrimp harvest was tested with one-way ANOVA and total bacterial cell numbers from DAPI counting of FL and PA bacterial communities were analyzed using MANOVA. As first-level *post hoc* test, the effect of significant predictor variable (here: sampling day) in MANOVA of FL and PA bacterial numbers was then tested with individual GLMMs per fraction. Furthermore, pairwise comparisons between sampling days were conducted as second-level *post hoc* test. Repeated measures correlation between FL and PA bacterial cell numbers was estimated using rmcorr R package (Bakdash and Marusich, 2018).

Principal component analysis (PCA) was conducted to examine the relationship among environmental parameters, as well as cultivable bacterial abundances, and to characterize shrimp ponds of the different farming systems over time. Bray–Curtis dissimilarity coefficients were calculated to investigate variation of OTU compositions in FL and PA communities among ponds, as well as within and between the systems. Patterns of BCC in both fractions was visualized by non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities using the function *metaMDS* of the *vegan* R package (Oksanen, 2017). Analysis of similarity (ANOSIM) was performed to test for differences in community composition in free-living and particle-attached fraction between systems over the time.

Redundancy analysis (RDA) was performed to explore relationships between bacterial communities and environmental variables in FL and PA fractions, for OTUs occurring in at least 10 samples, and with a sequence proportion of at least 1% in one sample. Data sets were separated by size fractions, and then centered log-ratio-transformed using *aldex.clr* (Fernandes et al., 2014) before being tested. Forward model selection based on the AIC, followed by collinearity and variance inflation inspection, was used to select the environmental parameters best suited to explain patterns in BCC. The significance of the individual parameters of the RDA models was assessed using restricted permutation tests to account for the repeated measurements within ponds. For the PA fraction, data from day 60 was excluded due to a missing value to ensure equal numbers of observation from each pond.

Population turnover, which occurred in the FL and PA fractions, was estimated for certain interesting OTUs of the genera *Salegentibacter, Psychrobacter, Halomonas, and Vibrio*, using the fraction of read abundance times cells method (FRAxC) proposed by Kevorkian et al. (2018), where bacterial cell numbers in the FL and PA of each selected sampling day were

64

multiplied with the relative sequence proportion of 16S rRNA genes. We assumed that the biases associated with sampling procedure, DNA extraction, amplification, and cell counts did not vary over time. FRAxC results were also used to estimate correlation between estimated *Vibrio* abundance and selected environmental parameters (SPM, salinity, pH, temperature, ammonium, phosphate, and nitrite).

All statistical analyses, as well as figure visualizations were performed in R (R version 3.4.2, R Core Team, 2017, using R Studio v.0.98.1056) with the packages vegan (Oksanen, 2017), nlme (Pinheiro et al., 2017), ALDEx2 (Fernandes et al., 2014), rmcorr (Bakdash and Marusich, 2018), and gplots (Warnes et al., 2016).

RESULTS

Shrimp Culturing Conditions

Total shrimp harvest differed significantly between semi-intensive and intensive systems (one-way ANOVA, $F_{1,4} = 111.43$, $p < 0.01$). The intensive system showed a twofold higher production than semi-intensive system, which were $3,950 \pm 284$ kg and $1,990 \pm 151$ kg, for intensive and semi-intensive final harvest (mean $\pm$ SD, $n = 3$ per system). There was a high variability among the ponds within each system for turbidity, chlorophyll a, pH, salinity, DO, and inorganic nutrients during rearing time, which increased over time (**Table 1**). Physical parameters such as salinity, pH, DO, chlorophyll a, and SPM were significantly different between the two systems (GLMM, $p < 0.05$, **Supplementary Table 4**). Among sampling days, we detected differences in temperature, salinity, pH, turbidity, chlorophyll a, SPM, cultivable heterotrophic bacteria and potential pathogenic *Vibrio* (GLMM, $p < 0.05$, **Supplementary Table 4**). The interaction between day and farming system was also significant for temperature, salinity, and turbidity. However, there were no significant differences between semi-intensive and intensive systems for dissolved inorganic nutrients, temperature, and turbidity (GLMM, $p < 0.05$, **Supplementary Table 4**).

In both systems, pH decreased rapidly after day 40, but when limestones were added into the ponds, it increased from 7.92 ± 0.13 to 8.38 ± 0.53 and 7.63 ± 0.46 to 8.11 ± 0.13, in semi-intensive and in intensive system, respectively. Salinity decreased from 35.37 ± 0.15 to 32.44 ± 3.26 PSU and 38.43 ± 0.78 to 33.57 ± 0.45 PSU, in semi-intensive and in intensive system, respectively, due to freshwater input. Overall, the intensive system showed significantly higher salinities, SPM, and chlorophyll a concentration than the semi-intensive system, as well as higher variability of inorganic nutrients (**Table 1**).

The abundances of cultivable heterotrophic bacteria (THB) and potential pathogenic *Vibrio* (TPPV) in semi-intensive and intensive systems increased with rearing time, and peaked at day 60, at concentrations of 3.4×10^7 CFU mL^{-1} of THB and 1.4×10^4 CFU mL^{-1} of TPPV, and 2.6×10^7 CFU mL^{-1} of THB and 2.65×10^5 CFU mL^{-1} of TPPV, respectively (**Table 1**). Forward model selection showed that SPM, salinity, turbidity,

temperature, and phosphate were major determinants for THB, while the determinants for TPPV were SPM, salinity, turbidity, pH, temperature, nitrite, and silicate.

Principal component analysis for environmental parameters and cultivable bacterial abundances was able to retrieve 42.61% of the variation among ponds on the first two principal components (**Figure 1**). According to water parameters and cultivable bacterial abundances, there was a clear separation by shrimp farming systems based on rearing time. At the beginning of rearing (days 10 and 20), ponds of intensive and semi-intensive systems were clustered with high values of the first principal component, which was driven mainly by high salinity. Afterward, they were separated by the second principal component with ponds of the intensive system characterized by high concentrations of chlorophyll a. The abundances of THB and TPPV highly correlated to SPM (*Spearman* correlation, *rho*: 0.87 and *rho*: 0.40 for THB and TPPV, respectively).

Aggregates containing bacterial cells were found in different sizes. At day 10, small aggregates appeared which were formed by bacterial cells and their exudates. After 40 days, aggregates were composed of plankton, bacterial cells and bacterial exudates, causing the increase of aggregate sizes in both systems, as well as bacterial cell numbers. Aggregates of sizes between 937.5 and 1,406.5 μm^2 were most abundant in both system over time, with the highest aggregate number at day 10 (511 ± 63 aggregates mL^{-1}) and at day 40 (494 ± 27 aggregates mL^{-1}), containing 86 ± 3 and 72 ± 6 bacterial cells per individual aggregate for semi-intensive and intensive pond waters, respectively (mean $\pm$ SD; $n = 3$; **Supplementary Figure 1** and **Supplementary Table 1**). Free-living (FL) bacterial cells of both systems increased and peaked at day 60 in concentration 3.8×10^7 cells mL^{-1} and 5.0×10^7 cells mL^{-1}, for semi-intensive and intensive systems, respectively. Total particle-attached (PA) bacterial cell numbers were steady after day 40, with 6.8×10^5 cells mL^{-1} and 6.4×10^5 cells mL^{-1}, for semi-intensive and intensive systems, respectively (**Table 2**). The FL cell numbers were positively correlated to the PA cell numbers (repeated measure correlation, *Spearman* correlation, rho = 0.50, $df = 23$, $p = 0.01$) and differed only among days (**Supplementary Tables 5, 6**).

Bacterial Community Analysis

After removal of low-quality reads, 8,994–78,615 sequences, with on average 34,471 sequences per sample, were obtained from 59 samples. One sample was removed due to low sequence counts (<500 sequences). A total of 77,433 OTUs was obtained, ranging from 426 to 2465 OTUs, with an average of 1,312 OTUs per sample (**Supplementary Figure 2**).

Most abundant bacterial OTUs in both systems belonged to the classes Acidimicrobiia, Actinobacteria, Alphaproteobacteria, Bacilli, Cyanobacteria, Flavobacteriia, and Gammaproteobacteria (**Figure 2**). Among them, the genera *Alteromonas, Erythrobacteraceae, Exiguobacterium, Halomonas, Vibrio, Pseudoalteromonas, Psychrobacter, Salegentibacter*, and *Sulfitobacter* were present in every sample. Cyanobacteria, such as Synechococcus and Cobetia, were frequently present in the semi-intensive system. The highest sequence proportion in the FL fraction of both systems belonged to Salegentibacter,

65

TABLE 1 | Physical parameters, in-organic nutrient concentrations, and cultivable heterotrophic bacteria abundances over 70 rearing days in the semi-intensive and intensive farming system.

Day	10		20		30		40		50		60		70	
Systemᵃ	S	T	S	T	S	T	S	T	S	T	S	T	S	T
Parametersᵇ														
SPM* (mg L⁻¹)	66.6 ± 8.0ᵃ	68.7 ± 5.2ᵃ	68.1 ± 4.9ᵃ	70.3 ± 7.7ᵃ	80.1 ± 12.2ᵇ	91.5 ± 8.3ᵇ	96.5 ± 5.7ᶜ	122.0 ± 22.2ᶜ	158.1 ± 4.0ᵈ	202.6 ± 18.5ᶠᵈ	167.3 ± 5.5ᵈ	187.6 ± 11.0ᵈ	173.1 ± 2.2ᵈ	171.3 ± 17.7ᵈ
Chl a* (mg L⁻¹)	13.34 ± 7.13ᵃᶜ	5.47 ± 3.82ᵃ	14.61 ± 1.49ᵃᵉ	10.11 ± 5.04ᵃᵇ	11.22 ± 0.96ᵃᵇ	22.8 ± 19.8ᵃᵉ	18.5 ± 5.5ᵃᵉ	77.9 ± 32.1ᵉ	20.7 ± 9.1ᵃᵉ	63.5 ± 29.1ᶜᵉ	30.9 ± 32.3ᵃᵉ	33.7 ± 14.2ᵇᶜᵉ	16.9 ± 14.9ᵃᶜᵈ	68.1 ± 27.9ᵈᵉ
pH*	8.24 ± 0.04ᵇ	8.18 ± 0.11ᵇ	8.20 ± 0.14ᵃᵇ	7.95 ± 0.07ᵃᵇ	8.26 ± 0.22ᵃᵇ	7.9 ± 0.1ᵃᵇ	8.5 ± 0.1ᵇ	8.0 ± 0.1ᵇ	8.3 ± 0.2ᵃᵇ	7.6 ± 0.5ᵃᵇ	7.9 ± 0.1ᵃ	7.8 ± 0.1ᵃ	8.4 ± 0.5ᵇ	8.1 ± 0.1ᵇ
DO* (mg L⁻¹)	6.13 ± 0.14	6.32 ± 0.1	6.4 ± 0.02	6.36 ± 0.13	5.90 ± 0.65	6.2 ± 0.1	6.1 ± 0.2	6.9 ± 0.8	5.9 ± 0.3	6.1 ± 0.2	5.7 ± 0.1	6.3 ± 0.2	5.8 ± 0.5	6.2 ± 0.3
Salinity* (PSU)	35.37 ± 0.15ᵇᵈ	38.43 ± 0.78ᵈ	35.20 ± 0.69ᵃᵈ	36.79 ± 0.50ᶜᵈ	32.44 ± 3.26ᵃ	35.9 ± 0.9ᵇᵈ	33.7 ± 0.7ᵃᵇᶜ	35.0 ± 0.9ᵃᵇᶜ	33.4 ± 0.3ᵃᵇ	35.8 ± 0.3ᵇᵈ	33.6 ± 1.4ᵃᵇᶜ	34.1 ± 0.8ᵃᵇᶜ	34.6 ± 0.9ᵃᵇᶜ	33.6 ± 0.5ᵃᵇ
Temperature (°C)	31.24 ± 0.41ᶜᵈ	30.19 ± 0.50ᵇᶜᵈ	28.08 ± 0.64ᵃ	30.44 ± 0.18ᵇᶜᵈ	31.61 ± 0.98ᵈ	30.4 ± 1.6ᵇᶜᵈ	29.6 ± 0.2ᵃᶜ	28.9 ± 0.5ᵃᵇ	31.0 ± 0.1ᶜᵈ	30.1 ± 0.4ᵇᶜᵈ	30.8 ± 0.8ᶜᵈ	30.2 ± 0.1ᵇᶜᵈ	30.7 ± 0.5ᵇᶜᵈ	30.5 ± 0.4ᵇᶜᵈ
Turbidity (NTU)	16.53 ± 10.69ᵃᶜ	3.9 ± 2.21ᵃ	24.5 ± 8.67ᵇᶜ	9.93 ± 8.44ᵃᵇ	35.87 ± 30.26ᵇᶜ	22.9 ± 14.6ᵇᶜ	27.0 ± 10.5ᵇᶜ	25.7 ± 2.7ᵇᶜ	25.9 ± 12.9ᵇᶜ	25.3 ± 5.6ᵇᶜ	27.6 ± 7.2ᵇᶜ	30.2 ± 14.4ᶜ	26.9 ± 9.2ᵇᶜ	35.7 ± 15.9ᶜ
Cultivable Bact. (log10 CFU mL⁻¹)														
THB	4.75 ± 0.08ᵃᶜ	4.42 ± 0.15ᵃ	5.35 ± 0.04ᵇᶜᵈ	4.65 ± 0.30ᵃᵇ	6.29 ± 0.15ᵈ	5.50 ± 0.16ᶜᵈ	5.99 ± 0.50ᵈᵉ	6.76 ± 0.20ᵉᵍ	6.87 ± 0.69ᶠᵍ	7.44 ± 0.19ᵍ	7.53 ± 0.19ᵍ	7.41 ± 0.36ᵍ	7.47 ± 0.18ᵍ	7.4 ± 0.1ᵍ
TPPV	2.03 ± 0.56ᵃ	2.13 ± 0.90ᵃ	4.35 ± 0.36ᵇᶜ	3.32 ± 0.32ᵃᵇ	4.51 ± 0.19ᵇᶜ	3.98 ± 0.18ᵇ	3.85 ± 0.29ᵇ	3.73 ± 0.41ᵇ	3.85 ± 0.48ᵇ	3.72 ± 0.15ᵇ	4.16 ± 0.69ᵇᶜ	5.41 ± 0.89ᶜ	4.18 ± 0.42ᵇᶜ	4.36 ± 0.7ᵇᶜ
Nutrient (mg L⁻¹)														
NH4⁺	0.60 ± 0.37	0.436 ± 0.671	0.46 ± 0.71	0.15 ± 0.08	0.31 ± 0.23	0.26 ± 0.34	0.23 ± 0.19	0.26 ± 0.22	0.50 ± 0.07	0.37 ± 0.30	0.61 ± 0.858	0.835 ± 0.248	0.33 ± 0.34	0.36 ± 0.25
NO2⁻	0.004 ± 0.004	0.183 ± 0.292	0.004 ± 0.004	0.008 ± 0.007	0.021 ± 0.025	0.005 ± 0.003	0.007 ± 0.007	0.201 ± 0.334	0.016 ± 0.006	0.001 ± 0.001	0.002 ± 0.001	0.079 ± 0.113	0.013 ± 0.009	0.004 ± 0.003
NO3⁻	0.048 ± 0.06	0.075 ± 0.11	0.007 ± 0.005	0.097 ± 0.16	0.004 ± 0.003	0.027 ± 0.042	0.028 ± 0.02	0.213 ± 0.184	0.034 ± 0.025	0.014 ± 0.013	0.052 ± 0.04	0.307 ± 0.456	0.028 ± 0.026	0.284 ± 0.484
PO4³⁻	0.33 ± 0.31	0.53 ± 0.41	0.29 ± 0.20	0.50 ± 0.37	0.22 ± 0.17	0.22 ± 0.27	0.31 ± 0.38	0.72 ± 0.07	0.39 ± 0.26	0.11 ± 0.12	0.35 ± 0.36	0.59 ± 0.47	0.61 ± 0.08	0.31 ± 0.22
SiO4⁴⁻	1.424 ± 1.453	0.206 ± 0.191	0.179 ± 0.047	0.315 ± 0.253	1.217 ± 0.918	0.343 ± 0.134	0.565 ± 0.576	0.324 ± 0.245	0.898 ± 1.193	0.45 ± 0.36	0.636 ± 0.792	0.11 ± 0.07	0.623 ± 0.668	1.157 ± 1.413

Significant differences between systems at a significance threshold of 0.05 are indicated by asterisks; superscript letters indicate significant differences for the interaction system and day (p < 0.05). Data are shown as mean ± standard deviation. ᵃS, semi-intensive system; T, intensive system. ᵇSPM, suspended particulate matter; Chl a, chlorophyll a; THB, total heterotrophic bacteria; TPPV, total potential pathogenic bacteria.

66
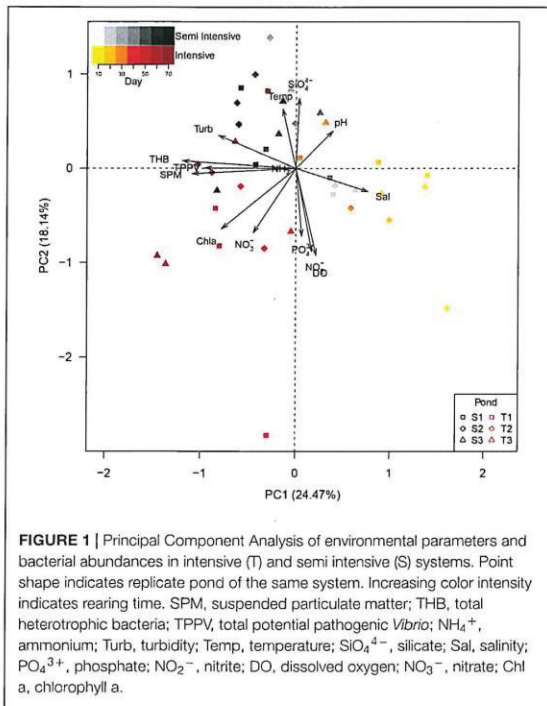
FIGURE 1 | Principal Component Analysis of environmental parameters and bacterial abundances in intensive (T) and semi intensive (S) systems. Point shape indicates replicate pond of the same system. Increasing color intensity indicates rearing time. SPM, suspended particulate matter; THB, total heterotrophic bacteria; TPPV, total potential pathogenic *Vibrio*; NH_4^+, ammonium; Turb, turbidity; Temp, temperature; SiO_4^{4-}, silicate; Sal, salinity; PO_4^{3+}, phosphate; NO_2^-, nitrite; DO, dissolved oxygen; NO_3^-, nitrate; Chl a, chlorophyll a.

TABLE 2 | Total bacterial cell numbers in the free-living (FL) and the particle attached (PA) fractions.

Day	Fractions			
	FL [$\times 10^7$ cells mL^{-1}]		PA [$\times 10^5$ cells mL^{-1}]	
	S	T	S	T
10	0.06 ± 0.01^a	0.09 ± 0.02^a	0.62 ± 0.08^a	0.50 ± 0.05^a
40	1.83 ± 0.13^b	3.09 ± 2.34^b	6.77 ± 0.50^b	6.40 ± 0.24^b
50	2.94 ± 1.04^b	2.13 ± 0.11^b	6.51 ± 0.79^b	6.55 ± 0.31^b
60	3.79 ± 0.89^c	5.01 ± 0.85^c	6.16 ± 0.67^b	6.09 ± 0.37^b
70	3.47 ± 0.94^{bc}	3.64 ± 1.22^{bc}	7.68 ± 0.36^c	7.42 ± 0.21^c

Data are shown as average ± standard deviation. Similar superscript letters after cell numbers in semi-intensive (S) and intensive (T) indicate no significant difference. Bacterial numbers in the FL and PA are interpreted separately. Complete statistical results were presented in **Supplementary Table 6.**

Sulfitobacter, and Halomonas, while in the PA fraction *Psychrobacter, Vibrio,* and *Halomonas* were dominant, where the latter comprised up to 80% of the sequences in samples from the semi-intensive system after 40 and 50 days (**Figure 2**). In the SILVA reference database (SILVA 128 version), *Halomonas* OTU1 has a sequence similarity of 98 and 100% to *H. aquamarina* and *H. meridiana*, respectively.

Free-living and particle-associated bacterial communities from the same water sample were very different from each other as indicated by Bray–Curtis dissimilarity coefficients (**Table 3**). In addition, there was high variability in BCC within the same

fraction among replicate ponds, which lead to a wide range in Bray-Curtis dissimilarity coefficients (0.27-0.97 and 0.33-0.83 for PA and FL fractions, respectively) between intensive and semi-intensive systems (**Table 4**).

NMDS showed a highly heterogeneous composition of the bacterial communities in the FL and the PA fraction for both systems (**Figure 3**). Analysis of similarity (ANOSIM) confirmed that there was no detectable pattern in FL or PA the BCC between system and day (**Supplementary Table 7**).

Redundancy analysis revealed that environmental variables explained 20.53 and 36.77% of the BCC in the FL and the PA fractions, respectively (**Supplementary Figure 3**). Among the observed environmental parameters, salinity was best suited to explain patterns in the composition of both FL and PA bacterial communities ($R^2 > 10\%$). Furthermore, chlorophyll a and nitrate had a minor effect on BCC in the FL fractions (**Table 5**).

