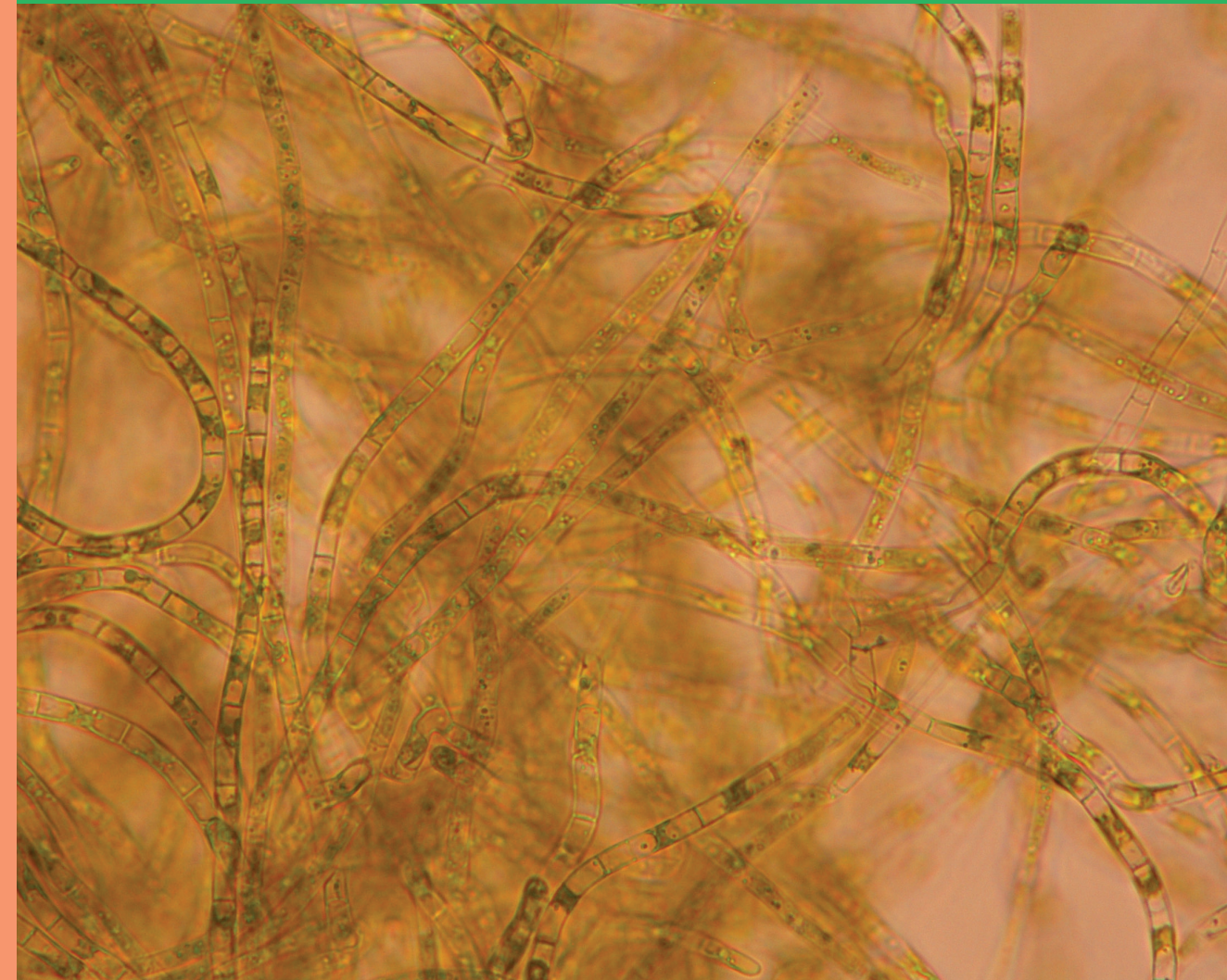


Microalgae-bacteria interactions: a key for improving water quality in recirculating aquaculture systems?

Microalgae-bacteria interactions: a key for improving water quality in recirculating aquaculture systems?

Norulhuda Mohamed Ramli



Norulhuda Mohamed Ramli

Invitation

On Monday,
December 10th 2018
at 13:30

in the Aula of
Wageningen University
& Research,
Generaal Foulkesweg 1a,
Wageningen.

Norulhuda Mohamed Ramli
will defend her PhD thesis:

**Microalgae-bacteria interactions:
a key for improving water
quality in recirculating
aquaculture systems?**

After the ceremony, there
will be drinks in the "Aula".
You are most welcome.

Your presence will be
highly appreciated

Paranymphs
Nazri Nayan
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Kabir Kazi
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Propositions

1. The shared role of microalgae and bacteria in ammonia removal increases the stability of recirculating aquaculture systems.
(this thesis)
2. In a recirculating aquaculture system, the presence of algae affects the composition of the bacterial community.
(this thesis)
3. The perception of farmers towards the risks of environmental disturbances for aquaculture is very important for policy makers to develop proper mitigation measures, as is shown by the Vietnamese pangasius industry.
4. An ecological regime shift could jeopardize the position of a country's ruling party.
5. University rankings affect small-scale farmers.
6. Presbyopia is a sign of leadership.

Propositions belonging to the thesis entitled:

“Microalgae-bacteria interactions:

a key for improving water quality in recirculating aquaculture systems?”