Toxin Gene Assay, *Vibrio* Occurrence and Correlation to Environmental Parameters

All tested samples were negative for *toxR, tlh,* and *tdh*. These results indicate that no common pathogenic *V. parahaemolyticus* occurred in both systems, even though the given samples displayed high sequence proportions of *Vibrio*, specifically OTU4. The sequence of *Vibrio* OTU4 was identical to 16S gene sequences in the SILVA reference database belonging to *V. mytili, V. diabolicus,* and *V. parahaemolyticus*. The estimated abundance (FRAxC) of the dominant *Vibrio* OTU differed strikingly from that of the other dominant OTUs, such as *Halomonas, Psychrobacter,* and *Salegentibacter,* suggesting an inverse relationship. When FRAxCs of *Vibrio* were overly high, the proportions of the other genera were low. In the PA fraction, FRAxCs of *Vibrio* were negatively correlated to FRAxCs *Halomonas* (**Figure 4**).

FRAxCs of *Vibrio* in both fractions were positively correlated to SPM, temperature, ammonium, NP ratio, and TPPV, but they were negatively correlated to pH and salinity. In addition, *Vibrio* was negatively correlated to inorganic nitrite and phosphate in the FL fraction, although in the PA fraction the opposite trend was observed. In general, all correlation coefficients did not suggest strong correlation with values between −0.36 and 0.38 (**Supplementary Table 8**).

DISCUSSION

No water discharge shrimp aquaculture resulted in an accumulation of organic matter, through feed pellets, shrimp feces and other organic materials. Previous studies reported that degradation of organic matter, leaching of feed pellets, and release of ammonium from feces increase inorganic nutrients in the water column (Funge-Smith and Briggs, 1998; Smith et al., 2002; Burford and Lorenzen, 2004). These nutrients support bacterial and phytoplankton growth, which then leads to simultaneous change of physical parameters such as pH, DO, SPM, and turbidity (Burford and Williams, 2001; Burford et al., 2003).

67

FIGURE 2 | Contribution of the most abundant bacterial operational taxonomic units (OTUs) in the semi-intensive (light gray to black) and the intensive systems (yellow to dark red). Taxonomic affiliation for OTUs is provided for genus (Right) and class (Left) levels. S1–3 and T1–3: replicate pond for the semi-intensive and intensive ponds, respectively. Days of rearing are indicated below the pond symbols. FL, free-living fraction; PA, particle-attached fraction.

In this study, shrimp pond water parameters and respective BCC in intensive and semi-intensive systems were compared. Contrary to our expectations, higher shrimp density in intensive farming systems did not significantly increase inorganic nutrients in the water column. We suggest that excessive nutrients were rapidly taken up by bacteria and phytoplankton as their abundance increased more strongly in the intensive system. This was also reflected in high SPM values in the intensive system due to the aggregation of phytoplankton cells. Phytoplankton can take up total ammonia nitrogen (TAN), while heterotrophic bacteria perform nitrification and nitrogen assimilation (Avnimelech,

2014). In pond water, photosynthesis by phytoplankton and cyanobacteria increases DO, while respiration and microbial activities, such as nitrification and sulfur oxidation decrease pH (Boyd and Tucker, 2014). The higher number of phytoplankton (which was indicated by Chl a) in intensive ponds might increase DO concentration. We supposed that photosynthesis might increase oxygen concentration. However, we are aware that our DO measurements only constitute daylight conditions and that due to the higher organic matter loading of the intensive system and in the absence of light, oxygen depletion via respiration processes may lower DO concentration below the levels of

68

TABLE 3 | Bray–Curtis dissimilarities of bacterial community composition between free-living (FL) and particle-attached (PA) fractions.

Pond	Day				
	10	40	50	60	70
S1	0.54	0.83	0.83	0.85	0.91
S2	0.53	0.48	0.77	NA*	0.54
S3	0.79	0.81	0.66	0.59	0.92
Average	*0.62*	*0.71*	*0.75*	*0.72*	*0.79*
T1	0.45	0.51	0.57	0.74	0.91
T2	0.68	0.87	0.91	0.49	0.69
T3	0.63	0.68	0.70	0.58	0.82
Average	*0.59*	*0.69*	*0.73*	*0.60*	*0.80*

S, semi-intensive system; T, intensive; NA* indicated a missing sample for particle-attached fraction.

TABLE 4 | Bray–Curtis dissimilarities of bacterial community composition within system (among ponds) and between the systems for each fraction over the time.

Fraction	Day	System	Within system		Between system	
			Minimum	Maximum	Minimum	Maximum
FL	10	S	0.68	0.73	0.33	0.83
		T	0.66	0.85		
	40	S	0.62	0.92	0.63	0.84
		T	0.47	0.69		
	50	S	0.50	0.68	0.40	0.76
		T	0.58	0.83		
	60	S	0.41	0.60	0.41	0.69
		T	0.46	0.64		
	70	S	0.44	0.59	0.45	0.63
		T	0.55	0.66		
PA	10	S	0.69	0.71	0.70	0.98
		T	0.78	0.96		
	40	S	0.38	0.82	0.50	0.98
		T	0.52	0.91		
	50	S	0.62	0.76	0.43	0.99
		T	0.50	0.95		
	60	S	0.86	0.86	0.50	0.79
		T	0.50	0.65		
	70	S	0.94	0.97	0.27	0.97
		T	0.62	0.65		

FL, free-living fraction; PA, particle-attached fraction; S, semi-intensive; T, intensive.

the semi-intensive system. Even though the intensive system resulted in higher abundances of phytoplankton and cultivable heterotrophic bacteria, we could show that the concentrations of harmful inorganic nutrients, such as ammonium and nitrite, DO, and pH were still far from lethal values for shrimps, as mentioned in other studies (Schuler, 2008; Avnimelech, 2014; Furtado et al., 2015).
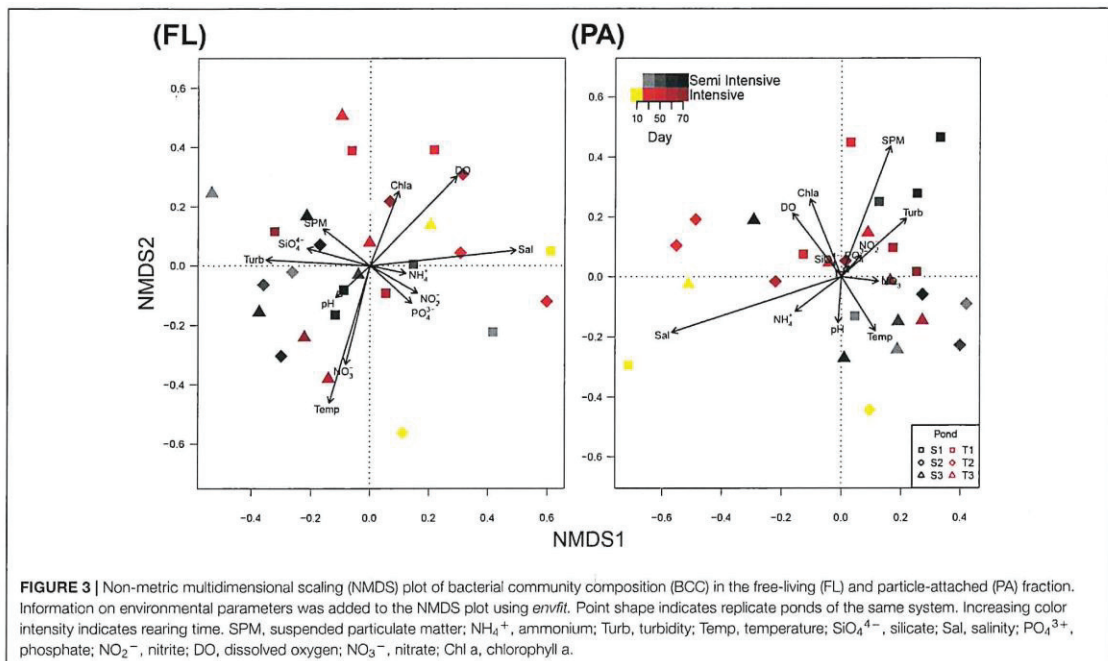
We observed fluctuation of particle (aggregate or flocs) number over rearing time. We supposed that lower pellet input, sinking or consumption of flocs might cause the fluctuation of particle numbers. Water movement and exudate secreted

either by bacteria or plankton lead to particle agglomeration (Grossart et al., 2006; Gärdes et al., 2011), generating bigger aggregates/flocs. When the aggregates sink to the bottom, particle numbers might decrease, resulting low turbidity/clearer water. Unfortunately, we did not measure daily feed input, sinking rate of aggregates and particle consumption by shrimp.

Environmental factors shape the structure and function of microbial communities (Allison and Martiny, 2008). Previous studies have shown that the succession of microbial communities was influenced by combinations of chlorophyll a, total nitrogen (TN), PO_4^{3-}, C/N ratio (Xiong et al., 2014), total phosphate, chemical oxygen demand (Zhang D. et al., 2014), and feed sources added into ponds (Qin et al., 2016). Of the parameters measured in this study, salinity was the most determinant variable which shaped BCC in the FL and particle-attached PA fractions, which concurred with previous studies (Yang et al., 2016; Hou et al., 2017; Kirchmann et al., 2017). We observed salinity fluctuation in both systems and supposed that rainfall and addition of sea water from reservoir pond to maintain rearing pond water level (~140–150 cm) decreased salinity. During our study, rainfall could increase 10–20 cm of water level, while sea water was added more frequent in intensive ponds due to regular mud removal. However, it should be considered that the influences of salinity on the BCC in the FL fraction do not completely exclude the influence of other water parameters, such as unmeasured sulfur compound in water column like hydrogen sulfide (H_2S) or sulfite which may explain the continually presence of *Sulfitobacter* in FL fraction.

Bacterial communities in intensive and semi-intensive systems were dominated by heterotrophic halophilic bacterial genera *Alteromonas*, *Erythrobacter*, *Halomonas*, *Pseudoalteromonas*, *Salegentibacter*, *Sulfitobacter*, and *Psychrobacter*. *Halomonas* and *Psychrobacter*, the salt-tolerant heterotrophic nitrifying bacteria (Chankaew et al., 2017), can oxidize ammonia in high concentration under saline conditions (Sangnoi et al., 2016). *Sulfitobacter* can oxidize sulfur and degrade high molecular weight dissolved organic matter (Bourne et al., 2004; Sosa et al., 2017). Other bacterial genera, such as *Salegentibacter*, *Exiguobacterium*, and *Erythrobacter*, were associated with shrimp shell degradation after molting (Sombatjinda et al., 2011). In our study, BCC differed from those reported by Sombatjinda et al. (2011) who analyzed *L. vannamei* earthen pond water with salinities close to freshwater. They identified *Nitrosomonas*, *Flavobacterium*, *Exiguobacterium*, *Synechococcus*, *Burkholderia*, *Nitrosospira*, and *Nitrobacter* as dominant bacteria (Sombatjinda et al., 2011). We assume that salinity caused the differences in BCC observed in our study, considering the salinity range of 32–38 PSU compared to 2–10 PSU in previous study (Sombatjinda et al., 2011). This is supported by a study of Chankaew et al. (2016), where common nitrifying bacteria, including *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus*, *Nitrospira*, *Nitrococcus*, and *Nitrobacter*, were absent in shrimp pond water with 20 PSU. Furthermore, the regular addition of commercial probiotics (personal communication with pond owners), which contained *Bacillus* sp., *Pseudomonas* sp, *Nitrosomonas* sp., *Aerobacter* sp., *Nitrobacter* sp., and *Nitrosococcus* sp. did not affect BCC in the FL and PA fractions of both systems, indicating

69

FIGURE 3 | Non-metric multidimensional scaling (NMDS) plot of bacterial community composition (BCC) in the free-living (FL) and particle-attached (PA) fraction. Information on environmental parameters was added to the NMDS plot using *envfit*. Point shape indicates replicate ponds of the same system. Increasing color intensity indicates rearing time. SPM, suspended particulate matter; NH_4^+, ammonium; Turb, turbidity; Temp, temperature; SiO_4^{4-}, silicate; Sal, salinity; PO_4^{3+}, phosphate; NO_2^-, nitrite; DO, dissolved oxygen; NO_3^-, nitrate; Chl a, chlorophyll a.
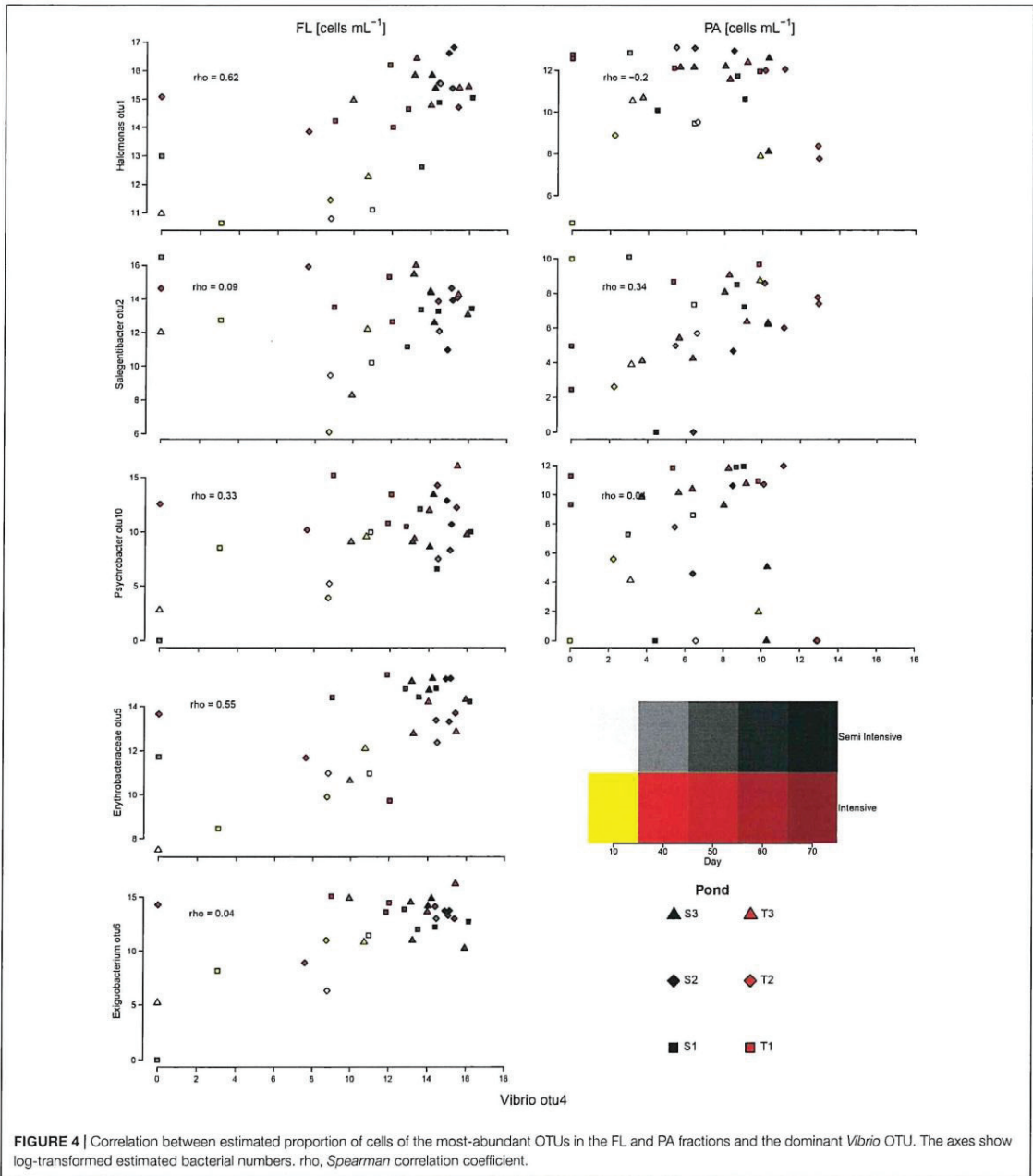
that nitrification processes were generated by other microbes. We propose that in our study the dominance of particular heterotrophic halophilic bacteria may result in higher uptake of inorganic nutrient such as ammonium and nitrite (Sangnoi et al., 2016). Therefore, even in the intensive pond waters inorganic nutrients remained low. In our study, BCC changed over time, following a "resilience scenario" (Allison and Martiny, 2008), in which a replacement of bacterial taxa occurred due to environmental change, followed by a quick return to its pre-disturbance composition. This phenomenon can be clearly seen in the particle-attached fraction of the intensive system. When pH decreased below 8 at day 40 and 50, *Vibrio* replaced *Halomonas* as the most abundant OTU, but the sequence proportions of *Halomonas* recovered, after the pH increased at day 60 and 70. In contrast, BCC in the FL and the PA fractions of the semi-intensive system during the same period was similar while experiencing stable pH (above 8). We propose that pH was a disturbing factor for heterotrophic halophilic bacteria in our shrimp systems and lead to the change of BCC only in the intensive system. This is supported by Krause et al. (2012) who reported that slight changes in pH caused compositional shifts in marine bacterial communities.

Vibrio, a potential opportunistic pathogen for *L. vannamei* (Heenatigala and Fernando, 2016; Rungrassamee et al., 2016), has been found in higher proportions in the PA fractions of the intensive system. This might indicate that the particulate fraction, specifically marine aggregates, can accumulate potentially pathogenic bacteria, as suggested by other studies (Lyons et al., 2005; Froelich et al., 2013). Our hypothesis that the intensive

TABLE 5 | Contribution and significance of observe environmental factors which explain the variation in community composition of the free-living (FL) and the particle-attached (PA) fractions based on redundancy analysis (RDA).

Source of variation	Adjusted R^2	df	F	P-value
FL				
Complete model (system + day)	0.103	5, 24	1.669	0.005
Sampling time (day)	0.070	4, 24	1.543	0.007
System	0.042	1, 24	2.172	0.204
Complete model (Sal + Chl a + NO_3^-)	0.153	3, 26	2.749	0.001
Salinity (Sal)	0.112	1, 26	4.558	0.001
Chlorophyll a (Chl a)	0.047	1, 26	2.510	0.005
Nitrate (NO_3^-)	0.030	1, 26	1.944	0.017
PA				
Complete model (system + day)	0.035	4, 19	1.207	0.184
Complete model (Sal)	0.128	1, 22	4.379	0.001

system seemed to be more vulnerable to *Vibrio* outbreaks is supported by high abundance and recurrent presence of *Vibrio* spp. only in the intensive system. Therefore, it is necessary to maintain SPM and aggregate abundance and to avoid massive *Vibrio* growth due to the fact that *Vibrio* can convert from non-virulent to virulent under certain cell density threshold or if dramatic environmental changes occur (Zhou et al., 2012). Nevertheless, toxin genes (*toxR, tlh,* and *tdh*) of *V. parahaemolyticus* (Makino et al., 2003), as well as *pirAB* toxins for AHPND (Xiao et al., 2017) were not present in this study. We argue that other halophilic, potentially probiotic, bacteria might inhibit chromosome II replication, where those genes are

70

FIGURE 4 | Correlation between estimated proportion of cells of the most-abundant OTUs in the FL and PA fractions and the dominant *Vibrio* OTU. The axes show log-transformed estimated bacterial numbers. rho, *Spearman* correlation coefficient.

located (Makino et al., 2003), as proposed by Defoirdt et al. (2011). As no other known probiotic taxa were detected in this study, we hypothesize that the presence of *Halomonas* in both systems might inhibit *Vibrio*. Recently, *Halomonas aquamarina* has been applied as probiotic in *L. vannamei* culture to oxidize

ammonium and to prevent *Vibrio* growth, which then leads to an increase of survival rates of *L. vannamei* (Zhang et al., 2009; Suantika et al., 2013; Sangnoi et al., 2016). However, other unobserved microorganisms, such as bacteriophage, *Bdellovibrio*, *Saccharomyces*, *Streptococcus*, *Streptomyces*, and protists might

71

also suppress *Vibrio* (Munro et al., 2003; Alagappan et al., 2010; Chavez-Dozal et al., 2013; Lakshmi et al., 2013; Kongrueng et al., 2017). Considering final shrimp harvests, the intensive system is the more economically promising system. However, shrimp pond management needs be improved to maintain water quality as well as beneficial bacterial communities. Regular feed control and mud discard were likely to have affected BCC in our study. Without regular mud discard, sludge and organic matter degradation might increase oxygen demand, and as a result oxygen might become depleted in the bottom of the shrimp pond. Under these conditions, anaerobic degradation of organic matter produces H_2S, and increases other toxic inorganic compounds such as ammonia and nitrite. Furthermore, sludge deposit in bottom pond might enlarge the anaerobic area which may reduce the habitable space for the shrimp. While strictly adjusted feeding in semi-intensive system seemed to lead to a more stable BCC, regular mud discard in the intensive system, which was conducted once to twice per day, might have prevented the accumulation of toxic compounds (i.e., hydrogen sulfide, ammonium, and nitrite), as indicated by the lower proportion of *Sulfitobacter*. We suggest that giving adequate feed, discarding sludge, maintaining pH, and salinity, might avoid abrupt water quality change, eliminate large anaerobic areas at the pond bottom, and may minimize proliferation of potential pathogens, especially *Vibrio*. Some aspects of shrimp pond management related studies are given in Hopkins et al. (1993); Funge-Smith and Briggs (1998), and Avnimelech and Ritvo (2003). Consequently, it is necessary for pond aquaculture to provide sufficient seawater and sludge reservoirs to avoid environmental degradation risks due to sludge removal.