Norulhuda Mohamed Ramli

Wageningen, 10 December 2018

**Microalgae-bacteria interactions:
a key for improving water quality in
recirculating aquaculture systems?**

Norulhuda Mohamed Ramli

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This research was conducted under the auspices of the graduate school of WIAS
(Wageningen Institute of Animal Sciences).

Microalgae-bacteria interactions: a key for improving water quality in recirculating aquaculture systems?

Norulhuda Mohamed Ramli

Thesis

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Prof. Dr A.P.J. Mol,
in the presence of the
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Norulhuda Mohamed Ramli

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Abstract

The roles of algae in improving aquaculture water quality are well-established. However, the integration of algae in a recirculating aquaculture systems (RAS) is less popular mainly due to the large area required for photosynthesis. As science progresses, a growing number of reports are available on the benefits of algae to water quality and fish health. This motivated the author to investigate the effects of algae on a RAS stability, by measuring the water quality and the effects on bacterial community composition in a RAS. A review was conducted on nitrogen removal by algae and the operation of an algae reactor in a RAS. This showed that a RAS configuration influence algae performance by affecting nitrogen loading and nitrogen species (ammonium versus nitrate), cultivation methods (suspended versus attached) and environmental conditions (light, temperature, pH, oxygen, and carbon dioxide). Next, a periphytic microalga, *Stigeoclonium nanum* was cultured in suspension or immobilized. The growth and nitrogen uptake of *S. nanum* was higher when immobilized than when cultured in suspension. *S. nanum* preferred ammonia rather than nitrate as nitrogen species. Further effects of *S. nanum* on the RAS water quality (total ammonia nitrogen (TAN), nitrite, nitrate, and phosphate) were also investigated. No difference of TAN between the RAS with algae (RAS+A) and the RAS without algae (RAS-A) was observed. However, nitrite, nitrate and phosphate were significantly lower in the RAS+A than in the RAS-A. When the RAS systems were perturbed by an acute pH drop (from pH 7 to 4 over three hours), no significant difference was observed between the RAS+A and the RAS-A on the resistance towards the stressor. This was shown by an increase in the TAN and the nitrite concentration in both treatments after the perturbation. However, the algae helped the RAS+A to regain a low nitrite level faster than the RAS-A. The diversity of bacterial community between the RAS+A and the RAS-A was not different, while the composition of bacterial community was significantly different between the RAS+A and the RAS-A, thus influencing the functioning of the RAS.

Table of contents

	Page
Chapter 1	General Introduction
	1
Chapter 2	Integration of algae to improve nitrogen waste management in recirculating aquaculture systems
	13
Chapter 3	Removal of ammonia and nitrate in recirculating aquaculture systems by <i>Stigeoclonium nanum</i> , a fresh water filamentous epiphyte, immobilized in alginate beads
	41
Chapter 4	Effects of <i>Stigeoclonium nanum</i> , a fresh water periphytic microalga on water quality in a small-scale recirculating aquaculture system
	59
Chapter 5	Resistance and resilience of small-scale recirculating aquaculture systems (RAS) with or without algae to pH perturbation
	81
Chapter 6	General Discussion
	109
	References
	123
	Summary
	138
	About the Author
	141
	Acknowledgement
	142
	List of publications
	143

Chapter 1

General introduction

1.1 Outline

In this study the author attempted to demonstrate that microalgae can improve water quality and the stability of a recirculating aquaculture system (RAS). The stability will be tested based on the function of nitrogen removal by perturbing a RAS with a pH stressor and observing the changes in the total ammonia, nitrite and nitrate concentration of the RAS before and after the perturbation. Therefore, this chapter will discuss; 1- the role of microalgae specifically in inorganic nutrient uptake and how it relates to microalgae-bacteria interactions; 2- the microbial community as a key player in maintaining RAS water quality and stability; 3- the nitrogen removal in a RAS and how microalgae can improve the nitrogen removal process. Finally in this chapter, the problem statements are discussed and the thesis outline is given.

1.2 Roles of microalgae in aquaculture

Microalgae are important microorganisms in aquaculture with many functions such as for feed and for the removal of inorganic nutrients, organic contaminants, and heavy metals (Fig. 1) (Becker, 2013; Eversole et al., 2008; Neori, 2011; Tucker et al., 2014). Microalgae contain high protein (between 40 to 70%), carbohydrates (between 10 to 65%) and lipids (between 5 to 45%) per microalgae dry weight which make them suitable for fish feed. In addition, microalgae contain another important range of polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5 n-3) and arachidonic acid (AA) which are valuable to boost growth and health for various fish species and invertebrate larvae (Becker, 2013; Roy and Pal, 2015; Ryckebosch et al., 2014; Sargent et al., 1997). Recently the use of microalgae in fish feed has become more significant as microalgae can potentially reduce the inclusion of fish meal and fish oil (Shah et al., 2018). Neori (2011) reported that microalgae used as feed through the technique of green water culture serve as an important drive for the production of world major planktivore species such as Nile tilapia, rohu carp, bighead carp, catla and shrimp.

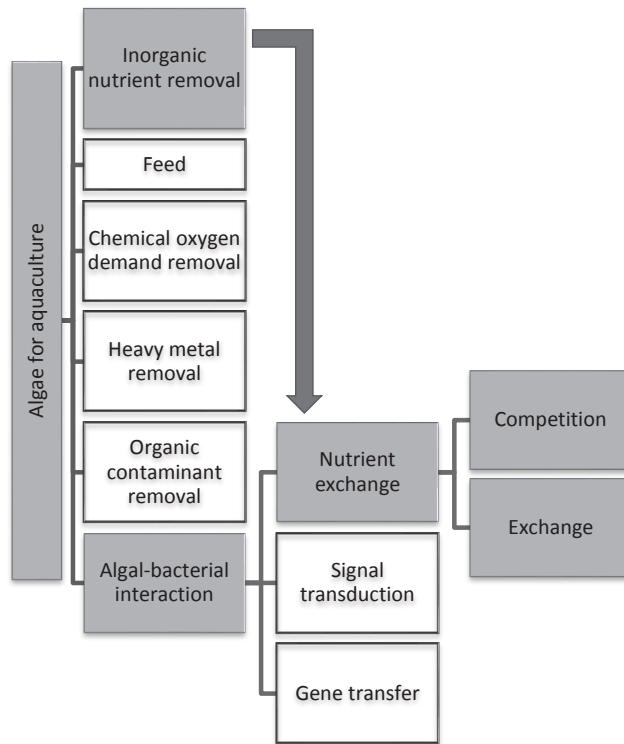


Fig 1 Roles of microalgae in aquaculture. Gray boxes show the research topics covered in the study.

The importance of microalgae as feed in green water culture indirectly demonstrates their importance for maintaining low nitrogen and phosphate because microalgae uptake these nutrients for their growth. Also, microalgae use carbon dioxide and produce oxygen which helps in reducing chemical oxygen demand (COD) from the water body and keeps the system in an oxidized condition. Additionally, the majority of aquaculture production volume comes from ponds where microalgae are present which translates to the significant role of microalgae in maintaining the balance of aquaculture ecosystem (FAO, 2016; Verdegem and Bosma, 2009). Nowadays, there are growing reports concerning the efficiency of microalgae in removing heavy metals and organic contaminants (Doshi et al., 2008; Matamoros et al., 2015; Shanab et al., 2012; Zhou et al., 2014). In addition, microalgae interact with aquatic microorganisms which have a direct influence on water quality (Cole, 1982; Glibert, 2012). Even though some challenges might limit their application such as the difficulty in harvesting and

disposal, the benefits offered by microalgae are very promising (de-Bashan and Bashan, 2010; Matamoros et al., 2015; Suresh Kumar et al., 2015).

1.2.1 Microalgal-bacterial interactions

Both positive and negative interactions co-exist between microalgae and bacteria, resulting in either inhibition or stimulation of co-occurring algae and bacteria (Cole, 1982; Fuentes et al., 2016; Joint et al., 2007; Natrah et al., 2014; Schumacher and Sekoulov, 2003; Vardi et al., 2006; Volk and Furkert, 2006). The types of interaction also can be categorized into signal transduction, gene transfer and nutrient exchange (Kouzuma and Watanabe, 2015). In signal transduction interaction, microalgae or bacteria produce chemicals which activate or inhibit gene expression and physiological activities, thus affecting their response to the environment and growth, while gene transfer occurs between microalgae and bacteria as part of an evolutionary process (Kouzuma and Watanabe, 2015). Meanwhile, nutrient exchange between microalgae and bacteria is considered as the most basic interaction (Cole, 1982; Cooper and Smith, 2015). In this thesis, the discussion is limited to the nutrient exchange category and effects of compounds produced by microalgae.

Microalgae, through photosynthesis produce oxygen and organic compounds. These products are used by bacteria for energy production and cell synthesis (Armstrong et al., 2000; Coveney and Wetzel, 1989). Bacteria degrade organic matter including dead microalgae cells which later could be used again by microalgae as a nutrient for growth (Rowe et al., 1975). One example of an application in wastewater treatment is microalga *Chlorella sorokiniana* that produces oxygen and bio surfactants to enhance phenanthrene degradation by *Pseudomonas migulae* (Muñoz et al., 2003).

Compounds produced by microalgae can either promote or inhibit bacterial growth. The inhibition is caused by the production of toxin or antibacterial compounds by microalgae (Anderson et al., 2012). For example extracellular polyunsaturated aldehydes produced by diatoms have been shown to inhibit the growth of 19 bacterial strains at concentrations between 3 to 145 mol L⁻¹ (Ribalet et al., 2008). On the other hand, bacteria too can promote microalgae growth, for example the *Azospirillum* species, known as a plant growth-promoting bacterium, increased the growth of fresh water microalga

Chlorella, when *Azospirillum* and *Chlorella* were cultured together (de-Bashan and Bashan, 2008). Bacteria are known to produce vitamin B12 which is essential for the growth of microalgae (Croft et al., 2005). On top of that, extracellular polysaccharides (EPS) produced from microalgae and bacteria are important for the development of biofilms. In a biofilm, bacteria perform important processes such as nutrient recycling, biodegradation and pollutant retention (Battin et al., 2003). Biofilm also can influence the morphology of residing microalgae and bacteria (Bernbom et al., 2011; Marshall et al., 2006; Spoerner et al., 2012).