In view of BCC in FL and PA fraction over rearing period and the absence of virulence genes, we propose to apply "bioflocs" in shrimp aquaculture. This can be generated by adding carbohydrates such as molasses, rice bran or tapioca. Bioflocs might become an alternative food source for shrimps and be able to maintain ammonia and nitrite concentration in shrimp pond (Avnimelech, 2014). Moreover, because bacteria from commercial probiotics were not detected in any samples, we suggest that the regular application of these probiotics in such high salinity rearing pond is not necessary to be done. At the end, operational shrimp rearing costs, especially for feed pellet and commercial probiotics, can be reduced.

CONCLUSION

Different stocking densities influenced water quality parameters, especially SPM, DO, chlorophyll a, and pH. High salinity shaped the BCC in the PA and FL fraction, favoring heterotrophic

halophilic bacteria which facilitate an optimum uptake of inorganic nutrients and prevent increase of *Vibrio* growth. Despite a wide variability of BCCs in intensive and semi-intensive systems, the abundance of *Vibrio* was higher in the intensive system, which may therefore be more vulnerable to disease outbreaks. Monitoring BCC, especially the PA fraction's, and larger particulates such as aggregates in shrimp pond waters may potentially prevent disease outbreaks such as Vibriosis, white feces disease, white-tale disease, and AHPND.

ETHIC STATEMENT

This study did not sacrifice any organism, nor introduce viable pathogenic bacteria to the environment. All sampling procedures were already informed to and have been agreed upon by shrimp pond owners.

AUTHOR CONTRIBUTIONS

YA, JH, and AG designed the project. YA collected the samples and conducted the *in situ* measurement with the logistic support of AT and AK. YA and CH completed the statistical analysis. YA prepared the manuscript with input from all co-authors.

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SUPPLEMENTARY MATERIAL

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74

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75

Bacterial abundance and community composition in pond water from shrimp aquaculture system with different stocking densities

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Supplementary Figures and Tables

1.1. Supplementary Figures
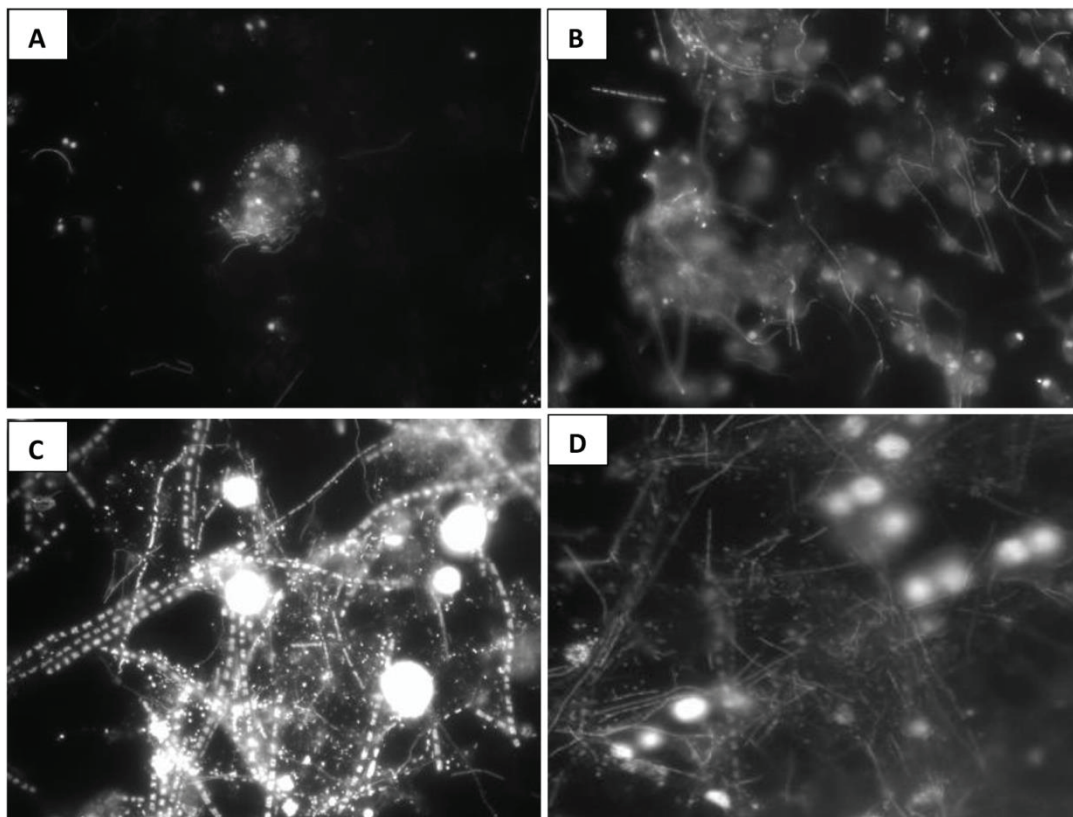
Supplementary Figure 1. Particle-attached bacterial cells from one of the water samples of intensive (A,C) and semi-intensive (B,D) ponds, at day 10 (A,B) and at day 40 (C,D). Bigger bright circles indicate plankton cells.
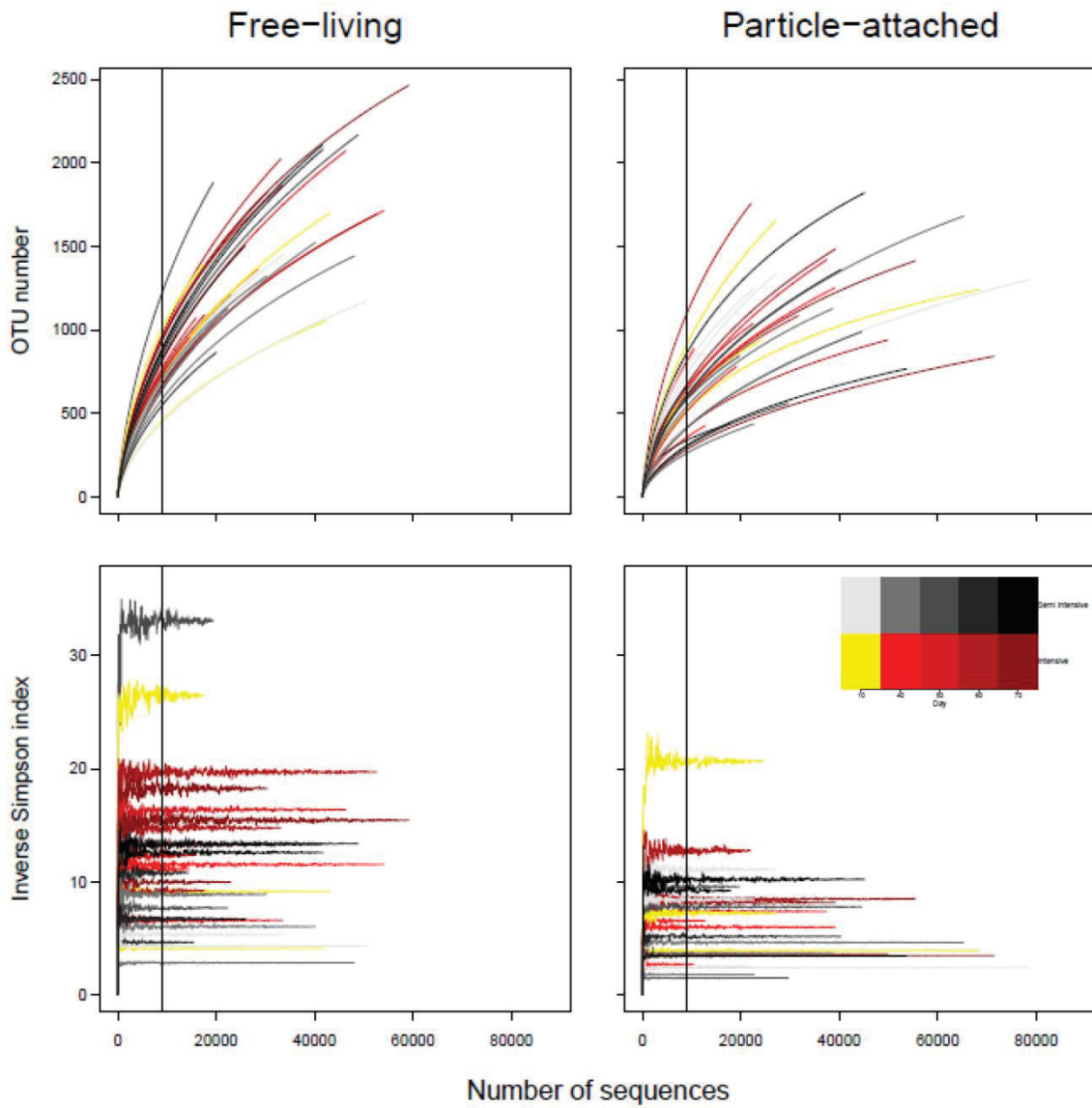
Supplementary Figure 2. Rarefaction curves of OTU number and inverse Simpson index from 59 samples. Increasing color intensity indicates rearing time. Color yellow till dark red indicates samples from the intensive ponds, while grey to black indicates samples from the semi-intensive ponds.
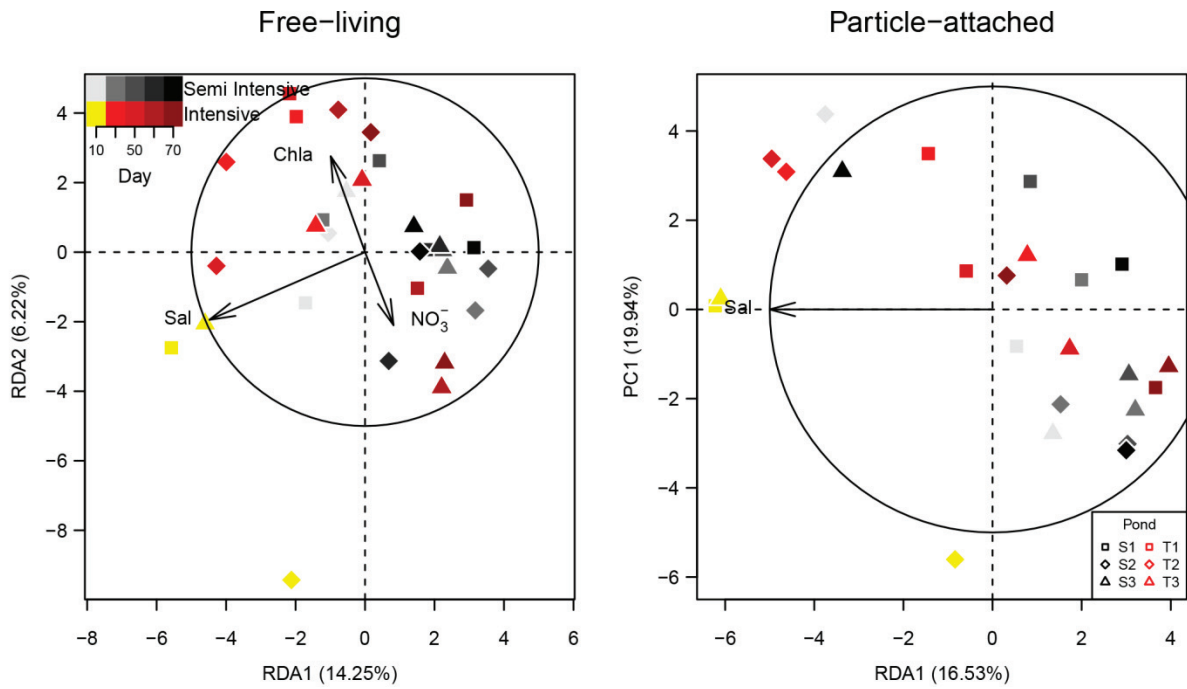
Supplementary Figure 3. Redundancy analysis (RDA) ordination showing bacterial community composition in relation to significant environmental variables. Point shape indicates replicate pond of the same system. Increasing color intensity indicates rearing time. Sal: Salinity, Chla: Chlorophyll a, NO_3^-: Nitrate.

1.2.   Supplementary Tables

Supplementary Table 1. Averages of bacterial cell concentrations (cell mL^{-1}) and aggregate numbers (aggregates mL^{-1}). Bacterial cell and aggregate numbers are presented in average ± standard deviation. Values without bracket indicate bacterial cell numbers. Values within bracket indicate aggregate numbers. Total bacterial cells were calculated by multiplication of the average of bacterial cell numbers, aggregates numbers, filter surface factor (x8) and then divided by filtered water (day 10: 10 ml, day 40,50 and 60: 1 ml, day 70: 0.5 ml).

Day	Aggregate sizes (µm²)									
	< 300		300 - 625		937.5 - 1,406.5		1,725 - 2,500		2,825 - 3,906.25	
	S	T	S	T	S	T	S	T	S	T
10	607±62	526±58	57±12 (296±7)	46±15 (338±23)	86±3 (511±63)	80±7 (398±13)	100±13 (124±17)	102±5 (111±11)	160±24 (24±5)	195±14 (20±2)
40	399±37	409±62	52±9 (246±41)	56±2 (298±14)	70±12 (494±27)	72±6 (433±62)	109±16 (182±10)	112±23 (159±13)	194±7 (90±4)	202±12 (70±11)
50	468±18	417±75	55±5 (273±19)	44±9 (290±31)	83±8 (345±25)	82±4 (420±37)	106±9 (201±10)	99±3 (185±29)	186±22 (85±9)	191±4 (84±8)
60	403±44	435±41	53±4 (255±22)	54±3 (251±8)	74±9 (343±23)	69±5 (353±8)	96±3 (193±11)	98±7 (198±14)	190±12 (99±8)	192±27 (96±6)
70	243±15	246±17	53±4 (142±14)	54±3 (133±22)	77±1 (275±13)	79±2 (270±19)	115±7 (85±7)	99±5 (77±11)	199±14 (46±3)	191±14 (52±10)

S: semi-intensive system (3 ponds), T: Intensive system (3 ponds).

Supplementary Table 2. Primer pairs for the toxin gene assay of *V. parahaemolyticus*

Primer sets[a]	Sequences	Length (bp)	T_M-value (°C)[b]	GC (%)[c]	Amplicon size (bp)	Source
*toxR*_F	5'-CAG CGT TGT GAA GCA ACA TTA G-3'	22	53	45.45	98	Our study
*toxR*_R	5'-CTC CAG ATC GTG TGG TTG TAT G-3'	22	54.8	50.0		
*tlh*_F	5'-CCGTCAGATTGGTGAGTATCAG-3'	22	54.8	50.0	99	Our study
*tlh*_R	5'-CGTTCAATGCACTGCTCAATAG-3'	22	53	45.45		
*tdh*_F	5'-CAGTATTCACAACGTCWGGTACTA-3'	24	54	41.67	200	Our study
*tdh*_R	5'-TGGAATAGAAYCTTCATCTTCACC-3'	24	54	37.5		
*PirA*_F	5'-GTCGGTCGTAGTGTAGACATTG-3'	22	57.4	50	147	Our study
*PirA*_R	5'-AGGGCGTTGTAAATGGTAAGT-3'	21	57	42.9		
*PirB*_F	5'-GGTGATGAATGGCTTGGTTATG-3'	22	56.9	45.5	124	Our study
*PirB*_R	5'-GCACATCAGAATCGGTGAAAC-3'	21	56.8	47.6		

[a]*tlh*: thermolabile hemolysin, *tdh*: thermostable direct hemolysin, *pirA* and *PirB*: Photorhabdus insect-related (Pir) toxins
[b] T_M: Melting temperature
[c] GC: Guanine-cytosine content

Supplementary Table 3. List of samples tested for toxin genes of *V. parahaemolyticus* (*toxR, tlh, tdh, pirAB*)

Number	Fraction	Pond	Day	System
1	FL	T1	10	Intensive
2	PA	T1	10	Intensive
3	FL	S1	10	Semi-intensive
4	PA	T2	40	Intensive
5	FL	T2	50	Intensive
6	PA	T2	40	Intensive
7	PA	T3	10	Intensive
8	FL	S1	60	Semi-intensive
9	FL	S2	50	Semi-intensive
10	FL	S2	40	Semi-intensive

Supplementary Table 4. General Linear Mix Models (GLMM) for physical parameters, the abundances of cultivable heterotrophic bacteria and inorganic nutrient concentrations.

Parameters[a]	System				Day				Interaction between system and day			
	dfn[b]	dfd[c]	F-value	p-value	dfn[b]	dfd[c]	F-value	p-value	dfn[b]	dfd[c]	F-value	p-value
Temperature	1	4	2.425	0.194	6	24	7.252	<0.001	6	24	5.706	0.001
Salinity	1	4	8.485	0.044	6	24	6.471	<0.001	6	24	3.086	0.022
pH	1	4	12.446	0.024	6	24	3.479	0.013	6	24	1.256	0.314
DO	1	4	10.501	0.032	6	24	1.814	0.139	6	24	0.885	0.521
Turbidity	1	4	1.258	0.325	6	24	7.885	<0.001	6	24	2.775	0.034
Chl a	1	4	7.315	0.054	6	24	6.228	<0.001	6	24	3.809	0.008
Phosphate	1	4	0.035	0.86	6	24	1.033	0.428	6	24	1.079	0.402
Nitrite	1	4	0.437	0.545	6	24	0.493	0.807	6	24	2.673	0.039
Nitrate	1	4	0.648	0.466	6	24	0.907	0.507	6	24	0.226	0.964
Ammonium	1	4	0.196	0.681	6	24	0.767	0.603	6	24	0.687	0.662
Silicate	1	4	2.858	0.166	6	24	0.802	0.578	6	24	1.163	0.358
SPM	1	4	13.197	0.022	6	24	121.978	<0.001	6	24	1.538	0.209
THB	1	4	0.99	0.376	6	24	102.191	<0.001	6	24	6.519	<0.001
TPPV	1	4	0.11	0.757	6	24	15.917	<0.001	6	24	2.694	0.038

[a]Chla: Chlorophyll a, SPM:Suspended particulate matter, THB: Total heterotrophic bacteria, TPPV: Total cultivable potential pathogenic *Vibrio*,
[b]dfn: degrees of freedom numerator,
[c]dfd: degrees of freedom denominator

Supplementary Table 5. Multivariate analysis of variance (MANOVA) for bacterial cell numbers from DAPI counting

Variables	Df	Pillai	Approx. F	Dfn	Dfd	Pr(>F)
Day	4	1.515	12.48	8	32	< 0.001
System	1	0.264	0.54	2	3	0.63
Interaction	4	0.274	0.64	8	32	0.74
Residual	16					

Df: degrees of freedom, dfn: degrees of freedom numerator, dfd: degrees of freedom denominator

Supplementary Table 6. General Linear Mixed Models (GLMM) for significant predictor variable (Day)

Parameters[a]	Day			
	Dfn	Dfd	F-value	p-value
PA cells	4	16	260.283	<0.001
FL cells	4	16	15.940	<0.001

Supplementary Table 7. R value of the post-hoc of analysis of similarity (ANOSIM) for bacterial community composition

Fractions	Day	Intensive				Semi-intensive			
		10	40	50	60	10	40	50	60
FL	10								
	40	0.22				-0.15			
	50	-0.11	-0.22			-0.37	0.04		
	60	0.11	0.26	0.15		-0.19	-0.04	-0.22	
	70	0.44	0.67	0.11	0.59	-0.04	0.07	-0.22	-0.26
PA	10								
	40	-0.04				-0.22			
	50	-0.04	-0.15			-0.07	-0.19		
	60	0.44	-0.19	0.19		0.00	0.25	-0.25	
	70	0.56	-0.04	0.11	-0.41	-0.11	-0.07	-0.07	-0.75

FL: Free-living, PA: particle-attached

Supplementary Table 8. Correlation between *Vibrio* FRAxC and selected biogeochemical parameters in free-living (FL) and particle-attached (PA) fractions

Parameters	*Spearman* correlation coefficients	
	FL	PA
SPM	0.38	0.21
Temperature	0.26	0.03
pH	-0.29	-0.36
Salinity	-0.28	-0.14
Ammonium	0.27	0.17
Nitrite	-0.18	0.14
Phosphate	-0.12	0.11
NP[a] ratio	0.18	0.13
TPPV[b]	0.27	0.24

[a] NP: Nitrogen-Phosphate
[b] TPPV: Total cultivable potential pathogenic *Vibrio*

Chapter 5. Structure and co-occurrence patterns of bacterial communities associated with white faeces disease outbreaks in the Pacific shrimp *Litopenaeus vannamei*

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Contribution to the manuscript:

Experimental concept and design	**90%**
Acquisition of experimental data	**100%**
Data analysis and interpretation	**70%**
Preparation of figures and tables	**70%**
Drafting manuscript	**80%**

Chapter 5. Structure and co-occurrence patterns of bacterial communities associated with white faeces disease outbreaks in the Pacific shrimp *Litopenaeus vannamei*

Abstract

A white faeces disease (WFD) occurs frequently in shrimp aquaculture. Understanding environmental conditions and bacterial community dynamics are essential to minimize rearing failure due to such bacterial disease. We analysed the pond water quality and compared water microbiomes as well as the intestinal microbiomes of healthy and infected-shrimps via 16S rRNA gene sequencing. We noticed that WFD occurred once water pH decreased to 7.71-7.84, and it concurred with bacterial shifts in the water microbiomes. The disease severity coincided with the degree of dominance of opportunistic pathogenic bacteria in the shrimp microbiome which constituted up to 60% and 80% of the sequences in samples from the early and advances stages of the disease outbreak, respectively. Bacterial co-occurrence of such opportunistic pathogenic bacteria included *Alteromonas*, *Arcobacter*, *Marinomonas, Photobacterium, Pseudoalteromonas* and *Vibrio*, which might therefore contribute to the WFD events. Notably, resilience of bacterial communities in the water microbiome occurred when the water pH was adjusted to above 8, followed by the recovery of the shrimps within 2 days of the disease event. Our results highlighted that pH is one of the major factors in preventing disease event, affecting not only the water microbiome, but also the shrimps and their intestinal microbiome.