Nutrient competition occurs between bacteria and microalgae. Bacteria and microalgae use the same nutrients such as phosphorus, nitrogen and carbon for growth (Bradley et al., 2010; Risgaard-Petersen et al., 2004; Thingstad et al., 1993). Through this function, they improve water quality. This shared function between bacteria and microalgae can be hypothesized to improve the stability of an ecosystem and could therefore help to maintain good water quality in an aquaculture system. Nonetheless, when different organisms use the same nutrients, competition may occur. For example, microalgae have been found to be superior under high phosphate conditions while bacteria were found to be superior under low phosphate conditions (Thingstad et al., 1993). Benthic microalgae too have been found to be more efficient at using ammonium than ammonia oxidizing bacteria (AOB) because the microalgae had higher N uptake rates and grew faster than AOB (Risgaard-Petersen et al., 2004). However, the competition might have a positive effect on a system as demonstrated by the microalgal-bacterial community which has been shown to be more efficient in treating ammonium ions than nitrifying bacteria alone during thiocyanate (SCN) degradation (Ryu et al., 2015). In short, microalgae demonstrate multiple roles for aquaculture that could potentially provide a synergistic effect for improving overall aquaculture practice.

1.3 Microbial community is the key player for water quality and stability in recirculating aquaculture system

Aquaculture production needs to increase in order to meet the demand of the world population. Due to limited resources such as water and land, a recirculating aquaculture system (RAS) is regarded as a superior alternative to flow-through or semi-flow through systems (Martins et al., 2010). A RAS has several advantages such as controlling the quantity of waste discharged into the environment, optimizing the volume of water per kg fish production, increasing biosecurity and reducing reliance on antibiotics (Martins et al., 2010; Piedrahita, 2003; Verdegem, 2013). Furthermore, some countries apply strict environmental regulations, thus making a RAS an ideal production system (SustainAqua, 2009).

In a RAS, water quality is controlled by mechanical (removal of solid waste) and biological (nutrient mineralization and recycling by microbial processes) means. While the mechanical process is more manageable, the biological process is more complex. Therefore, the understanding of these microbial processes is a prerequisite for proper management of a RAS (Blancheton et al., 2013). Nitrification is regarded as a key process in a RAS. However other important processes such as denitrification and anaerobic ammonium oxidation (anammox) may also occur in a RAS (Table 1). Although most of the processes are aerobic, anaerobic processes can occur in the solid wastes and the thick biofilm envelope (Timmons and Ebeling, 2007).

Bacterial communities maintain water quality and they relate to the functional stability of a RAS. In microbial ecology, biodiversity has been identified as one of the important community properties which affect ecosystem stability (Shade et al., 2012). Ecosystem stability can be specified as the functional stability of a system to maintain its function under changing conditions (Orwin and Wardle, 2004; Wang et al., 2011). Stability relates to system resistance (the ability to withstand a disturbance) and resilience (the speed of recovery of a system to its pre-disturbance state) (Griffiths and Philippot, 2013; Loreau et al., 2001; Pimm, 1984).

Table 1 Microorganisms associated with bio-filtration in recirculation aquaculture systems bio-filtration (Adapted from: Brown et al., 2013; Rurangwa and Verdegem, 2014; Schreier et al., 2010).

Process	Phylum and Genus
Nitrification	
Ammonium-oxidizing bacteria	<i>Nitrosomonas</i> , <i>Nitrosococcus</i> , <i>Nitrosospira</i>
Ammonium-oxidizing archaea	<i>Nitrosopumilus</i>
Nitrite-oxidizing bacteria	<i>Nitrospira</i> , <i>Nitrobacter</i> .
Denitrification	
Autotrophic	<i>Thiomicrospira</i> ., <i>Thiothrix</i> , <i>Rhodobacter</i> ., <i>Hydrogenophaga</i>
Heterotrophic	<i>Pseudomonas</i> , <i>Paracoccus</i> , <i>Comamonas</i> .
Dissimilatory nitrate reduction to ammonia	Various Proteobacteria and Firmicutes
Anaerobic ammonium oxidation (Anammox)	<i>Planctomycetes</i> , <i>Brocadia</i>
Sulphate reduction	<i>Desulfovibrio</i> , <i>Dethiosulfovibrio</i> , <i>Fusibacter</i> , <i>Bacteroides</i> .
Sulphide oxidation	<i>Thiomicrospira</i> .
Methanogenesis	Methanogenic Archaea

Meanwhile, biodiversity creates an insurance or capability for the system to stabilize against environmental fluctuations because different species may react in a different manner to the fluctuations (Loreau et al., 2001; McNaughton, 1977; Tilman et al., 2006; Yachi and Loreau, 1999). Other than the structure and diversity of the community, stability also depends on the interaction between the abiotic factors and physiological responses of organisms (Griffiths and Philippot, 2013; Griffiths et al., 2003). Although the diversity-stability theory has been found applicable in forest ecology and in many soil and aquatic microbial ecologies, it is important to note that the results of one community in an ecosystem might not be applicable to other ecosystems (McCann, 2000; Shade et al., 2012). Therefore, it is important to test the theory on an untested ecosystem such as a RAS. Currently, knowledge of improving RAS stability by the function of the bacterial communities in the system is limited.

1.4 Nitrogen removal in a RAS

Removal of total ammonia nitrogen (TAN: unionized ammonia, NH_3 + ammonium ions, NH_4^+) which will be referred to as ammonia, is one of the most important processes in a RAS because of the toxicity of ammonia for fish. In a RAS, nitrification is the key player in this process. It is a dissimilative process where ammonia is converted to nitrite and nitrite is converted to nitrate under an oxidized condition. Nitrification could result in nitrate accumulation (van Rijn, 1996). Although nitrate is less harmful than ammonia, high amounts of nitrate can cause growth retardation, abnormal swimming behavior and chronic health issues in fish (Davidson et al., 2014). Therefore, nitrate must be removed from the system (be it at much higher concentration levels than ammonia) and this is normally achieved by a periodic water discharge from a RAS. However, in doing so, the water quality of the receiving water bodies can deteriorate. So, another dissimilative process which is denitrification can be introduced and implemented in a RAS (Van Rijn et al., 2006). Although nitrification and denitrification have been found to be efficient in maintaining the water quality of a RAS, the efficiency of nitrogen use in a RAS is not very advantageous because the ammonia and nitrate are converted and not used. To optimize the overall nitrogen utilization efficiency of a RAS, alternative modes for controlling ammonia and nitrate concentrations in a RAS have to be researched.