Keywords: bacterial disease, aggregates, shrimp pond, intestinal microbiome, bacterial assemblage

Introduction

Bacterial diseases are major problems for *Litopenaeus vannamei* pond aquaculture in Asia and Latin America. They have been causing severe annual economic losses reaching approximately USD 1 billion over last decade[1,2]. Among reported bacterial diseases, acute hepatopancreatic necrosis disease (AHPND) and white faeces disease (WFD) are the most infectious and lethal ones[3–5]. The latter has been frequently occurring in Asian shrimp aquaculture since 2009[3,4,6], which reduced shrimp survival to 20-30%[4].

WFD events in shrimp aquaculture are observed by the presence of white faecal strings which float in the rearing water[3,7]. In contrast to AHPND which eradicates shrimp juveniles at the beginning of rearing[8–10], WFD usually occurs after approximately two or more months of

culture[4], resulting in malformation or retarded shrimp growth, unprofitable harvests, and even mass mortality[11]. *Enterocytozoon hepatopenaei* (EHP), vermiform bodies resembling protozoan gregarines and certain cultivable *Vibrio* species, such as *V. parahaemolyticus*, *V. fluvialis*, *V. mimicus*, *V. alginolyticus*, and *Vibrio sp.* were reported as the causative agents of the disease[3,4,15]. Furthermore, deteriorated water quality with low oxygen concentrations below 3.0 mg L^{-1} and alkalinity below 80 ppm caused peak mortality rates during WFD outbreaks[12].

Often antibiotics and viable beneficial bacterial cells (probiotics) are applied in shrimp farming to enhance shrimp growth and to avoid disease outbreaks[13–16]. However, these practices have been unsuccessful to prevent WFD in *L. vannamei* shrimp aquaculture. Also common assessment of the concentration of cultivable heterotrophic bacteria as well as presumptive *Vibrio* failed to predict pathogenic bacteria. Surprisingly, the WFD still happened even though cultivable presumptive *Vibrio* counts were 3-fold lower than cultivable heterotrophic bacteria (pers.comm. with shrimp pond owners). The cultivation method may not be able to grow *Vibrio* that have entered the viable but non-culturable (VBNC) phase[17] which then lead to miss prediction of the disease occurrence.

While the microbiomes of the intestine (MI) of healthy shrimps are well described[16,18–22], there is a paucity of information about the microbiome of diseased shrimps. Moreover, little is known about the water microbiome (WM) and rearing water parameters during WFD outbreaks. In this study, we analysed WM and separated between free-living (FL) and particle-associated (PA) bacteria in the pond water. Particles, especially larger aggregates can potentially harbour more pathogenic microorganisms[23–25], becoming hotspots of opportunistic pathogens, while they additionally serve as an alternative feed for shrimps and fishes[26,27]. During WFD events, once faecal strings containing, among others, opportunistic pathogenic bacteria disintegrate in the rearing water, faecal microbes are released, which may then enrich the WM[28] resulting in a high load of potential opportunistic pathogenic bacteria predominantly in the particulate fraction. We therefore hypothesize that the outbreak of the disease can be facilitated by consumption of contaminated aggregates. Thus, a detailed monitoring of FL and PA bacteria is necessary to predict disease transfer among shrimp in a closed shrimp aquaculture system.

To obtain comprehensive perspective of bacterial dynamics as well as water quality in shrimp ponds over the course of the WFD events, we (i) investigated water quality parameters, (ii) elucidated bacterial community compositions (BCC) in rearing water (WM),

87

in the intestines of healthy *L. vannamei* (MI) and on the white faecal strings (MFS), (iii) detected and estimated pathogenic *Vibrio* through their virulence gene copy numbers, and (iv) analysed bacterial assemblage characteristics for WFD beyond the presence of known causative agents, via co-occurrence network.

Results

The WFD events investigated in this study occurred in shrimp ponds, whose water parameters and WM during and after the WFD events have been reported elsewhere[29]. The WFD events were observed in ponds with moderate (P2) and high stocking densities (P3 and P4) at 52nd, 63th and 67th days of rearing, respectively, suggesting that the disease occurred regardless of the density of the reared shrimps. The WFD event was initiated by a shift in the WM and stress in the shrimps, indicated by a decrease of appetite 2-3 days prior to the onset of the disease, which was induced by a sudden change in pond water parameters.

Biogeochemical characteristics of the shrimp pond water. Ponds with infected shrimps (P2, P3, and P4) were characterized by lower pH (7.71-7.84), higher turbidity (38.0-41.7 NTU) and contained more cultivable non sucrose-fermenting presumptive *Vibrio* colonies (green colonies between 4000-4700 CFU mL^{-1}). In contrast, the pond with healthy shrimps (P1) had higher pH (> 8) and lower turbidity and count of non sucrose-fermenting *Vibrio* colonies (0-400 CFU ml^{-1}) (**Table 1**). Considering the low pH during the WFD events, the shrimp pond owners added limestone at night right after they observed symptoms of disease. This treatment was performed until the symptoms of the disease disappeared. Approximately, they added 0.4-1.5 ton per pond for 3 days (pond water volume were approximately 3500-3700 m^3). This treatment affected the water quality, particularly pH which increased to above 8, while numbers of non sucrose-fermenting presumptive *Vibrio* decreased 3 to 6-fold after the WFD outbreaks (**Table 1**).

Environmental parameters in P1 at day 60th and in P2, P3, and P4 at WFD outbreaks were plotted in a PCA to characterize the shrimp ponds (**Figure 1**). The ponds with healthy and diseased shrimps were separated along PC1, which accounted for 60% of the variation in the data, and was determined mostly by the abundances of cultivable presumptive *Vibrio*, ammonium and phosphate concentrations, pH, temperature, and turbidity. Nitrate and silicate concentrations, and salinity were among the water parameters which contributed most to PC2.
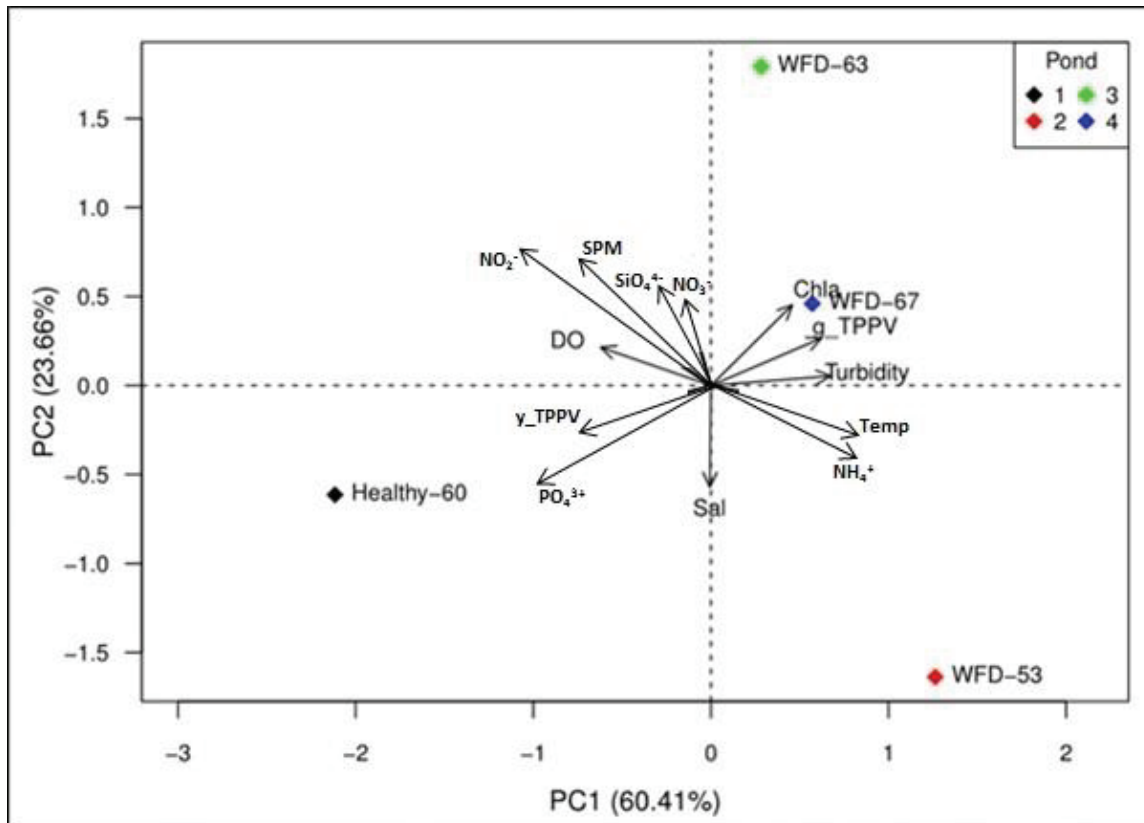
Figure 1 : Principal component analysis (PCA) of observed environmental parameters. Point labels indicate disease state and rearing day. SPM: suspended particulate matter, y_TPPV: yellow colonies of total potential pathogenic *Vibrio* (presumptive *Vibrio*), g_TPPV: green colonies of presumptive *Vibrio*, NH_4^+: ammonium, Turb: turbidity, Temp: temperature, SiO_4^{4-}: silicate, Sal: salinity, PO_4^{3+}: phosphate, NO_2^-: nitrite, DO: dissolved oxygen, NO_3^-: nitrate, Chla: chlorophyll a.

Table 1. Biogeochemical parameters of the pond with healthy shrimps (P1) and the ponds which experienced white faeces disease (P2, P3, P4) before, during and after disease event.

| Parameters	Pond 1			Pond 2			Pond 3			Pond 4		
Sampling time (day)	50	60	70	50	**53**	60	60	**63**	70	60	**67**	70
pH	8.12	8.40	8.21	8.18	**7.79**	7.93	8.02	**7.84**	7.96	7.74	**7.71**	8.17
Temperature (°C)	30.32	29.91	30.05	31.14	**30.94**	30.94	29.56	**30.61**	30.87	30.29	**30.23**	30.57
DO (mg L^{-1})	6.10	6.10	6.20	5.60	**5.57**	5.80	6.32	**5.98**	6.02	5.93	**5.68**	6.58
Turbidity (NTU)	19.60	25.60	18.50	19.10	**41.70**	25.60	25.70	**38.50**	38.60	30.70	**38.00**	49.90
Chl a (mg L^{-1})	88.66	21.19	39.60	28.96	**45.76**	10.22	70.28	**69.76**	69.79	31.64	**45.97**	94.90
Salinity (PSU)	35.53	34.47	33.36	32.92	**34.73**	34.02	36.12	**34.17**	34.09	35.64	**34.02**	33.27
SPM (mg L^{-1})	181.17	194.82	151.88	161.49	**184.34**	168.74	193.03	**196.38**	186.52	174.94	**183.47**	175.60
TPPV (CFU mL^{-1})												
- green colonies	0	0	400	400	**4000**	600	300	**4700**	700	300	**4000**	1100
- yellow colonies	7800	6700	3000	4100	**1700**	6400	3700	**1700**	9700	4400	**2000**	8800
Inorganic nutrients (mg L^{-1})												
PO$_4^{3-}$	0.063	0.886	0.086	0.914	**0.795**	0.095	0.245	**0.793**	0.524	0.031	**0.770**	0.333
NO$_2^{-}$	0.001	0.219	0.001	0.019	**0.199**	0.002	0.001	**0.217**	0.006	0.002	**0.205**	0.005
NO$_3^{-}$	0.029	0.076	0.006	0.046	**0.061**	0.061	0.011	**0.078**	0.003	0.003	**0.087**	0.842
NH$_4^{+}$	0.662	1.017	0.186	0.482	**1.088**	0.088	0.373	**1.060**	0.650	0.062	**1.041**	0.236
SiO$_4^{4-}$	0.185	0.643	2.775	0.394	**0.554**	1.554	0.859	**0.803**	0.494	0.297	**0.566**	0.197

SPM: suspended particulate matters, Chl a: chlorophyll a, TPPV: total cultivable potential pathogenic *Vibrio* (presumptive *Vibrio*), values observed at disease event are highlighted in bold.

BCC variation among samples. A total of 80 samples from commercial probiotic, bacterial strains, WM, MI, and MFS were sequenced, resulting in 3,917,111 high quality sequences ranging from 7,892 to 200,098, with a mean of 48,963 sequences per sample. After merging the OTU profiles of the technical replicates that were collected for the FL and the PA fractions in P2, P3, and P4 at WFD events, the sequencing data set for bacterial community analyses consisted of 70 samples with on average 58,336 sequences per sample. Cluster analysis and NMDS of these samples showed highly heterogeneous bacterial communities which were grouped in 7 clusters at a Bray-Curtis dissimilarity threshold of 0.95. Within each cluster average of Bray-Curtis dissimilarities ranged from 0.51 to 0.72. The WM in FL and PA fraction at non-disease events clustered in two groups, which were distinct from WM at disease events. Bacterial strains grown on the *Thiosulfate-citrate-bile salts-sucrose (TCBS) agar* from P1 clustered together with MI (**Supplementary Information Figure 1**).

Despite the high heterogeneity, WM in P1 had a similar composition of dominant bacterial taxa. Taxonomic identification of cultivable strains from P1 water indicated that all strains belonged to the genus *Vibrio*. The WM in P1 was predominantly comprised of the bacterial classes *Bacteroidia* (*Salegentibacter*), *Bacilli* (*Exiguobacterium*) and *Gammaproteobacteria* (*Halomonas* and *Psychrobacter*). These taxa were also found in the WM of the FL and the PA fractions of P2, P3, and P4 before and after the disease event (**Figure 2A**). During the disease event, the WM of P2, P3, and P4 were altered with *Bacteroidia* (*Mesoflavibacter*), *Campylobacteria* (*Arcobacter*) and *Gammaproteobacteria* (*Alteromonas*, *Marinomonas*, *Photobacterium*, *Pseudoalteromonas* and *Vibrio*) dominating BCC (**Figure 2A**). Those genera exhibited only low proportions in the WM of P1 at all sampling points and in both fractions, with the exception of *Vibrio*.

Dominant members of MI were *Gammaproteobacteria* consisting of the genera *Acinetobacter*, *Pseudomonas*, and *Vibrio*, while MFS samples were dominated by *Campylobacteria* (*Arcobacter*) and *Gammaproteobacteria* consisting of the genera *Alteromonas*, *Marinomonas*, *Photobacterium*, *Pseudoalteromonas* and *Vibrio* (**Figure 2B**). Interestingly, neither *Acinetobacter* nor *Pseudomonas* affiliated sequences were found in MFS. Conversely, *Alteromonas*, *Marinomonas*, *Photobacterium* and *Pseudoalteromonas* were absent in healthy shrimp samples. This clear distinction between MI and MFS was further supported by pairwise ANOSIM test, which showed that MI differed from the MFS of P2, P3, and P4, while MFS among ponds with infected shrimps were more similar (**Table 2**). Especially in the MFS from P2, *Alteromonas* made up more than 50% of all sequences in 7

out of 10 faecal string (FS) samples, while in the remaining 3 samples *Alteromonas* still constituted up to 40%.
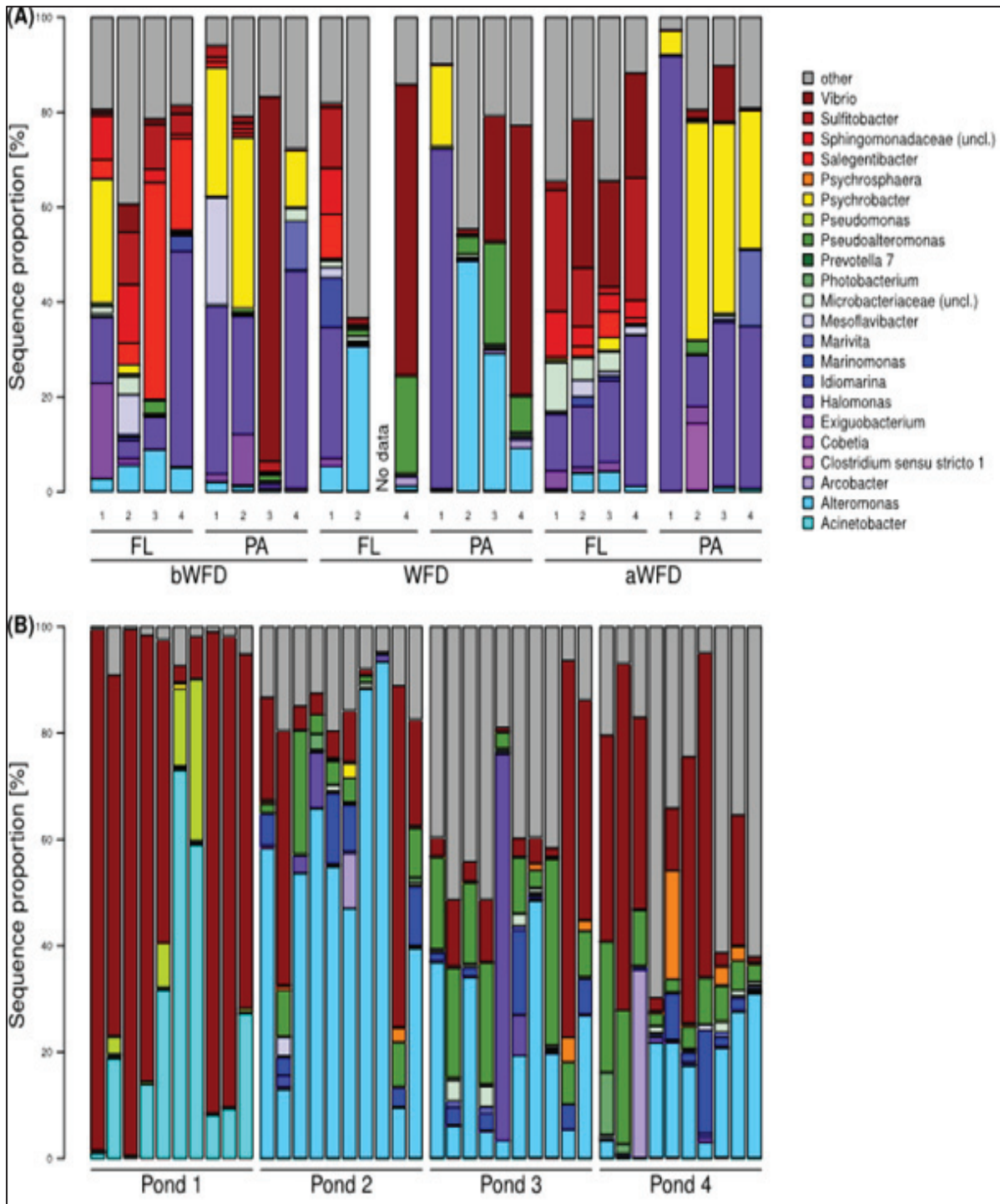
Figure 2. Contribution of the most abundant bacterial operational taxonomic units (OTUs) in water microbiome (A) and microbiome of shrimp intestine and faeces (B). Samples were collected from pond with healthy shrimps (P1) and pond with infected shrimps (P2, P3, and P4). Numbers in panel A indicate ponds. In panel B, 10 samples per pond were collected from the healthy shrimp intestine (P1), and fresh faecal strings (P2, P3, and P4). Taxonomic affiliation is provided in genus levels. FL: Free-living fraction, PA: Particle-associated fraction, bWFD: before WFD event, WFD: during WFD, aWFD: after WFD event. Intestine and faecal strings were taken at day 60, 53, 63, and 67 for Pond 1, 2, 3, and 4, respectively.

Table 2. Pairwise analysis of similarity (ANOSIM) and Bray-Curtis dissimilarity values comparing the intestinal microbiome of healthy shrimps (MI) and that of white faecal strings (MFS).

Microbiomes	MI P1	MFS P2	MFS P3	MFS P4
MI P1	0.63	0.92	0.95	0.93
MFS P2	**(0.80**, 0.002**)**	0.65	0.75	0.80
MFS P3	**(0.87**, 0.002**)**	**(0.22**, 0.006**)**	0.70	0.73
MFS P4	**(0.75**, 0.002**)**	**(0.24**, 0.002**)**	(-0.01, 0.523**)**	*0.75*

P: pond, where P1 was pond with healthy shrimps, while P2, P3 and P4 were ponds with infected shrimps (N = 10 samples per pond). The lower triangle of the tables shows ANOSIM R values (p-values adjusted for multiple testing using the Benjamini-Hochberg correction in parentheses). Significant results are highlighted in bold. The diagonal and upper triangle shows average of Bray-Curtis dissimilarity within and between ponds, respectively.