Ammonia and nitrate can be removed by plant (e.g. vegetables and macrophytes) and algae (macroalgae and microalgae) immobilization (Troell et al., 2003). When plant or algae immobilize ammonia, the amount of ammonia available for nitrification is reduced, thus reducing the conversion of ammonia to nitrite, and nitrite to nitrate. The possibility of applying these methods in a RAS is beneficial not only for improving water quality but also for improving nutrient use efficiency in a RAS. The uptake of nutrients by plants and algae is an assimilative process which produces biomasses that can be utilized for human and fish consumption. Plants can be further divided into vegetables and macrophytes. Integrating vegetable farming with a RAS is also called an “aquaponic” system. Alternatively, one of the most used macrophyte species used for water treatment is duckweed (Muradov et al., 2014).

According to the literature, the use of macroalgae (*Ulva* spp.) (Cahill et al., 2010), periphyton which contain microalgae (Valeta and Verdegem, 2015), aquaponic systems (Graber and Junge, 2009; Tyson et al., 2011) and aquatic macrophytes (Velichkova and Sirakov, 2013) could improve nitrogen utilization efficiency in a RAS. The uptake kinetics of algae is size dependent, which is a reason why microalgae take up nitrogen faster than macroalgae per unit biomass both at low and high nitrogen concentrations (Hein et al., 1995). Microalgae also have a higher nitrogen removal efficiency than macrophyte (duckweed) when used in a waste stabilization pond (Zimmo, 2003). An estimation which compared the performance of microalgae, macroalgae, duckweed, strawberry and tomatoes showed that ammonia removal in a RAS was best performed by the microalgae (Ojanen et al., 2017).

In terms of biomass management, macroalgae, vegetables (in aquaponics) and duckweed are all easy to manage and to harvest, producing low amounts of suspended solids (Troell et al., 2003). On the other hand, microalgae are difficult to manage and harvest due to their microscopic size and they also produce a high amount of solids (Benemann et al., 1977; de-Bashan and Bashan, 2010). In a RAS, too many suspended solids may hamper the efficiency of biofilters. Therefore, in a RAS, it is advisable to use a periphytic type of microalgae instead of planktonic microalgae to reduce the risk of accumulation of suspended solids by the microalgae. A nice example was given by Valeta and Verdegem (2015), who introduced microalgae by an algal turf scrubber and therefore, the management of microalgae was relatively easy and the accumulation of suspended solids remained limited.

A large surface area is required by macroalgae, duckweed, and aquaponics as these cultures have an aerial nature of light-dependency (Graber and Junge, 2009; Love et al., 2015; Troell et al., 1997; Xu and Shen, 2011). On the other hand, microalgae can occupy a volumetric culture unit instead of an aerial culture unit. This makes the integration of microalgae in a RAS more area efficient than in the case of macroalgae, duckweed, or aquaponics. Taking into account that microalgae provide better ammonia uptake and space utilization than macroalgae, duckweed, or an aquaponic system, it can be concluded that the incorporation of microalgae in a RAS should be explored for further improvement of RAS water quality.

1.5 Problem statement

A recirculating aquaculture system is a system for aquaculture intensification due to its ability to produce more fish per unit area and water volume than a flow-through system. However, a RAS discharges inorganic nitrogen, particularly nitrate, as a result of nitrification. High concentrations of nitrate hamper fish growth and therefore, nitrate must be removed from the RAS.

This thesis contributes by integrating knowledge from other domains and extends the application to a RAS and subsequently provides insight into how microalgae will affect nitrogen removal and the stability of a RAS. Many methods are available to remove nitrogen in a RAS, but N removal through the use of microalgae is the least explored for application in a RAS despite the method being more sustainable and offers various benefits/advantages such as less surface area required etc. (Section 1.4). Studies of microalgae are abundant for non-RAS systems (Section 1.2), and findings in these studies indicate that microalgae can regulate the bacterial community in systems other than a RAS. In a soil system, the diversity of the bacterial community was shown to maintain the stability of the system (Griffiths and Philippot, 2013). This led to the inference that the stability of a RAS may potentially be controlled by influencing the diversity of the bacterial community by introducing microalgae in a RAS.

The hypothesis developed by the author considers that for a typical RAS, incorporating microalgae will improve water quality, and when the stability of the system was perturbed, for instance, a sudden drop in pH, microalgae and their interactions with bacteria will stimulate the recovery of RAS stability, leading to a better resistance and resilience of the system to perturbations.

To test the hypothesis, this thesis is aimed at achieving the following study objectives:

- 1- To review the state of the art of algae incorporation in a RAS.
- 2- To measure the ammonia and nitrate removal by *Stigeoclonium nanum*, a periphytic microalga selected for this study.
- 3- To observe the effects of microalgae inclusion on the bacterial community in a RAS.
- 4- To study the effects of microalgae inclusion on water quality and on the stability (resistance and resilience) of the RAS under both normal conditions and perturbed conditions.