During disease outbreaks, MFS and WM from ponds with diseased shrimps (P2, P3 and P4) shared similar bacterial communities. Furthermore, the WM of the FL and the PA fractions from ponds with infected shrimps at non disease event was highly dissimilar to the MFS. This was also observed between MI and WM of P1 (**Figure 3**). These trends in Bray-Curtis dissimilarities were highly significant.
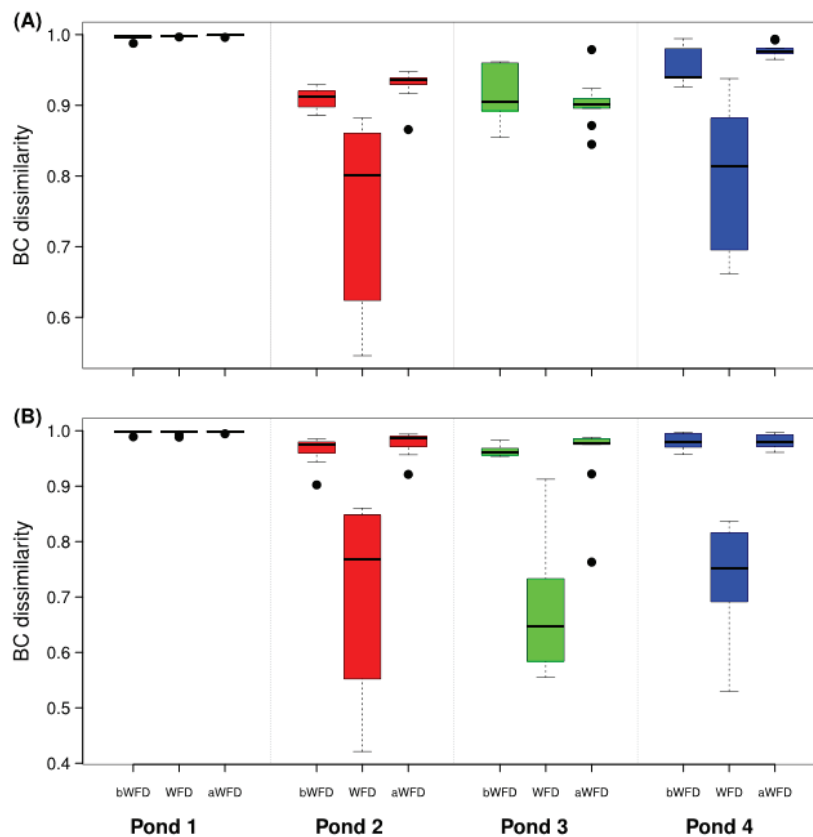
Figure 3. Bray-Curtis dissimilarity values between the free-living (A) and particle-associated (B) fraction compared to the intestinal (MI) or faecal microbiome(MFS). bWFD: before white faeces disease, WFD: during white faeces disease, aWFD: after white faeces disease.

Virulence gene detection and quantification. Our primer pairs detected *toxR, tlh,* and *tdh* genes from positive control *V. parahaemolyticus* DSM 10143 with a limit of quantification of 26 cells mL^{-1} (**Supplementary Table 1 and 2**). We targeted these three genes in WM, MI, MFS samples, but only two virulence genes (*toxR* and *tlh)* could be detected and quantified (**Table 3**).

Concentrations (copy numbers) of the *toxR* and *tlh* gene in MI and MFS did not differ from each other (**Table 3**), and varied in a range from 3.7 to 4.5 and 3.5 to 4.3 log gene copies fecal string^{-1} or intestine^{-1} for *toxR* and *tlh*, respectively. However, concentration of *toxR* and *tlh* genes differed significantly among ponds and sizefractions. Particle-associated and free-living fraction from P2 water contained higher *toxR* gene copy numbers (14.9 ± 1.3 and 13.2 ± 1.3 log copies L^{-1}, respectively), which differed significantly compared to the respective fractions of the two other ponds with infected shrimps (TukeyHSD Post-hoc test, **Table 3**). In contrast, no virulence genes were detected in P1 water at all sampling points as well as in the water of the remaining ponds (P2, P3, and P4) at non-disease sampling points.

Table 3. Concentration of *toxR* and *tlh* gene in intestine of healthy shrimps, faecal strings of WFD infected shrimps, pond water samples in free-living (FL) and particle-associated (PA) bacterial fractions

| Sample	Pond	N	% presence (n(+)/N) *toxR, tlh*	Range level *toxR*	*tlh*	Mean ± SD *toxR*	*tlh*	ANOVA *toxR*	*tlh*	Unit
Intestine	P1	10	90 (9/10)	1.2-6.9	0.9-6.9	4.5 ± 1.8	3.9 ± 2.5			Log copies intestine^{-1}
Faecal string	P2	10	90 (9/10)	1.5-5.0	0.9-5.5	3.7 ± 1.0	3.7 ± 1.2	df: 3, 36 F-value: 0.71 p: 0.55	df: 3, 36 F-value: 0.14 p: 0.93	
Faecal string	P3	10	100 (10/10)	2.1-6.6	1.6-6.6	3.7 ± 1.4	3.5 ± 1.5			Log copies string^{-1}
Faecal string	P4	10	90 (9/10)	1.6-6.4	2.1-6.5	3.9 ± 1.5	4.3 ± 1.6			
Water										
PA	P1	3	0	< LoQ	< LoQ	< LoQ	< LoQ			
	P2	3	100 (3/3)	13.9-15.9	11.7-13.8	14.9 ± 1.3^{ab}	13.0 ± 1.2^{a}			
	P3	3	100 (3/3)	3.4-6.7	6.1-7.0	4.6 ± 1.9^{bc}	6.7 ± 0.5^{b}	df: 5, 12 F-value: 49.05 p: < 0.001	df: 5, 12 F-value: 126.08 p: <0.001	Log copies L^{-1}
	P4	3	100 (3/3)	6.4-6.8	5.8-6.4	6.7 ± 0.2^{c}	6.0 ± 0.3^{b}			
FL	P1	3	0	< LoQ	< LoQ	< LoQ	< LoQ			
	P2	3	100 (3/3)	11.7-14.2	14.2-16.1	13.2 ± 1.3^{a}	14.9 ± 1.0^{a}			
	P3	3	100 (3/3)	2.9-4.7	1.5-2.6	5.0 ± 0.8^{ac}	2.2 ± 0.6^{c}			
	P4	3	100 (3/3)	4.3-5.9	4.0-5.4	3.8 ± 0.9^{c}	4.7 ± 0.7^{d}			

N: sample size, SD: standard deviation, PA: particle-associated fraction, FL: free-living fraction. LoQ: limit of quantification. Significant different groups of samples based on TukeyHSD post-hoc tests are indicated by different superscripts. Copy numbers of *toxR* and *tlh* genes were tested separately.

Bacterial co-occurrence networks. After filtering of rare and low sample coverage OTUs, 269 OTUs were retained from MI and MFS samples for co-occurrence network analysis using SPIEC-EASI. Louvain clustering was able to generate 15 bacterial co-occurrence modules (**Figure 4** and **Supplementary Table 3**). Among 15 modules, 2 modules (M2 and M14) represented co-occurring OTUs unique to MI samples of healthy shrimps. They consisted of *Acinetobacter*, *Pseudomonas*, as well as two *Vibrio* OTUs. Interestingly, these *Acinetobacter*, *Pseudomonas*, and *Vibrio* OTUs were absent in all WMs including those from P1. Ten modules (M3, M4, M5, M7, M8, M9, M10, M11, M12 and M15) were identified as characteristic for infected shrimps. Module 3 and 5 consisted exclusively of single genus which was *Alteromonas* and *Photobacterium*, respectively, while remaining modules consisted of more than three genera. Notably, *Vibrio* OTUs appeared in the healthy and the diseased shrimp samples and contributed to four network modules (M1, M6, M12, and M14). M1 and M6 consist uniquely of *Vibrio* which represented bacterial co-occurrence networks in both MI and MFS, while in M12, *Vibrio* co-occurred with *Arcobacter* and *Pseudoalteromonas*, which characterized bacterial co-occurrence for infected shrimps.

Random forest test informed module accuracy between pairwise tested ponds (**Supplementary Table 4**) which confirmed specific modules for healthy and diseased shrimp samples based on their frequent in the tested samples. It produced 5 modules (M2, M3, M4, M9, and M14) which frequently occurred between MI and MFS where M2 and M14 were characteristic for healthy shrimp, while M3, M4, and M9 were characteristic for infected shrimps. Representative modules and its members which indicate the microbiome of healthy and infected shrimp as well those for both stages were visualized in a heat map (**Figure 5**).
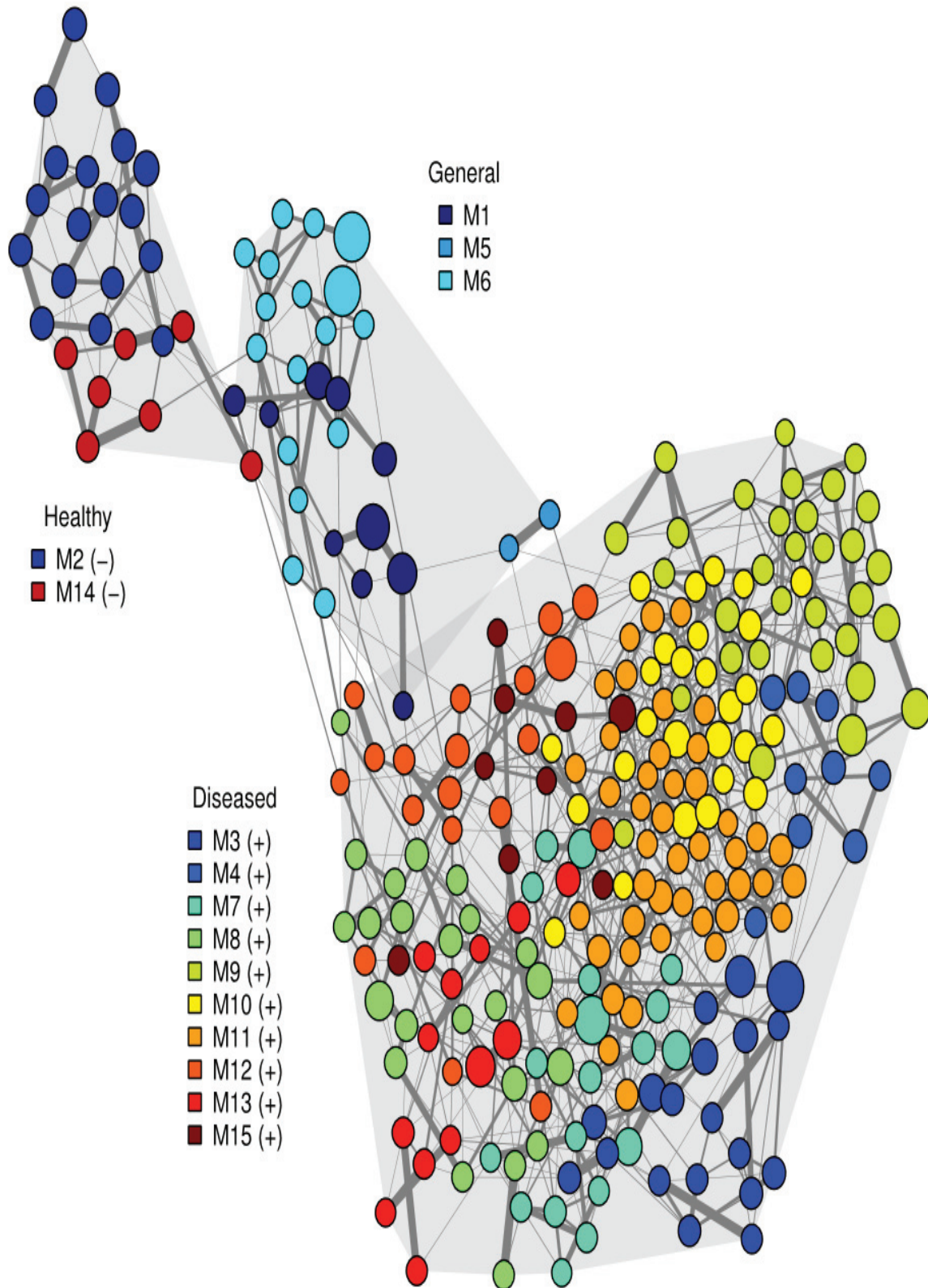
Figure 4. Bacterial co-occurrence network generated by SPIEC-EASI. Network modules (M) detected by Louvain clustering are shown in different colours. Node size corresponds to overall OTU sequence proportion in the MI and MFS samples. Modules which are present in healthy and diseased shrimps is grouped as general modules.
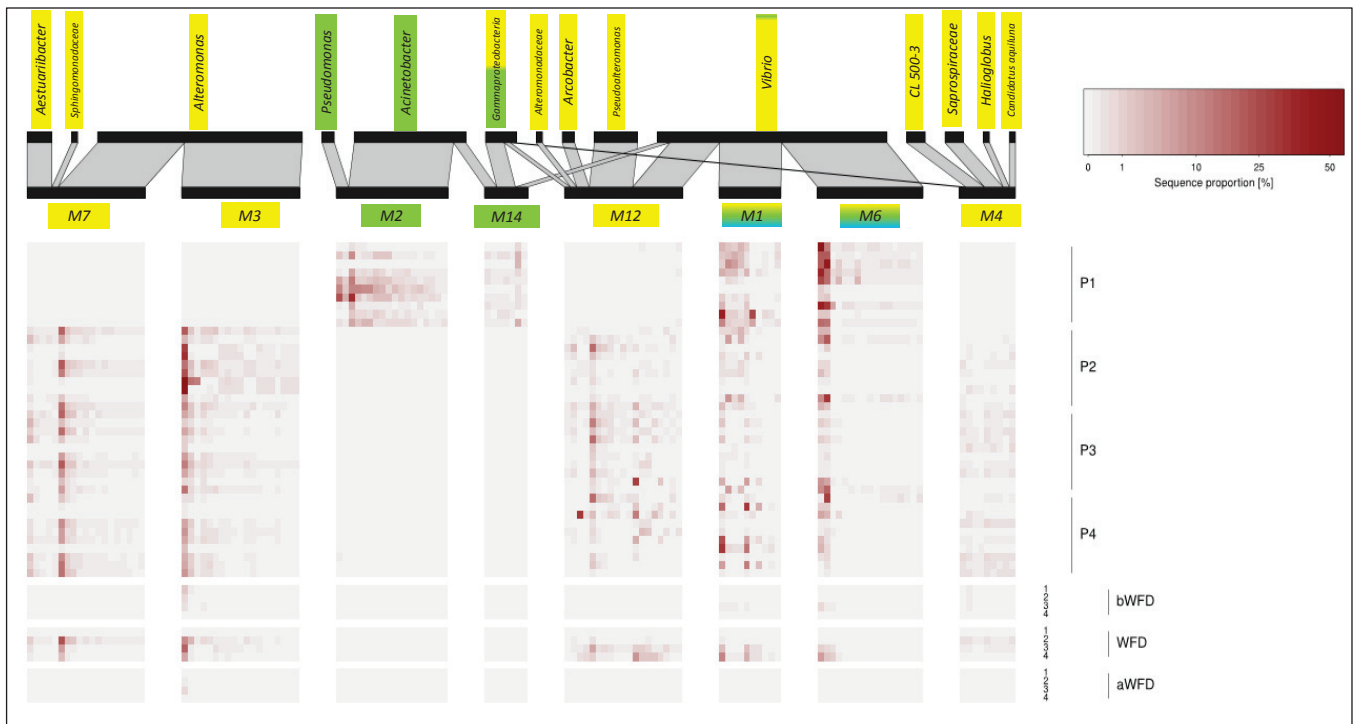
Figure 5. The sequence proportion of the members of the most dominant and most distinguishing network modules between healthy shrimps (P1 and green) and diseased shrimps (P2, P3, P4, and yellow) as well as their contribution to the particle-associated fraction from the respective ponds and sampling points. Their taxonomic affiliation is provided on genus level. Water samples were not used for the network construction.

Discussion

To better understand WFD in the Pacific white leg shrimp *Litopenaeus vannamei* aquaculture, we measured water quality and analysed bacterial community dynamics. Based on the visual estimation of white faecal string (FS) numbers in the ponds, we discriminated WFD event into two phases: start of disease (early symptoms) represented by P3 and P4 with lower numbers of FS, and early-outbreak which occurred in P2 with greater FS numbers. Because the microbiome of fresh shrimp faeces and that of the intestines of healthy *L. vanamei* have been shown to be comparable[16,28], we only dissected the intestines of healthy shrimps and analysed them together with the fresh faecal strings collected from diseased shrimp. In addition, if the shrimp already defecated, it was difficult to distinguish healthy and infected shrimps since the shrimp intestine was already empty.

Continuous nutrient availability in shrimp ponds supports microbial growth[30,31]. In addition, environmental factors, such as salinity, temperature, pH, phytoplankton, chemical oxygen demand and inorganic nutrients, affect bacterial community dynamics in shrimp ponds[16,29,32]. A high salinity range of 32.7-34.6 ppt shapes bacterial community compositions in shrimp pond water, resulting in the dominance of halophilic bacteria[29,32]. Dominant halophilic heterotrophic bacteria, such as *Exiguobacterium*, *Halomonas*, *Salegentibacter*, *Psychrobacter*, and *Sulfitobacter* in the WM of ponds at non disease events may play roles in organic matter degradation, nitrification, and sulfite oxidation[33,34]. They may also inhibit growth of potential pathogenic bacteria, for example *Pseudoalteromonas* and *Vibrio*, as reported in previous studies[15,29,35]. *Halomonas* and *Psychrobacter* might be involved in nitrification in pond[33,34,36], while organic matter degradation might be done mainly by *Marinomonas*, *Salegentibacter*, and *Sulfitobacter*[37].

Regular feed input causes unintended negative effects on water quality which eventually limits shrimp growth. Large nutrient proportions from the feed, which are not incorporated by shrimps, together with organic matter waste (i.e. uneaten food and faeces) stimulate biological activities which exert an oxygen demand inside the pond[38]. Moreover, excess of inorganic nutrients may cause phytoplankton and bacterial blooms, which then affect oxygen demand and influence other physical parameters such as pH, suspended particulate matter, and turbidity. Without external intervention, for example addition of limestone, calcium carbonate or dolomite, water pH tended to decrease as could be observed in P2, P3, and P4 during disease event.

Sudden change of pH in shrimp pond affects shrimps[39–41], including its intestinal microbiome. In this study, the decrease in pH might affect shrimp physiology, causing stress in shrimp which was indicated by low appetite in shrimps prior to disease onset. The stress event then induced changes in the shrimp intestine microbiota, whereby beneficial bacteria, such as *Pseudomonas* and *Acinetobacter*[42–44] decreased in proportion. When shrimp stress level increased, as could be simulated in P2 samples, dramatic change of the MI occurred, of which opportunistic pathogenic bacteria colonized the intestine, became the dominant one and made up MFS. The relationship of pH, stress, and disease was also reported for cases of AHPND occurring in shrimp ponds with a pH range of 7.4-7.7[10]. Furthermore, the gradual shift from presumably beneficial bacteria-dominated to potential pathogen-dominated microbiomes, which coincided with progression of the disease from early symptoms to early outbreak might indicate that the changes in intestinal bacterial communities are closely associated with the severity of the shrimp disease. This notion corresponds to previous study[16], which reported that changes in shrimp intestinal microbiome occurred in parallel with changes in disease severity, reflecting the transition from a healthy to a diseased state. In this study, MI of the healthy shrimp were correspond to previous study[16,18,19,45,46], which found that *Acinetobacter*, *Pseudomonas* and *Vibrio* were present in the intestine of healthy shrimp.

Decrease in pH was reported to change bacterial community compositions in shrimp pond water[20]. We observed shifts of WM in both FL and PA fractions during disease events, resulting in a dominance of opportunistic pathogenic bacteria such as *Alteromonas*, *Pseudoalteromonas* and *Vibrio*. Moreover, MFS enriched WMs which contributed to dominant proportion of *Alteromonas* in FL and PA fraction, as could be observed in the WM of P2. Shrimp faeces were easily disintegrated (up to 27% within 12h)[28], and could be unravelled faster in ponds due to water movement and mechanical aeration. Disintegration of faeces will facilitate bacterial dispersion, as well as protein and inorganic nutrient enrichment from feces[28]. The enrichment of the WM by opportunistic pathogenic bacteria may follow the level of disease and the number of infected shrimps. If greater pathogenic bacteria are released in pond water and attached to particulate matter, it will facilitate disease widespread among shrimps. Healthy shrimps may consume pathogen laden particulate matter and be then intoxicated. Thus, in this scenario, shrimp faecal bacteria not only contribute to bacterial abundance, structure and function of the WM, but also influence shrimp health because composition of shrimp intestinal microbiome was partly linked with the water in which they reared[16,20,45].