1.6 Thesis outline

This thesis consists of a general introduction (**Chapter 1**), a review of the state of the art of using microalgae in recirculating aquaculture systems (**Chapter 2**), experimental chapters (**Chapter 3, 4, and 5**), and a general discussion (**Chapter 6**).

Chapter 2 highlights the management of microalgae in a RAS. In **Chapter 3** the selected microalga (*Stigeoclonium nanum*) immobilized in alginate beads are tested for their preference for ammonia or nitrate as a nitrogen source. The result of this study is used to predict the behavior of *S. nanum* when it is incorporated in a RAS for the subsequent experiment. In **Chapter 4**, the effect of microalgae in a RAS on water quality and bacterial community is tested under normal conditions. In **Chapter 5**, the effect of microalga in a RAS on stability and the bacterial community is tested when the RAS is perturbed. Finally in **Chapter 6** the results from these experiments, especially contributions of microalgae in terms of improving the RAS stability are discussed. The overall conclusion and research implications are also discussed.

Chapter 2

Integration of algae to improve nitrogen waste management in recirculating aquaculture systems

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J.A.J Verreth

Abstract

The integration of phototrophic organisms (such as algae) for removal of inorganic nitrogen in a recirculating aquaculture system (RAS) has mainly been restricted to outdoor systems due to the large area required for photosynthesis. Recent studies have shown that algae can improve the stability of a RAS, as well as help to control harmful bacteria, or remove heavy metals and organic contaminants from the water. Therefore, algae should be part of a RAS so that the health of the RAS can be improved. The objective of this paper is to review nitrogen removal by algae and algae reactor operation in a RAS. This review reveals that to improve algae performance in a RAS, the species selection and algae cultivation method should match the RAS configuration. Finally, although currently the cost might hinder the application of algae integration in a RAS, it is believed that future technological advancement of algae cultivation methods will allow algae integration to become more economically feasible.

2.1 Introduction

Recirculation aquaculture systems (RAS) are intensive systems which rely on formulated feed to provide all nutrient requirements for the cultured organisms (FAO, 1988). A RAS includes a self-cleaning-conditioning system after which the water is reused for the culture (Timmons et al., 2002). In a RAS, fish are stocked at high densities, which can reach up to 150 to 350 kg m⁻³ depending on the species and average fish size. Waste generated in a RAS depends on fish metabolic activities and feed composition (Amirkolaie, 2005; Bovendeur et al., 1987; Eding et al., 2006; Heinsbroek et al., 2007). Analyzing information in the literature, Schneider et al., (2005) concluded that between 50 to 70% of feed nitrogen (N) and 35 to 85% of feed phosphorus (P) became waste in the culture system. Fish feeds usually contain high concentrations of protein (30 to 60% crude protein). According to Ebeling et al., (2006), when introducing 1 kg of feed containing 32% crude protein in a 1 m³ RAS, 30 g ammonia will be released, which in this case would mean that the ammonia concentration in the water could equal 30 mg L⁻¹. In a RAS, due to the harmful effect of total ammonia, its concentration should be maintained below 1 mg L⁻¹ (Timmons et al., 2002). Therefore, the waste must be treated before the water recirculates in the system.

In a RAS, recycling reduces the amount of water exchange necessary. The rate of water exchange in a RAS is usually between 0.1 to 3 m³ kg⁻¹ feed (Bregnballe, 2015; Martins et al., 2010). In order to maintain the water quality in a RAS while keeping water renewal per day limited, a series of water purifying units can be installed, such as a solid removal unit, a biological filtration unit for inorganic nitrogen removal, and a reservoir where water conditioning may take place (heating, oxygenation, and disinfection) (Timmons et al., 2002). The biological filtration unit controls the concentration of total ammonia, one of the most harmful forms of inorganic nitrogen produced by fish. The key process of controlling the total ammonia level is by autotrophic nitrification which converts ammonia into nitrite and nitrite into nitrate. However, the end product of nitrification, nitrate, accumulates in the RAS. The concentration of nitrate-N (NO₃-N) can be as high as 400 to 500 mg NO₃-N L⁻¹ and can also cause adverse effects on the growth of farmed organisms (Davidson et al., 2014; Van

Rijn et al., 2006). Therefore, in some system configurations, denitrification is additionally applied to control the level of nitrate and to avoid high levels of nitrate waste to be discharged to the environment.

However, denitrification is not a sustainable process in the sense that the inorganic nitrate-N, while useful as fertilizer, is converted to N_2 gas, a non-readily useful form of nitrogen. At the same time, producing inorganic N fertilizers from N_2 gas is an energy intensive process (Bartels, 2008). Therefore, to improve the sustainability of a RAS, alternative approaches for ammonia, nitrite, and nitrate conversion need to be explored, such as assimilating nitrogen (N) by organisms which can be subsequently harvested. An example is assimilation by algae. Few reports are available on the integration of algae in a RAS. Van Rijn (2013) reported that integration of phototrophic organisms (such as algae) in a RAS was mainly restricted to outdoor RAS due to the large areas required for photosynthesis. In contrast, the dissimilative processes (nitrification and denitrification) are more suitable for a compact indoor RAS. However, recent studies show many benefits of integrating algae in an aquaculture production system. They improve the stability of a RAS (Mohamed Ramli et al., 2018), as well as possibly help to control harmful bacteria in the culture water (De Schryver and Vadstein, 2014; Defoirdt et al., 2004; Natrah et al., 2014; Tendencia and dela Peña, 2003), or remove heavy metals and organic contaminants from the water (Matamoros et al., 2015; Muñoz and Guieysse, 2006; Suresh Kumar et al., 2015).

This paper reviews nitrogen removal by algae and algae reactor operation in a RAS. Nitrogen removal is the topic selected as it is a major cause of water quality deterioration in aquaculture systems, in addition to its role in determining successful algae growth in an aquaculture production system.

2.2 RAS with algae –definition

The concept of a RAS was originally designed for indoor systems (Timmons et al., 2002), however, this concept has been broadened to pond systems (outdoor) (Bosma and Verdegem, 2011). The main processes for water treatment in a RAS are solid separation and biological treatment processes mainly for transforming inorganic nitrogenous waste into nitrate or nitrogen gas through nitrification/denitrification or for ammonia assimilation through algae and bacteria. In an outdoor RAS

the biological processes occur simultaneously in ponds, whereas, in an indoor RAS, bacterial and algal processes are compartmentalized and managed specifically to support the purification process in each compartment.

In this article, this review focuses on a RAS which has at least one algae reactor as a bio-filtration unit separated from the main culture unit be it outdoor or indoor. Seven studies were selected as examples of a RAS with an algae reactor (Fig. 1; the details of the studies are supplied in Supp. Table 1 and Table 2). Discussions in this review include other algae related studies whenever appropriate.

2.3 Estimation of nitrogen loading into algae reactors and removal rate by algae or algae reactors

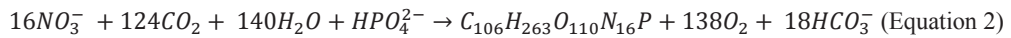
2.3.1 Nitrogen removal rate

Different methods and assumptions have been used to estimate nitrogen loading into algae reactors and removal rates by algae (reactors). The nitrogen loading rate is the amount of nitrogen received per unit area of the algae reactor per unit of time ($\text{g N m}^{-2} \text{ day}^{-1}$). Nitrogen loading rates were reported in the studies of Cahill et al., (2010), Valeta and Verdegem, and SustainAqua (2009) (Fig. 1). However, in studies by Pagand et al., (2000) and Deviller et al., (2004) the nitrogen loading rate was not given and therefore was estimated using the nitrogen concentration and flow rate into the algae reactor. Similarly, Huang et al., (2013) did not report nitrogen loading rates. In this case, it was impossible to calculate the nitrogen loading rate since the mussels under study were fed live algae and no information was given concerning the amount of microalgae fed.

Methods to estimate the nitrogen removal rate include; 1) nitrogen removal estimated from algal growth /productivity (Brune et al., 2003); 2) nitrogen removal determination by measuring nitrogen differences between the influent and the effluent of an algal reactor (Pagand et al., 2000); 3) nitrogen removal estimation by comparing the differences of nitrogen between a system with algae and system without algae (Metaxa et al., 2006).

The basis of the first method is that the rate of photosynthesis reflects the rate of nitrogen assimilation of algae ($\text{g N m}^{-2} \text{ day}^{-1}$). The effect of nitrogen assimilation by algae could reduce the

nitrogen concentration in water, thus nitrogen assimilation is regarded as nitrogen removal. The nitrogen removal rate by algae is normally expressed per unit area considering the light distribution which is expressed per unit area. During photosynthesis, inorganic carbon in the form of CO_2 or HCO_3^- and nitrogen are used in the form of ammonium (NH_4^+) (Equation 1) or nitrate (NO_3^-) (Equation 2) as an N source (Ebeling et al., 2006; Stumm and Morgan, 1996).



According to Equation 1, one g ammonium nitrogen assimilated by algae produces 15.84 g of algae biomass. Also, in this formula, carbon is 35% and nitrogen is 6% of the algal biomass, thus the percentage ratio of carbon to nitrogen (C/N) ratio of algae biomass is 5.6. This formula can be used to estimate nitrogen assimilation when the algae biomass (given as dry weight or as carbon content) in a system is known. There is also another ratio used for carbon content in algae whereby from the measured algae dry solids, 50% is considered to be carbon (Chisti, 2007). Meanwhile, a C/N ratio of algae of 10 is also used (Boyd, 1985; Gál et al., 2003). The use of a C/N ratio of 5.6 could lead to a higher estimation of nitrogen removal by algae than using a C/N ratio of 10. However, to compare between studies the same C/N ratio must be applied. The nitrogen content of algae can be also be directly determined by nitrogen composition analysis of the algae (SustainAqua, 2009). Where algae productivity is not available, algae standing biomass (g m^{-2} or g L^{-1}) is sometimes used. This method is normally used in combination with the calculation of the nitrogen budget of a system. A disadvantage is that by using the algae standing biomass, the nitrogen removal rate cannot be determined.

The second method to estimate nitrogen removal was reported in Valeta and Verdegem (2015), Pagand et al., (2000) and Deviller et al., (2004). This method is useful to estimate the nitrogen removal rate in an alga reactor, which is different from the removal rate by the algae themselves because nitrogen can also be taken up by nitrifying bacteria and can be lost through ammonia volatilization.

The third method was used when no information on algae was available. For instance, in Huang et al., (2013) the removal rate of nitrogen by the use of a periphyton turf scrubber (PTS) was estimated using the nitrogen difference between a RAS with a PTS and a RAS without a PTS (control).