The presence of toxin genes such as *toxR* and *tlh* belonging to *V. parahaemolyticus* in the intestines and faecal strings, as well as in FL and PA fraction of water samples during disease outbreaks indicates that pathogenic bacteria are available in those samples. However, their action to provoke disease, only occur if environmental condition support their outbreaks or if the abundance of beneficial bacteria in the shrimp intestine is lower than that of pathogen. *V. parahaemolyticus* has greater toxicity in brackish-neutral pH condition, a sudden change of environmental parameters such as temperature and pH or acquire plasmid from other pathogen[47–50]. Once *V. parahaemolyticus* activates their toxin genes, they will produce haemolysin which cause infection in shrimp tissues[51–53].Bacterial co-occurrence network may explain the interaction between beneficial and pathogenic bacteria in shrimps. Bacterial assemblage of healthy shrimps could be clearly distinguished from those of infected-shrimps. We propose that *Acinetobacter* and *Pseudomonas* composing module 2, as well as *Acinetobacter* and the two *Vibrio* OTUs composing module 14 are part of the indigenous beneficial bacterial community of the healthy shrimps. The lack of these bacteria, as can be observed in MFS may increase aggressiveness of pathogen in shrimps. Some strains of *Acinetobacter*, *Pseudomonas* and *Vibrio* were reported as beneficial bacteria for shrimp farming, either for nitrification process, for accumulation or degradation of poly-ß-hydroxybutirate (PHB) compound or as antagonist bacteria against pathogens[22,26,42–44,54,55]. Since *Acinetobacter* and *Pseudomonas* are intolerant to high salinity[42,43], they cannot survive in such saline shrimp pond water. Therefore, they did not enrich WMs. The presence of *Vibrio* in healthy and infected shrimps might indicate different *Vibrio* ecotypes. While some *Vibrio* OTUs might represent opportunistic pathogens, others may not fall into that group and even be beneficial in low proportions[56,57], as can also be observed in this study

Evidence of the presence of pathogenic *Vibrio* in healthy shrimps might alert shrimp farming processes. Shrimp pond managements, specifically maintenance of water and sediment quality as well as keeping intestinal microbiota steady, is one of key factors in shrimp farming. In addition, laboratory experiment with controlled water parameters proved that *L. vannamei* are sensitive to low (< 6.5) and high pH (> 9) and that combination of lethal pH value and intoxication by pathogenic *Vibrio* increased shrimp mortality rate[39,58,59]. However, our study informed that when rearing water had higher pH (>8), resilience in the WMs occurred and WFD stopped.

Water quality has a large impact on the health status and growth of the shrimps[20]. There was a close connection between water at disease event, WM and MFS. Our findings on

the application of commercial probiotics to cure WFD in shrimps revealed that probiotics bacteria such as *Lactobacillus* were absent in WM. This means that application of probiotics in shrimp pond water was not effective. *Lactobacillus* was no longer detectable after they were diluted in the shrimp pond water. Instead of spreading the probiotics into the pond water, we propose to add it into feed pellet which will be eaten by shrimps. With this method, colonization of probiotic bacteria in the shrimp intestine may occur effectively. In term of prediction of pathogenic bacteria, cultivation method using TCBS agar to estimate the presence of pathogenic bacteria were failed to culture *Alteromonas, Photobacterium* and *Pseudoalteromonas*, which were also present in pond water at non-disease event.

We observed that environmental stressors, such decrease in pH, increase in turbidity and SPM, induced substantial shift in the water microbiomes, and affected shrimp physiology which in turn resulted in imbalances in the intestinal microbiota and subsequently, the emergence of disease. Alternatively, environmental pressures such as change of temperature, salinity, inorganic phosphate and pH, may enhance pathogenic virulence and increase host susceptibility to infection[40,48,60]. Our data support the notion that disease in aquaculture is the result of complex interactions among water biogeochemical variables, the microbiota of the habitat in which the shrimps are reared and the host [61,62].

We conclude that the WFD event was initiated by a shift of the WM and stress in the shrimps, which was induced by a sudden change in water quality with pH and dissolved oxygen concentrations dropping below 8 and 6 mg L^{-1}, respectively. Prolonged exposure to these conditions will increase disease severity and may lead to mass mortality. Moreover, we report several opportunistic bacteria besidessuch as *Arcobacter*, *Alteromonas, Marinomonas, Photobacterium* and *Pseudoalteromonas* that might contribute and/or cause WFD. Conversely, even though WM changed during periods of low water quality, they recovered when environmental conditions recuperated to pre-disturbance conditions. To avoid shrimp loss, shrimp farming management should consider microbial dynamics in rearing water, as well as in the reared shrimps.

Methods

Sample collection and sampling sites. Water samples were collected between 9-11 a.m. from 1 pond with healthy shrimps (P1 which served as control) and 3 shrimp ponds (P2, P3, and P4) that experienced WFD event between 50 to 70 days of rearing in October-November 2016. All ponds were chlorinated 2 weeks before shrimp rearing. Initial population densities

were 40 (P2) and 90 post-larvae m^{-3} (P1, P3, and P4), with the same origin of shrimp fries (PL15, specific pathogen free, Central Pertiwi Bahari Firm, Rembang, Central Java, Indonesia). Shrimp ponds were located in Rembang Regency, Central Java, Indonesia (-6°37`41.13" S 111°30`1" E and -6°42`11.66" S 111°21`54" E). Water sampling as well as measurements of environmental parameter were described in a previous study[29], which can be accessed in https://doi.pangaea.de/10.1594/PANGAEA.889316.

For bacterial community analysis, ten fresh white faecal strings were collected from feeding trays of each pond with infected shrimps. Ten healthy shrimps from P1 were collected using the feeding tray and put on ice in the cold storage immediately. They were then dissected in the laboratory to retrieve their filled intestines with sterile dissecting tools. Before dissection, shrimps were swabbed with ethanol 70% to sterilize their body and to avoid contamination from the carapace. All samples were immediately put in Eppendorf tubes, frozen and stored at -20°C until DNA extraction.

Cultivable potential pathogenic bacterial strain enumeration and identification from pond water. To obtain cultivable potential pathogenic bacteria from all ponds, 100 µL of undiluted to 10^{-4} diluted water sample were plated onto selective Thiosulfate Citrate Bile Salts Sucrose (TCBS) medium (Roth, Karlsruhe, Germany), followed by incubation at 35°C for 24 hours. Colonies which grew on the TCBS media were then calculated to determine cultivable potential pathogenic numbers. Strains which grew on TCBS plates from P1 at 60th day sampling were pooled by swabbing and collected into Eppendorf tubes containing 100 µl sterile sea water, and stored at -20°C until DNA extraction and analyses for its taxonomic identity. Totally, there were 6 pool strain tubes from 6 TCBS plates.

Molecular analysis of bacterial communities. 500 mL of water samples were filtered to collect bacterial cells. To distinguish between free-living (FL) and particle-associated (PA) bacterial communities, a serial filtration was conducted through 3.0 µm and 0.2 µm polycarbonate filters (ø 47 mm, Whatman, Dassel, Germany) for the PA and the FL bacterial fractions, respectively. Genomic DNA from water samples was extracted according to Nercessian *et al.*[63], while bacterial cells from intestines, white fecal strings, and isolates were extracted using phenol-chloroform methods [64]. DNA pellets were dissolved in 40 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5). DNA concentrations were measured photometrically and checked for purity (ratio of light absorption at 260 to 280 nm) using a nanoquant plate reader (Infinite M200 Pro, Tecan, Germany).

103

16S rRNA gene amplification was performed from genomic DNA extracts. DNA sequences of the V3-V4 hypervariable region of the 16S rRNA gene were obtained from amplicon sequencing with the primer set S-D-Bact-0314-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') / S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAAKCC-3')[65]. Sequencing at LGC genomics (Berlin, Germany) was done on an Illumina MiSeq using the V3 Chemistry (Illumina) in a 2x300 bp paired-end run. Demultiplexing, i.e. grouping of sequences by sample, and the removal of the primer sequences from the raw paired-end reads were performed by LGC genomics (Berlin, Germany). Sequences from genomic DNA from water samples at non disease period in P2, P3, and P4, as well as P1 genomic DNA were retrieved from a previous study[29] with an accession number PRJEB26390. Triplicate of each sample were pooled before sequencing, while water samples from P2, P3, and P4 at disease period were sequenced in triplicate. However, in order to obtain comparable results for WMs analysis, triplicate OTU profiles from the FL and PA fractions of P2, P3, and P4 at the WFD event were merged by taking the sum of the sequence counts per OTU.

Sequences were quality trimmed with a sliding window of 4 bases and a minimum average quality of 15 with *trimmomatic* v.033[66]. Quality trimmed sequences were merged using PEAR v0.9.8 [67]. Then, Minimum Entropy Decomposition (MED) was used to cluster sequences [68,69]. MED applies the principle of oligotyping [68], which uses the Shannon entropy to iteratively partition amplicons at single nucleotide resolution, thereby providing more accurate descriptions of closely related but distinct taxa[70]. During MED, we used a minimum substantive abundance (-M) of 50 to filter low-abundant OTU with the decomposition of one nucleotide position at a time (-d 1). For each OTU (oligotyping node), one representative sequence was taxonomically classified with SINA (SILVA Incremental Aligner) v1.2.11 using the SILVA rRNA project reference database (SILVA v.128) at a minimum alignment similarity and quality of 0.9 and a last common ancestor consensus of 0.7[71]. Unwanted lineages (such as archaea, chloroplasts, and mitochondria) were removed.

Detection and quantification of virulence genes. Several primer pairs were designed to detect toxin genes in *Vibrio parahaemolyticus*, such as transcriptional regulator (*toxR*), thermolabile haemolysin (*tlh*), thermostable direct haemolysin (*tdh*), and *photorhabdus* insect-related (*pirA* and *pirB*) gene. The DNA sequence from Genbank (accession number AY578148.1) was used to design primer sets for *toxR*, *tlh*, and *tdh* genes, while the sequence from AB972427.1 was used to design primer pairs for *PirA* and *PirB* gene. Primer pairs were

then evaluated according to Bustin *et al.*[72] before quantitative PCR application. Primer pairs were listed in **Supplementary Table 5**.

Detection and quantification of gene copy numbers from positive and negative control, as well as collected samples was performed in a quantitative PCR machine (CFX Connect Real-time System Bio-Rad, München, Germany) using the primer sets described previously. qPCR conditions were as follows: a reaction mixture consisted of 10 µL 2X SensiFast SYBR® No-ROX (Bioline, Luckenwalde, Germany), 1 µL of 25 mM $MgCl_2$ (Roboklon EURx, Berlin, Germany), 0.2 µL of 0.5 mM forward and reverse primer (Biomers, Ulm, Germany), 8.8 µL sterile distilled water, and 2 µl of DNA template (concentration 0.5-10 ng $µL^{-1}$). 3 steps qPCR amplification was performed as follows: pre-denaturation at 95°C for 3 minutes, followed by 40 elongation cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 20 s, and a dissociation step after final elongation was added to improve amplification specificity. *V. parahaemolyticus* DSM 11058 (DSMZ, Braunschweig, Germany) was used as positive control for *toxR, tlh, tdh, pirA* and *pirB* toxin genes, while *V. vulnificus* DSM 10143 (DSMZ, Braunschweig, Germany) served as negative control. A serial dilution of the positive control (known concentration) was used to estimate gen copy numbers from environmental samples (**Supplementary Information Table 1**). Gene copy numbers for *toxR* and *tlh* were determined with the equation y = -3.5542x + 44.891 with R²: 0.994 and y = -3.3003x + 42.982 with R²: 0.996, respectively.

Data analysis and co-occurrence bacterial network. A principal component analysis (PCA) was conducted to examine the relationship among environmental parameters and to characterize shrimp ponds during the WFD outbreaks. For further statistical analyses, DNA sequence samples were categorized into water microbiomes (23 samples), shrimp microbiomes (40 samples), cultivable *Vibrio* strains from pond with healthy shrimp (6 samples), and probiotics (1 sample). Patterns of bacterial community compositions in all samples were visualized by non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities using the function *metaMDS*, while a pairwise ANOSIM tests were performed to observe significant differences between MI and MFS. ANOVA was performed for Bray-Curtis dissimilarity values of WM and MI/MFS as well as toxR and tlh gene copy numbers.

Bacterial OTUs from intestine and white faecal string (FS) were analysed to identify subpopulations (modules) of co-varying bacteria using SPIEC-EASI (Sparse inverse covariance estimation for ecological association inference) version 1.0.2 [73]. The statistical

method SPIEC-EASI comprises two steps, firstly a transformation for compositionality correction of the OTUs matrix and secondly an estimation of the interaction graph from the transformed data using sparse inverse covariance selection[73]. Pre-filtering of OTUs was performed before SPIEC-EASI to exclude rare and low sample coverage OTUs in the data sets, retaining only OTUs which occurred in at least 5 samples with a proportion of least 0.1%. Regression coefficients from the SPIEC-EASI output were extracted and used as edge weight to generate a bacterial co-occurrence network using *igraph* package [74]. Negative values, which indicated inverse trends among OTUs were excluded for Louvain clustering, which was then performed to extract network modules. Modules characteristic for the MI of the healthy pond and the MFS of each of the diseased ponds were identified using pairwise random decision forests based on module eigengenes. Module eigengenes and Random Forests were calculated using the R packages WGCNA[75] and randomForest[76], respectively. The sequence proportions of the members of the modules related to healthy shrimp or the WFD events (based on the highest mean decrease Gini and accuracy) were visualized in a heatmap.

All statistical analyses, as well as figure visualizations were performed in R (R version 3.4.2, R Core Team, 2017, using R Studio v.0.98.1056) with the packages vegan[77], nlme[78], ALDEx2[79], gplots[80] and packages mentioned previously. DNA sequences were deposited in the ENA, while biogeochemical parameters and R scripts for statistical analyses were deposit into PANGEA using the data brokerage service of the German Federation for Biological Data/GFBio[81]. Accession number for DNA sequences and DOI are still pending.

Author Contributions

YRA, JH, AG designed the project, YRA collected the samples and conducted the in-situ measurement. YRA and CH completed the statistical analysis with the input from JH and AG. YRA prepared the manuscript with input from all co-authors. CH and AG shared equal responsibility as last authors. All authors reviewed the manuscript.

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Supplementary Table 1. Genomic DNA concentration and estimation of virulence gene copy numbers in *V. parahaemolyticus* from two cell collection assays

Bacterial Culture	Cell numbers (cell/ml)	DNA concentration (ng/μl)*	*toxR*		*tlh*		*tdh*	
			Copy numbers (per ml culture)**	Cycle (ct)	Copy numbers (per ml culture)**	Cycle (ct)	Copy numbers (per ml culture)**	Cycle (ct)
V. parahaemolyticus DSM 11058	2.28×10^8	[a]Filtration: I. 155.1 II. 93.9 III. 62.0 [b]Cells: I. 690.2 II. 154.7 III. 616.6	4.45×10^8 2.69×10^8 1.78×10^8 6.20×10^9 1.34×10^9 5.55×10^9	14.59 14.89 14.61 15.24 14.65 14.82	4.45×10^8 2.69×10^8 1.78×10^8 NA NA NA	14.80 15.12 14.88 NA NA NA	4.45×10^8 2.69×10^8 1.78×10^8 NA NA NA	14.55 15.37 14.95 NA NA NA

*Volume of extracted *V. parahaemolyticus* culture from two cell collection/preparation assay; 1st assay: cells were filtered in a polycarbonate filter (porosity: 0.2 μm, diameter: 47 mm) and 2nd assay: centrifuged cell, of which [a]: 5 ml, DNA extract was eluted in 80 μl of TE buffer, [b]: 2 mL, DNA extract was eluted in 100 μL of TE buffer; **Copy number calculation according to an equation from ThermoFischer scientific (https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html) where Molar mass per base pair is 650 (g/mol)/bp, Fragment length (nt) is 5165770 (total genome of *V. parahaemolyticus*, Makino *et al*., 2013), Measured concentration (ng/uL) is concentration of the DNA extracts. All virulence genes are located in chromosome II (Makino *et al*., 2013). Underlined value in DNA concentration indicated the genomic DNA which was used for qPCR test and quantification.

115

Supplementary Table 2. Gene copy from serial dilution of the *V. parahaemolyticus* (*Vp)* DNA as positive control for the detection of *toxR*, *tlh*, and *tdh* gene

Dilution	DNA concentration (ngμL^{-1})	Estimation of *Vp* cell	*toxR*		*tlh*		*tdh*	
			Copy numbers (log copy number mL^{-1})	Cycle (ct)	Copy numbers (log copy number mL^{-1})	Cycle(ct)	Copy numbers (log copy number mL^{-1})	Cycle(ct)
10^0	93.9	2.28 x 10^8	8.43	14.9	8.43	14.9	8.43	14.9
10^{-1}	9.39	2.28 x 10^7	7.43	17.7	7.43	17.8	7.43	17.0
10^{-2}	9.39 x 10^{-1}	2.28 x 10^6	6.43	21.7	6.43	22.0	6.43	20.9
10^{-3}	9.39 x 10^{-2}	2.28 x 10^5	5.43	25.2	5.43	25.8	5.43	24.9
10^{-4}	9.39 x 10^{-3}	2.28 x 10^4	4.43	28.6	4.43	28.8	4.43	29.4
10^{-5}	9.39 x 10^{-4}	2.28 x 10^3	3.43	31.9	3.43	31.9	3.43	33.3
10^{-6}	9.39 x 10^{-5}	2.28 x 10^2	2.43	34.8	2.43	34.9	2.43	36.7
10^{-7}	9.39 x 10^{-6}	2.28 x 10^1	1.43	37.9	1.43	37.8	< LoQ	< LoD

LoQ: Limit of quantification, LoD: Limit of detection

Supplementary Table 3. Modules generated by Louvain clustering

Modules	Members (OTUs)
1	*Vibrio* (10)
2	*Acinetobacter* (16), *Pseudomonas* (2)
3	*Alteromonas* (19)
4	*Candidatus aquilina* (1), CL 500-3 (3), *Gammaproteobacteria*_unclassified (1), *Halioglobus* (1), *Saprospiraceae*_unclassified (3)
5	*Photobacterium* (2)
6	*Vibrio* (17)
7	*Aestuariibacter* (4), *Alteromonas* (14), *Sphingomonadaceae*_unclassified (1)
8	*Alteromonadaceae*_unclassified (5), *Gammaproteobacteria*_unclassified (2), *Pseudoalteromonas* (6), *Psychrosphaera* (3), *Salinomonas* (1), *Vibrio* (4), *Vibrionaceae*_unclassified (2)
9	*Alteromonas* (1), *Caldilineaceae*_unclassified (1), *Cyanobium* PCC-6307 (1), GWA2-38-13b_unclassified (2), *Halomonas* (3), *Ilumatobacteraceae*_unclassified (2), *Marivita* (2), *Microbacteriaceae*_unclassified (3), *Nautella* (1), *Nitriliruptoraceae*_unclassified (8), PeM15_unclassified, *Pirellulaceae*_unclassified (1), *Planctomicrobium* (1), *Rhodobacteraceae*_unclassified (2), *Ruegeria* (1), *Synechococcus* cc9902 (1)
10	*Aestuariibacter* (1), *Alphaproteobacteria*_unclassified (1), *Candidatus actinomarina* (1), CHAB-XI-27_unclassified (1), Clade III_unclassified (1), Crocinitomix (1), Flavobacteriaceae_unclassified (3), Halioglobus (6), HIMB11 (1), *Idiomarina* (1), *Marivita* (1), *Nitriliruptoraceae*_unclassified (1), OM43 clade (1), *Peredibacter* (1), *Rhizobiaceae*_unclassified (1), *Rhodobacteraceae*_unclassified (1), *Robiginitalea* (1), *Salinihabitans* (1), Sva0996 marine group (1), *Winogradskyella* (1)
11	*Alteromonas* (1), *Aurantivirga* (1), *Bacteroidia*_unclassified (1), *Balneola* (1), *Balneolaceae*_unclassified (1), *Candidatus actinomarina* (1), *Chitinophagales*_unclassified (2), *Crocinitomicaceae*_unclassified (1), *Cryomorphaceae*_unclassified (3), *Flavobacteriaceae*_unclassified (7), *Kiloniella* (1), Litoricola (2), *Marinagarivorans* (1), NS11-12 marine group_unclassified (2), NS7 marine group_unclassified (1), NS9 marine group_unclassified (1), *Oleibacter* (3), *Oleiphilus* (1), *Owenweeksia* (3), *Phaedactylibacter* (1), *Pseudoalteromonas* (1), *Rhodothermaceae*_unclassified (1), *Saccharospirillaceae*_unclassified (3), *Saprospiraceae*_unclassified (2), SAR 324 clade (Marine group B)_unclassified (1), SM2D12_unclassified (1), *Tenacibaculum* (1)
12	*Alteromonadaceae*_unclassified (1), *Arcobacter* (2), *Gammaproteobacteria*_unclassified (1), *Pseudoalteromonas* (7), *Vibrio* (8)
13	*Alteromonadaceae*_unclassified (1), *Marinomonas* (7), *Pseudoalteromonas* (1), *Salinomonas* (3), *Vibrio* (1)
14	*Acinetobacter* (2), *Gammaproteobacteria*_unclassified (1), *Vibrio* (2)
15	*Aestuariibacter* (1), *Alteromonadaceae*_unclassified (1), *Gammaproteobacteria*_unclassified (1), *Pseudoalteromonas* (5), *Vibrio* (1)

Supplementary Table 4. Importance value of each module retrieved from Random Forest test.

Modules	P1-P2		P1-P3		P1-P4	
	MDA	MDG	MDA	MDG	MDA	MDG
1	9.708e-03	0.441	0.012	0.523	0.003	0.319
2	**7.840e-02**	**1.586**	**0.072**	**1.490**	**0.086**	**1.720**
3	**7.582e-02**	**1.527**	0.016	0.461	0.008	0.366
4	**2.944e-02**	**0.815**	**0.026**	**0.706**	**0.078**	**1.637**
5	7.880e-05	0.032	0.0003	0.055	0.0006	0.044
6	**1.816e-02**	**0.669**	**0.017**	**0.615**	**0.020**	**0.736**
7	**5.828e-03**	**0.2632**	**0.033**	**0.823**	**0.008**	**0.336**
8	1.471e-02	0.544	0.002	0.077	0.006	0.185
9	2.956e-02	0.843	0.029	0.798	0.023	0.898
10	5.942e-03	0.352	0.027	0.865	0.018	0.531
11	4.589e-03	0.186	0.004	0.137	0.013	0.462
12	**1.381e-02**	**0.411**	**0.040**	**1.098**	**0.010**	**0.357**
13	5.279e-03	0.142	0.002	0.087	0.003	0.114
14	**7.993e-02**	**1.550**	**0.080**	**1.600**	**0.078**	**1.611**
15	2.387e-03	0.137	0.005	0.165	0.003	0.183

MDA: Mean decrease accuracy, MDG: Mean decrease gini, Value written in bold were used for bacterial co-occurrence network visualization.

Supplementary Table 5. Primer pairs for the toxin gene assay of *V. parahaemolyticus*

Primer sets[a]	Sequences	Length (bp)	T_M-value (°C)[b]	GC (%)[c]	Amplicon size (bp)	Source
*toxR*_F	5'-CAG CGT TGT GAA GCA ACA TTA G-3'	22	53	45.45	98	Our study
*toxR*_R	5'-CTC CAG ATC GTG TGG TTG TAT G-3'	22	54.8	50.0		
*tlh*_F	5'-CCGTCAGATTGGTGAGTATCAG-3'	22	54.8	50.0	99	Our study
*tlh*_R	5'-CGTTCAATGCACTGCTCAATAG-3'	22	53	45.45		
*tdh*_F	5'-CAGTATTCACAACGTCWGGTACTA-3'	24	54	41.67	200	Our study
*tdh*_R	5'-TGGAATAGAAYCTTCATCTTCACC-3'	24	54	37.5		
*PirA*_F	5'-GTCGGTCGTAGTGTAGACATTG-3'	22	57.4	50	147	Our study
*PirA*_R	5'-AGGGCGTTGTAAATGGTAAGT-3'	21	57	42.9		
*PirB*_F	5'-GGTGATGAATGGCTTGGTTATG-3'	22	56.9	45.5	124	Our study
*PirB*_R	5'-GCACATCAGAATCGGTGAAAC-3'	21	56.8	47.6		

[a]*tlh*: thermolabile hemolysin, *tdh*: thermostable direct hemolysin, *pirA* and *PirB*: Photorhabdus insect-related (Pir) toxins

[b] T_M: Melting temperature

[c] GC: Guanine-cytosine content

Supplementary Figure 1. Non Metric Multidimensional Scaling (NMDS) plot of water microbiomes (triangles), shrimp microbiomes (squares), bacterial strains (grey rhombus) and probiotic (orange rhombus). Pond 1 is pond with healthy shrimps, while pond 2, 3 and 4 are ponds with diseased shrimps.
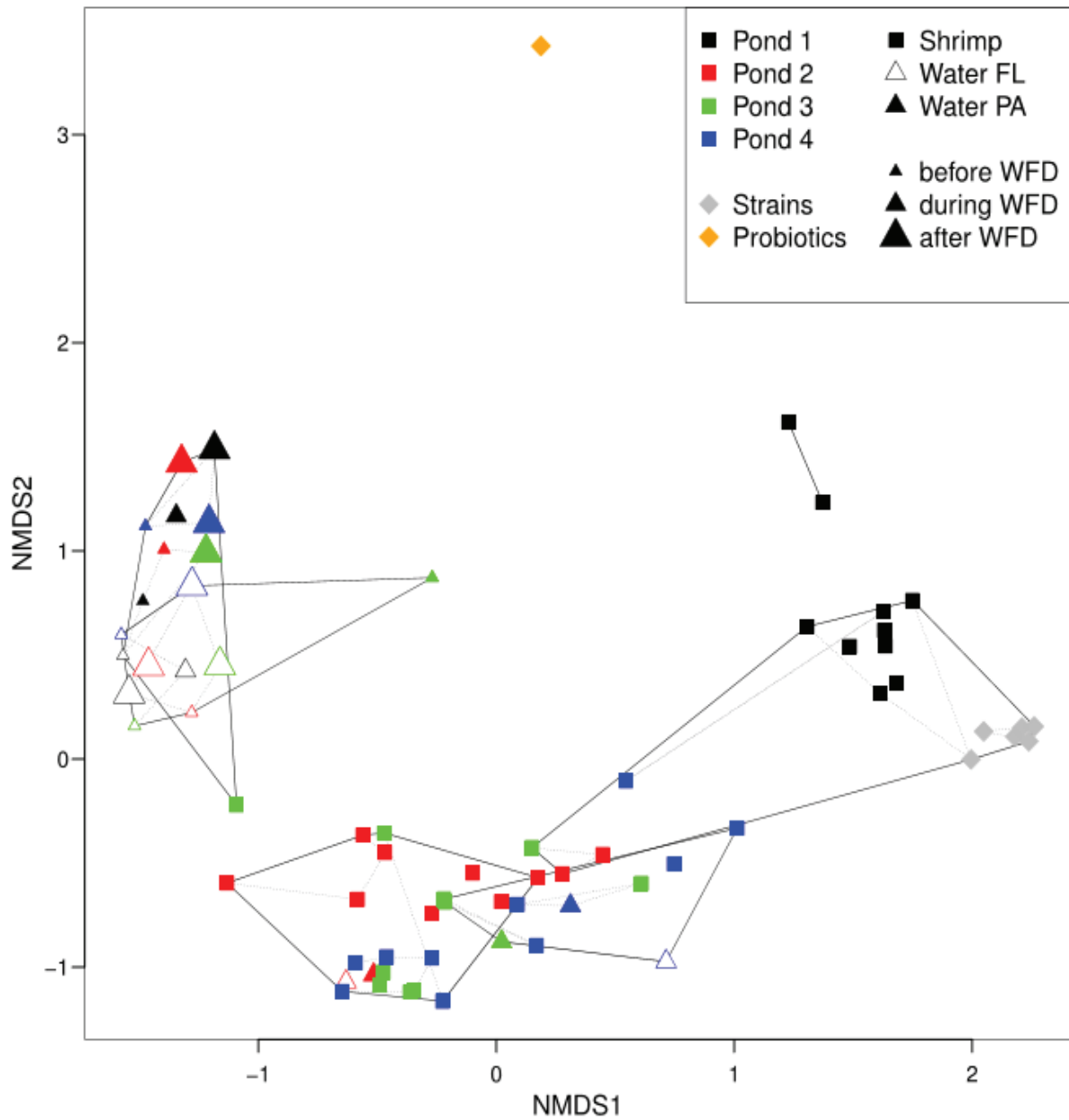
Chapter 6. General discussion

This thesis contains detailed investigations on water quality and bacterial dynamics in Indonesian shrimp (*Litopenaeus vannamei*) aquaculture systems. In this chapter, the main highlights of this PhD thesis will be discussed by considering their applicability for increasing sustainability in shrimp pond aquacultures. Therefore, farming processes in different shrimp aquaculture systems will be evaluated, and the importance of water quality and bacterial dynamics for successful shrimp rearing will be discussed. The emphasis lies on bacterial community compositions (BCC) of different bacterial lifestyles, called free-living (FL) and particle-associated (PA) stages at non-disease and at white feces disease events. Moreover, this chapter considers the effect of the addition of molasses, an easily dissolvable carbon source, into the shrimp pond water. Finally, the potential applications of aggregates (bio-flocs) in shrimp pond aquacultures are discussed together with an outlook for more sustainable pond aquaculture.

6.1. Semi-intensive (SI) and intensive (I) shrimp farming systems

Shrimp farms in Rembang Regency have operated since 1990. Initially, they had grown black tiger shrimps, *Penaeus monodon* (local name: udang windu), in traditional and in extensive farming systems either in monoculture or in polyculture with milk fish (*Chanos chanos*). In 2006, they switched to *L. vannamei* (local name: udang vanami/vaname/panami) because *P. monodon* harvests decreased due to diseases. Yielding higher amounts of harvests in shorter periods than with *P. monodon*, shrimp farmers decided to permanently culture *L. vannamei*. Recently, apart from the side effect of eutrophication, *L. vannamei* farming has been faced with culturing problems, such as white spot disease (Taslihan *et al*., 2013) and bacterial diseases, such as vibriosis and white feces disease.

Shrimp aquacultures in Rembang Regency operate four types of farming systems: traditional, extensive polyculture, semi-intensive (SI) and intensive (I) systems. SI and I pond systems were studied in this thesis. The SI and I ponds are located at a distance of 40 km from each other and use Java Sea seawater for shrimp cultivation. Before rearing processes, they performed pond water sterilization using chlorine and saponin to eradicate fishes and crabs larvae which might be available in the seawaters and might threaten rearing processes. Tang (1961) reported that saponin can control predaceous fishes in shrimp pond. Neither of the two farming systems allowed for water-exchange. For the continuous oxygen supply, they placed 6 paddle wheels into the pond system. The SI ponds cultured 40 post-larvae (PL) m^{-3}, while the I ponds reared 90 PL m^{-3}. Rearing processes take place for 70 to 90 days (but can be

extended up to 110-120 days depending on shrimp prices). During the rearing process, SI and I used the sort of feed pellets and probiotics, which were obtained from Central Proteina Prima (CPP) Firm, Semarang, Central Java, Indonesia. The SI pond owner preferred to have a dense phytoplankton population from the beginning of rearing, while the I pond owners preferred clearer water (less phytoplankton), when they started shrimp cultivation. To enhance phytoplankton growth, the operators of the SI ponds added 30-50 kg urea per pond, three days after water sterilization. Riegman *et al.* (1998) reported that phytoplankton are able to take up 10 % of nitrogen from urea. Thus, the addition of urea to 'sterile' pond waters during pond preparation may enhance phytoplankton growth. Possible reasons for the decision of SI pond operators to increase phytoplankton abundances may be the following: The uptake of inorganic nutrients by phytoplankton (and bacteria) contributes to the regulation of ammonium and nitrite concentrations in pond waters. Three to four days after the addition of urea, pond operators of SI system released the post-larvae (PL) into the nurtured ponds. In contrary, the I ponds did not add urea to the system and released the PL into the ponds seven day after water sterilization.

Shrimp pond farming differs from indoor shrimp aquaculture. In the indoor shrimp farming, feeds are usually given in a constant amount. Water supply is carefully treated to eradicate pathogenic bacteria and to minimize toxic inorganic nutrient. Moreover, water quality parameters are measured regularly (Van Wyk, 2001). In contrary, the shrimp pond farming does not have strict water surveillance procedures. The shrimp pond operation has usually focused on shrimp satiation (shrimp diet), total suspended particles and phytoplankton concentrations. The level of shrimp satiation is usually checked by providing certain amount of feed pellets into feeding trays (2-4 trays per pond) and observing shrimp feeding behavior for 30-60 minutes. If the tray is empty, the pond operators will increase the amount of feed pellets for next feeding time. In contrary, if there are left-over pellets in the feeding tray, the operator will then decrease the quantity of the following feed or even skip the subsequent feed event.

Average shrimp weight is measured regularly to evaluate shrimp growth. Shrimp weight gain is firstly measured at 30 days of rearing and then repeated every 2 weeks by sampling of 20 individuals per pond. Pond operators then determine food conversion ratio (FCR) by calculating the ratio between given feed with weight gain of cultured shrimp. Even though this practice seems to be a rough estimation for whole population, it has become part of feed management in pond farming (Patnaik and Samocha, 2009). The reported FCRs of *L.*

vannamei in Indonesian aquaculture were in between 1.0 to 2.0 (Wijayanto *et al.*, 2017; Samadan *et al.*, 2018). The FCR is depended on the density of the shrimps and the amount of provided pellet.

Despite the lack of inorganic nutrient monitoring in either of the systems (SI or I), the operators tried to maintain water quality during rearing. For this purpose, the operators provided feed pellets by considering the FCR rates that they obtained. Furthermore, they added 30-50 kg of lime stones or dolomite into the ponds every two weeks in order to maintain pH, suspended particles, and plankton densities. In I ponds, the operators discharged sludge every week and after 60 days of rearing even every day. Therefore, the I pond owners need sludge reservoirs close to their ponds (**Appendix I**). From the initial density of PL shrimps, the I ponds usually obtain harvests 2-4 fold higher than the SI's harvest, as also observed here with 3,950 ± 284 kg and 1,990 ± 151 kg, for I and SI, respectively. In addition, the I ponds can be harvested 4-5 times from a complete rearing cycle by doing 3 to 4 partial harvests, while the SI ponds only allow for 2 or 3 harvests.

6.2 Biogeochemical parameters of shrimp pond waters

Biogeochemical parameters, such as pH, salinity, suspended particulate matter, chlorophyll a, and inorganic nutrients, varied between and within systems (SI and I) due to different rearing strategies. Within system variations (i.e. different ponds of the same systems) can be caused by differences in daily operational practices. These differences include for example provision of different amounts of feed as well as additional treatments, such as addition of lime stones, commercial probiotics, fermented rice brans and molasses. These different treatments also cause temporal changes in ponds.

Abundances of phytoplankton increased with rearing time. The phytoplankton ranges in this study was slightly lower than those observed in China (Zhang *et al.*, 2014) and India (Sahu *et al.*, 2013). I supposed that chlorination during pond preparation reduced phytoplankton concentrations at the beginning of shrimp rearing. The SI ponds were characterized by slightly higher phytoplankton concentrations at the beginning of rearing (6.3-20.6 mg chlorophyll a L^{-1} versus 2-9.6 mg chlorophyll a L^{-1} in the SI and I ponds, respectively). We supposed that urea which was added by the SI pond operators after water sterilization affected the phytoplankton abundances. Evaporation rates and addition of water to maintain pond water level which lose due to evaporation and sludge discharge may affect salinity in shrimp pond. The addition of fresh water in SI system decreased salinity, which may influence bacterial community composition. To avoid drastic change on salinity,

sufficient amount 'sterile' seawaters is needed. Therefore, I propose to prepare sea water reservoirs which are close to rearing ponds. Despite different amount of provided feed pellets and cultured shrimps in SI and I systems, the inorganic nutrient concentrations in the investigated ponds, particularly ammonium and nitrite, were always far from lethal concentrations for *L. vannamei* shrimps.

During this study, the pH values decreased gradually from its initial value which was around 8.4 to 7.8 (**chapter 4**). During WFD events (**chapter 5**), pH values were considerably low (pH: 7.71-7.84). This suggests that pH may play a role in disease occurrence. It has previously been shown that diseases in *L. vannamei*, including e.g. AHPND and vibriosis, occurred at a pH lower than 7.7 (Chen *et al*., 2017; Heenatigala and Fernando, 2016). This highlights the importance of monitoring/ managing pH values during shrimp rearing. Since constantly low pH values can threaten shrimps (see also section on microbial dynamics), limestone or similar structures (i.e. dolomite) were added to the ponds to increase alkalinity, whenever necessary. Furthermore, the addition of molasses (**chapter 3**) may also increase pH as well as affect the inorganic nutrients, such as ammonium, nitrite and nitrate.

6.3 Bacterial dynamics in shrimp ponds and shrimp intestines

In this study, free-living (FL) and particle-associated (PA) bacterial fractions were different in numbers and community structure. This is in line with numerous previous studies, which also suggest different abundances and bacterial community composition (BCC) (Kiørboe *et al*., 2003; Kramer *et al*., 2013; Lyons *et al*., 2010; Rieck *et al*., 2015). Particulate matter in shrimp ponds harbour bacterial cells resulting in high bacterial concentrations in particle-associated fraction. Moreover, environmental parameters influence the bacterial community composition (BCC) of FL and particle-associated (PA) fractions. According to redundancy analyses (RDA, **chapter 4**), the water parameters, such as salinity and pH, drive the BCC in the particle attached fraction, while salinity, phytoplankton abundances, and nitrate shape the BCC in the FL fraction.

a. Bacterial dynamic in shrimp pond waters

Bacterial populations in shrimp pond waters own different lifestyles. This is in accordance to the notion of aquatic bacterial lifestyle proposed by Grossart (2010). In this study, certain bacteria can be categorized as 'truly' FL bacteria, including *Sulfitobacter*, *Erythrobacteraceae*, and *Exiguobacterium*, and PA bacteria such as, *Psychrobacter*. Moreover, other bacterial taxa, such as *Alteromonas*, *Halomonas*, *Pseudoalteromonas*, *Salegentibacter* and *Vibrio*, may frequently alternate between free-living and particle-

associated stages. The FL bacteria spend their whole life cycles as in individual cells in water column, while PA bacteria spend most of their life cycle attached to living or non-living particles (Grossart, 2010). These findings are important to regulate bacterial community dynamics in shrimp ponds, especially in PA fraction. Since purely FL bacteria cannot escape environmental variable fluctuations (Grossart, 2010) and many bacteria colonizing aggregates show a high production of antibiotics (Grossart *et al*., 2004; Long and Azam, 2001), sudden drastic changes in water quality variables must be avoided to keep the dominancy of non-pathogenic bacteria in the FL and PA fractions.

Aquaculture related processes, such as regular feed supply may influence bacterial community composition in rearing water (Farzanfar, 2006; Vargas-Albores *et al*., 2017). The numbers of cultivable total heterotrophic bacteria (THB) and total potential pathogenic *Vibrio* (TPPV or so called total presumptive *Vibrio*) in our study were in line with previous study (Lavilla-Pitogo *et al*., 1998). In contrast to the previous study, which reported massive shrimp mortalities after 45 days of culturing due to an infection of the hepatopancreas by luminescent *Vibrio,* there was no disease outbreak during our sampling campaign (**chapter 4**). During non-disease sampling points the BCC was always dominated by heterotrophic halophilic bacteria, such as *Exiguobacterium*, *Halomonas*, *Psychrobacter*, and *Salegentibacter*. However, when water pH decreased below 8, the dominant bacteria shifted to opportunistic pathogenic bacteria, which included *Alteromonas* and *Vibrio*. If the pH remained below 8 for several days, those bacteria, along with other pathogenic bacteria, such as *Alteromonas*, *Pseudoalteromonas*, and *Arcobacter* became the dominant bacteria, especially in PA fraction. In this study, shrimp disease coincided with low pH, high numbers of cultivable green colonies presumptive *Vibrio* (up to 4000 CFU mL^{-1}) and the above opportunistic pathogens. Moreover, higher concentration of virulence genes, such as *toxR* and *tlh* genes, in the PA rather than the FL fraction suggests particles represent a hotspot for pathogenic bacteria. This is in line with previous studies, which have highlighted the role of particles in disease ecology (Froelich *et al*., 2013; Lyons *et al*., 2005). The high occurrence of contaminated aggregates in shrimp pond waters may increase intoxication among cultured shrimps since shrimps may eat the contaminated particles.

Molasses can be added to pond systems to help sustain dominance of non-pathogenic heterotrophic bacteria, such as *Exiguobacterium*, *Halomonas*, *Psychrobacter*, *Salegentibacter* and *Sulfitobacter* (**chapter 3**). These laboratory results are highly relevant for shrimp aquacultures because these bacteria are also dominant in shrimp pond waters at non-disease

events. Furthermore, the addition of molasses can regulate inorganic nutrient concentrations in shrimp pond waters since they improve bacterial growth, which uptake inorganic nutrients, such as ammonium, nitrate and nitrite. Therefore, I propose to use molasses not only to generate aggregates as feed source for shrimps, but also to maintain the dominance of non-pathogenic heterotrophic bacteria. However, since pathogenic bacteria, particularly *V. parahaemolyticus*, may be available in shrimp pond waters and thrive on microalgae aggregates (**chapter 3**), I recommend to regularly monitor abundances of pathogenic *Vibrio* in PA fraction using rapid-sensitive tests, such as real-time PCR or Loop-mediated isothermal amplification (LAMP) assays (Nemoto *et al*., 2011; Wang *et al*., 2015, 2016) before the addition of molasses into the rearing ponds.

b. **Bacterial dynamic in shrimp microbiome**

Change in pH, phytoplankton concentrations and bacterial shifts may occur naturally in shrimp ponds. These changes may lead to physiological stress in shrimps, which can cause shrimp mortality if the stress sustains for too long. In contrary, if the intervention aims to improve shrimp well-being (e.g. via the addition of probiotic bacteria), shrimp may have better performance. In **chapter 5**, we observed physiological stress in shrimp which was indicated by low appetite for more than 3 days. Concomitantly, we detected decrease of pH, which led to a shift of shrimp microbiome. Dominant beneficial-candidate bacteria in the intestine and fresh feces of healthy shrimps, such as *Acinetobacter*, *Bacillus*, *Lactobacillus*, *Pseudomonas* (Sha *et al*., 2016; Vargas-Albores *et al*., 2017; Xiong *et al*., 2016; Zheng *et al*., 2017), were absent in diseased shrimps. They were replaced by opportunistic pathogenic bacteria, such as *Arcobacter*, *Alteromonas*, *Pseudoalteromonas*, *Photobacterium*, and *Vibrio*, which also became the dominant bacteria the PA fraction of pond water at disease events. Thus, changes in water quality parameters, especially sudden decrease of pH and increase of the abundance of opportunistic pathogenic bacteria in PA fraction, will affect shrimp microbiome (**chapter 5**).

6.4 Aggregate formation and potential application

Aggregates in shrimp ponds can be formed by giving water movement. In shrimp farming, agglomerated particles can be used as additional and natural shrimp feeds. Total aggregate volume in an observed I pond was estimated to be about 1.9-2 x 10^4 L aggregates in about 4.2 x 10^6 L pond waters (**chapter 3**). This is equivalent to 266 to 367 kg of wet weight aggregates. If the aggregates are converted in accordance with carbohydrate, lipid, and protein content estimation (**chapter 3**), the estimated values will be 30 kg J, 183 kg J, and 93 kg J of

carbohydrates, lipids and proteins, respectively. This suggests that pond water serve nutritious natural feeds, which are sufficient for two days pellet diet.

The nutritional value of these aggregates increases by the addition of molasses. Shrimps will get nutrient from aggregated particles which also harbour bacteria. This allows shrimp to get nutrient not only from organic particles, but also from its associated bacterial cells. If beneficial bacteria exist on aggregates, they can be ingested and then reside in the shrimp gut, which may ameliorate shrimp physiology via the production extracellular enzymes and involved in the degradation of substrates. They may also produce and accumulate intracellular polyhydroxybutyrate (PHB) which increase shrimp immune against pathogens (Defoirdt *et al*., 2011; Liu *et al*., 2010; Suantika *et al*., 2013; Zhang *et al*., 2009).

Before intense generation of aggregates in shrimp farms, information on the biogeochemistry of the system is required. Firstly, the C:N ratio in pond water has to be measured to determine the appropriate quantity of additional carbon sources. Secondly, the carbon sources have to be chosen based on physical parameters, such as the quantity of suspended particulate matter, pH, dissolved oxygen, and salinity. Thirdly, the PA BCC has to be estimated to determine whether beneficial-commercial bacterial strains need to be added to the aggregates or if optimizing the indigenous bacterial communities is sufficient.

6.5 Estimation of total ammonium nitrogen (TAN) in shrimp ponds

Semi-intensive and intensive ponds with regular feed supply may accumulate inorganic nutrients, especially ammonium, nitrite, and nitrate. High concentrations of ammonium have been proven to be toxic for shrimps (Schuler *et al*., 2008). TAN can be estimated using two different equations (T- and E-equation) with slightly different results as proposed by Timmons *et al*. (2002) and Ebeling *et al*. (2006). Timmons *et al*. (2002) proposed the equation based on the feeding rate, as $P_{TAN} = F*PC*0.092$, while Ebeling *et al*. (2006) proposed the equation based on zero-exchange marine shrimp production system, as $P_{TAN} = F*PC*0.144$, where P_{TAN} is production rate of total ammonium nitrogen (kg day^{-1}), F is feed rate (kg day^{-1}), and PC is protein concentration in feed (decimal value).

After several days of rearing, SI and I systems may have different concentrations of TAN. Estimated TAN production from SI system at day 60 amounted to 2.2 and 3.5 kg TAN day^{-1}, while estimated TAN production in I system were 3.9 and 6.0 kg TAN day^{-1} for T-equation and E-equation, respectively. This great amount of TAN can be taken by heterotrophic bacteria and phytoplankton for their growth. Furthermore, daily sludge discard in I ponds, which reached 1.7-2.2 x 10^3 L day^{-1} may have reduced the TAN production in I

ponds. Thus, the TAN uptake by heterotrophic bacteria and phytoplankton and regular sludge discard may cause low ammonium and nitrite concentrations in I pond waters.

Outlook

This thesis proves that nutritious aggregates can be generated from shrimp pond waters. Identification of particles, which compose aggregates, is necessary to be performed to understand the main composition of aggregates. For this purpose, microscopic observation and bacterial community identification from single aggregate is required to elucidate aggregate composition and bacterial community structure, respectively. It is possible that different dominant phytoplankton (or microalgae) and bacterial cells compose aggregates. Therefore, identification of phytoplankton species and its abundances in shrimp pond water as well as in aggregates is necessary (**Appendix II**). In addition, further analyses on essential amino acids, such as lysine, and fatty acids in aggregates, followed by laboratory experiments about the doses of aggregate diets for shrimps are still required.

Considering potential presence of nutritious aggregates as well as high numbers of heterotrophic bacteria and phytoplankton in shrimp ponds, I propose three recommendations for shrimp pond aquaculture. Firstly, it may be necessary to reduce the amount of provided pellets into certain constant level (i.e. 100 kg feed pellets per day) after 90 days of rearing until the final harvest. To substitute the pellets and to keep sufficient amount of food, shrimp farmers can add other carbon sources, such as molasses, rice-bran or tapioca. This approach will provide other forms of shrimp meals, which are carbohydrates-rich particles and bio-flocs. At the end, operational costs for feed pellets can be saved without reducing shrimp performance or the final shrimp harvest. Secondly, since effluents of shrimp ponds contain important amount of particulate matter including phytoplankton and bacteria, direct discharge of these effluents will contribute to eutrophication and deterioration for the adjacent ecosystems (Herbeck *et al*., 2013; Kautsky *et al*., 2000). Instead of discharging these effluents directly, post-harvest shrimp ponds can be used to rear herbivorous fishes, such as milk fish (*Chanos chanos*) or rabbitfish (*Siganus* sp) (**Appendix III**). These two fishes are commonly grown in sea cage nets (Marte, 2010; Yousif *et al*., 2005) and can be potentially reared in saline pond waters. Finally, it is possible to try polyculture systems between *L. vannamei* with fishes, either *Siganus* sp. or *Chanos chanos* since they do not prey on shrimps. However, further detail research on biota interactions, feed competition and requirements, as well as potential disease transfer between two different organisms are still prerequisite to achieve a successful cultivation method.

Conclusion

This thesis provides an overview of shrimp farming in semi-intensive and intensive systems with particular emphasis on water parameters and bacterial community dynamics, including disease events. The four chapters, which consist of a literature review, one laboratory experiment and two field studies, emphasize the importance of biogeochemical parameters, especially pH, and the microbial community for successful shrimp rearing. The results of this thesis show that pH value should be kept above 8 to avoid shifts towards pathogenic bacteria in the microbiome. Phytoplankton and bacterial cells have to be maintained carefully to govern ammonium and nitrite concentrations in shrimp pond waters. Secondly, bacterial community dynamics including investigation and estimation of the potential pathogenic bacteria are vital to avoid shrimp disease. Thereby, the BCC should be separated into FL and PA bacteria also in aquaculture systems. This is useful because the PA fraction may contain more pathogenic bacteria. Since the shrimps may consume particles from the water column, particles represent a direct vector for disease in aquacultures. Furthermore, aggregates containing high amounts of pathogens may increase probability of intoxication in shrimp, which increase the widespread of shrimp disease within pond. Thirdly, regular feed and additional feed diets should be provided to shrimp pond by not only considering the expected average weight gain, but also the feed conversion ratio (FCR), average daily growth (ADG), and water biogeochemistry. If necessary, the regular feed diet can even be stop for few days because the results show that there are sufficient natural food sources in shrimp pond waters to sustain growth.

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Appendix I. Map of Indonesia and imagery satellite pictures of shrimp ponds in Rembang Regency
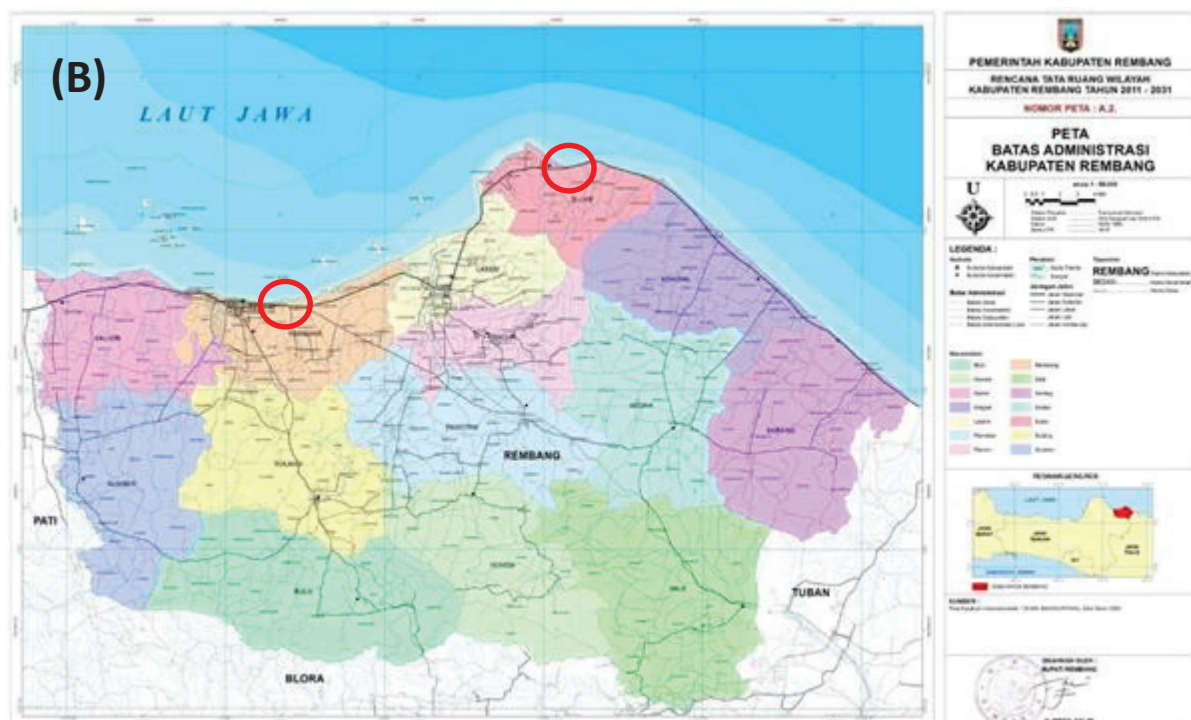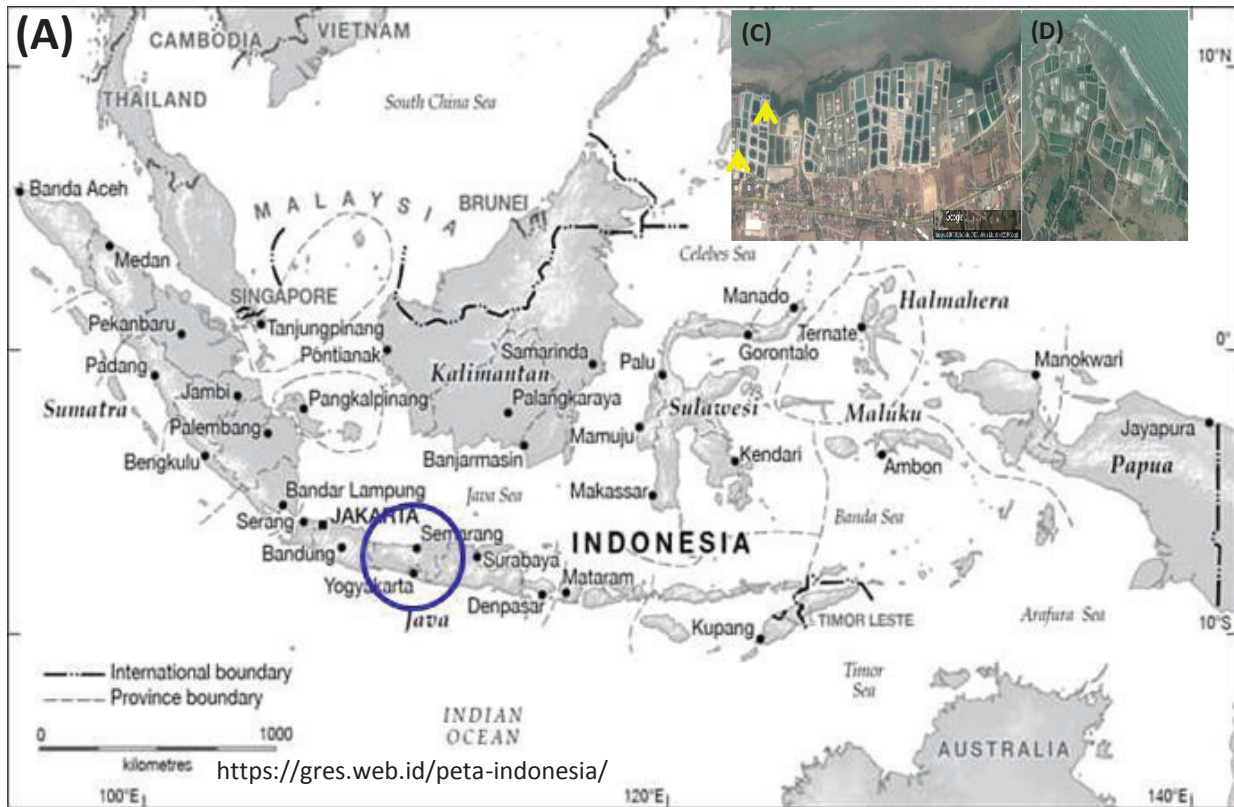

Figure 1. Map of Indonesia (panel A), blue circle indicates Central Java Province. Panel B indicates Rembang Regency with two pond areas in Rembang district (left red circle) and Sluke district (right red circle). Shrimp ponds in Tireman, Rembang (C) and in Sluke (D). Yellow arrows indicate sludge reservoirs.

Appendix II. Shrimp feed pellets and phytoplankton compositions in shrimp pond waters.

https://twitter.com/cpprima/status/1062931951856713728

Figure 2. Feed pellets from Central Proteina Prima (CPP) Firm. Ingredients of feed pellets: fish flour, shrimp flour, squid flour, fish oil, wheat, soybean, cholesterol, phospholipid, vitamins and minerals.
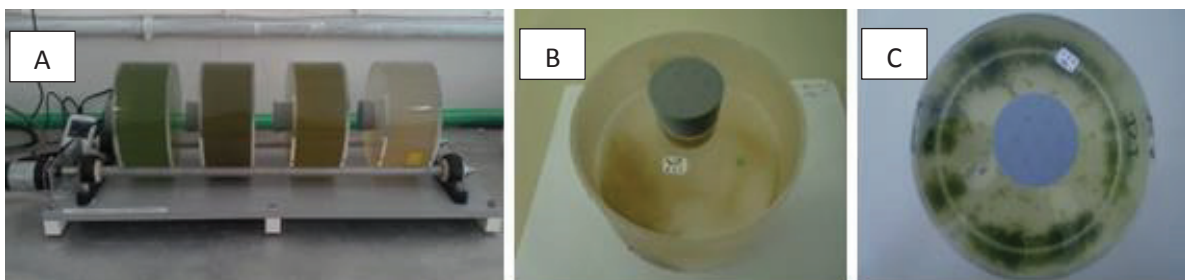

Figure 3. Aggregate formations from different shrimp pond waters

Table 1. Dominant phytoplankton community compositions in pond waters which were used in aggregate formation experiments.

Figure 4B (Species: cell mL^{-1})	Figure 4C (Species: cell mL^{-1})
Biddulphia sp. : 5.2×10^6	*Brachionus sp.* : 3.5×10^4
Coscinodiscus sp. : 1.3×10^5	*Planaria sp.* : 5×10^4
Euglena sp. : 7.0×10^4	*Chlorella sp.* : 7.6×10^6
Chlorella sp. : 4.3×10^6	*Arthrodesmus sp.* : 9.6×10^5
Microcystis sp. : 2.0×10^6	*Chroococcus sp.* : 1.6×10^5
Gonyaulax sp. : 5.0×10^4	*Monoraphidium sp.* : 4.8×10^5
Micrasterias sp. : 4.5×10^3	*Ulothrix sp.* : 9.9×10^5
	Coscinodiscus sp. : 6.8×10^5
	Micrasterias sp. : 8×10^4

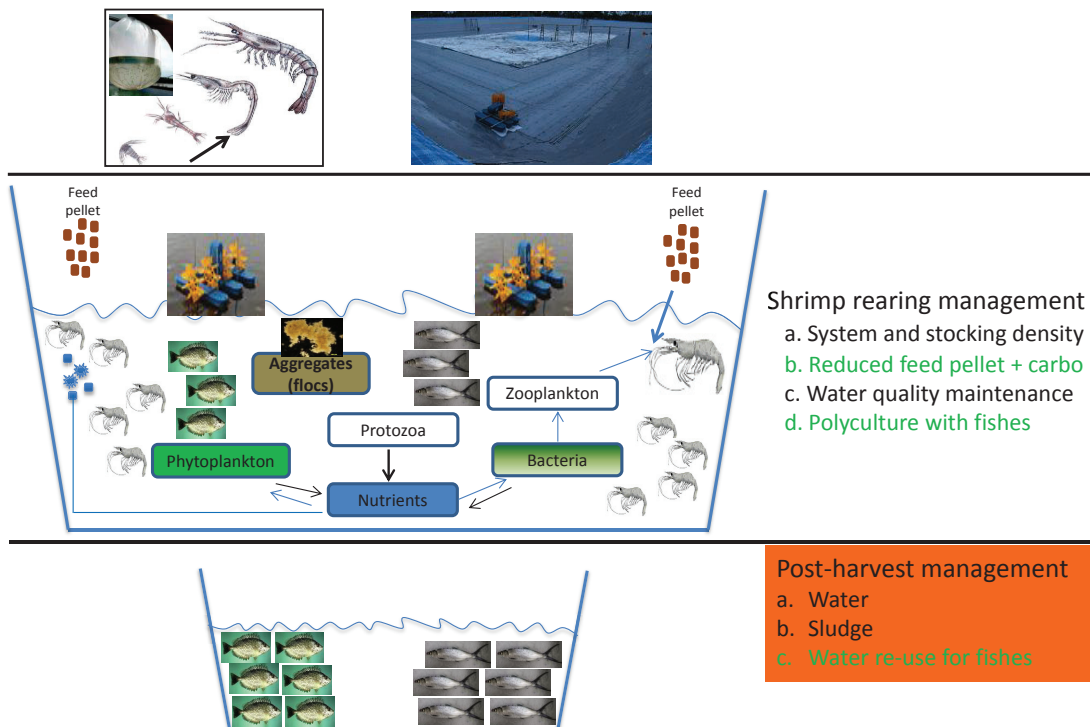**Appendix III. Schema of sustainable shrimp pond aquaculture**

Figure 4. Modified shrimp aquaculture processes which include feed managements by substituting feed pellets with other carbohydrate meals and application of polyculture systems, either with rabbitfish (*Siganus* sp.) or milkfish (*Chanos chanos*). Alternatively, the fishes can be reared after final harvest of shrimps. Source pictures: *Siganus* sp. (https://www.dunia-perairan.com/2017/03/ikan-baronang-siganus-sp.html), milkfish (by Yustian Rovi Alfiansah).

Appendix IV. List of publications

Publication related to the PhD thesis

- **Alfiansah Y.R.**, Hassenrück C, Kunzmann A., Taslihan A., Harder J., Gardes A., 2018. Bacterial abundance and community composition in pond water from shrimp aquaculture systems with different stocking densities. Frontiers in Microbiology 9:2457. doi:10.3389/fmicb.2018.02457.

Co-author for publications which are not included into the PhD thesis

1. Danyun Ou, Bin Chen, Tri Aryono Hadi, Suharsono, Wentao Niu, **Yustian Rovi Alfiansah**. Next-generation sequencing revealed specific microbial symbionts in *Porites lutea* with pigment abnormalities in North Sulawesi, Indonesia. Acta Oceanologica Sinica,2018, Volume 37, Issue 12, pp 78–84.

2. Kegler P., Kegler H.F., Gärdes, A., Ferse S.C.A., Lukman M., **Alfiansah Y.R.**, Hassenrück C., Kunzmann A., 2017. Bacterial biofilm communities and coral larvae settlement at different levels of anthropogenic impact in the Spermonde Archipelago, Indonesia. Frontiers in Marine Science 4:270. doi: 10.3389/fmars.2017.00270.

3. Baum G., Kegler P., Scholz-Böttcher B.M., **Alfiansah Y.R.**, Abrar R., Kunzmann A. 2016. Metabolic performance of the coral reef fish *Siganus guttatus* exposed to combinations of water borne diesel, an anion surfactant and elevated temperature in Indonesia. Marine Pollution Bulletin 110: 735-746.

4. Herz N., Ferse S., **Alfiansah Y.R.**, Kunzmann A. 2016. High-performance liquid chromatography to detect thiocyanate in reef fish caught with cyanide: a practical field application. SPC Live Reef Information Bulletin No.21: 8-16.

Manuscript in preparation which is not part of this thesis

Bachmann, J., Hassenrück, C., **Alfiansah, Y.R**., Barz, J., Busche, T., Iversen, M.H., Grossart, H-P., Gärdes, A. Particles in the spotlight: a measure of aquaculture-induced impacts on aquatic ecosystems.

Reviewer

Ling Qiao, Zhiqiang Chang, Jian Li, Zhao Chen, Ligan Yang, Qiang Luo, Phytoplankton community structure and diversity in the indoor industrial aquaculture system for *Litopenaeus vannamei* revealed by high-throughput sequencing and morphological identification. *Journal of Aquaculture Research*. Reviewed on April 2019**.**

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