2.4 Factors that affect nitrogen removal rates by algae

The nitrogen removal rate by algae reactors in different RAS varies and from the examples the removal rates range between 0.01 to 1.4 g N m⁻² day⁻¹ (Fig. 1). From these examples, factors that affect nitrogen removal rate are discussed.

2.4.1 Algae growth rate or algae biomass

Algae growth rate or biomass is normally in proportion to the nitrogen removal rate. However, since experimental conditions differ for each study and different methods and assumptions have been used to estimate the growth rate or biomass and nitrogen removal, a high algae biomass did not guarantee a high nitrogen removal rate, or vice versa (Fig. 2). From the selected studies, the macroalgae biomass (g algae m⁻² algae reactor) was higher than the microalgae/periphyton biomass (Fig. 2). However, the removal rate of nitrogen per g algae per day (mg N removed g⁻¹ algae day⁻¹) by microalgae/periphyton systems was higher than by macroalgae systems (Fig. 3), probably because the periphyton biomass also comprised of other types of microorganisms and detritus (SustainAqua, 2009; Valeta and Verdegem, 2015). The microorganisms played important roles for nitrogen removal in the periphyton community. Additionally, Hein et al., (1995) reported that the uptake kinetic by algae is size-dependent, which is the reason microalgae have a higher uptake rate than macroalgae.

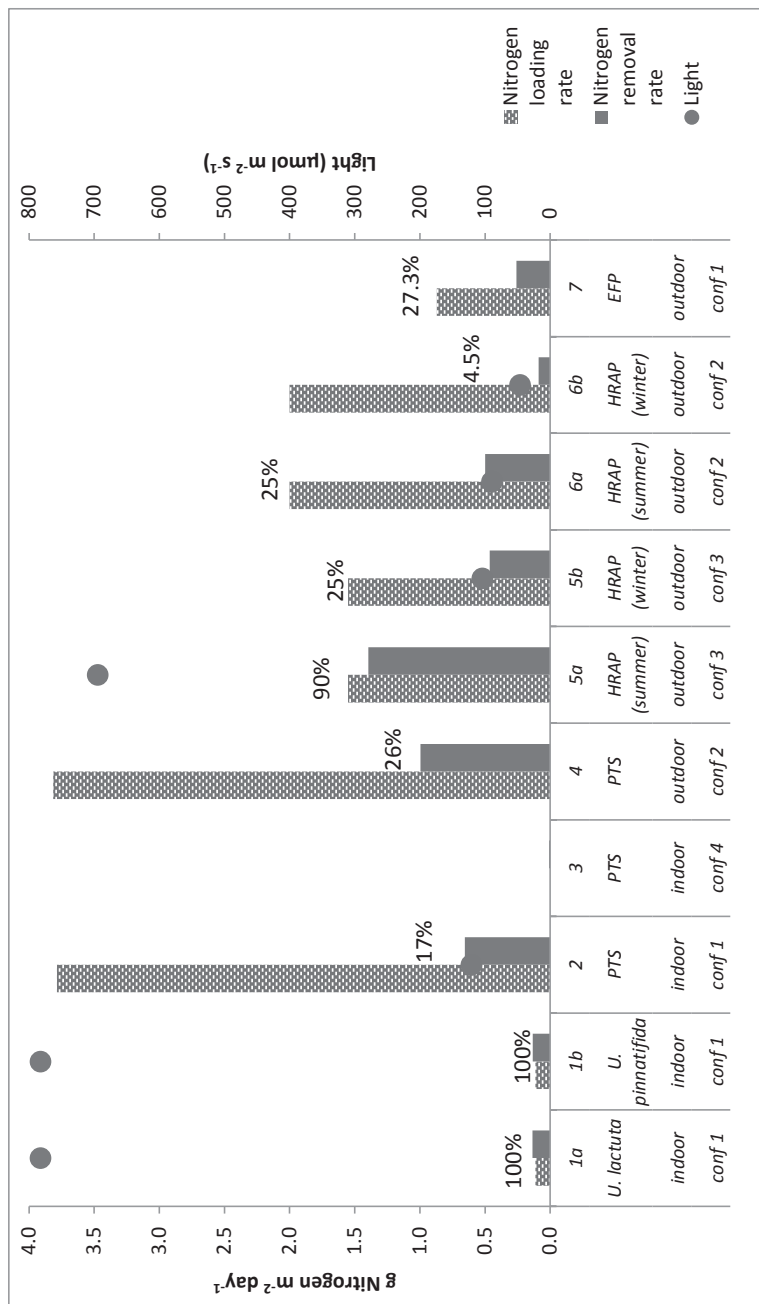


Fig 1 Nitrogen removal and loading rate by algae reactors in recirculating aquaculture systems (RAS). % value refers to the percentage of nitrogen removed. Indoor and outdoor labels in the figure indicate the location of the algae reactors. The algae reactor in study 1 used a mono-algal species (Cahill et al., 2010), *Ulva lactuta* and *Ulva pinnatifida*. Study 2 (Valeta and Verdegem, 2015), study 3 (Huang et al., 2013), and study 4 (SustainAqua, 2009) used a periphyton turf scrubber (PTS), study 5 (Pagand et al., 2000), and study 6 (Deviller et al., 2004) used high rate algal ponds (HRAP) and study 7 (Gál et al., 2003) used an extensive fish pond (EFP) as methods to integrate algae in the RAS. Maximum photosynthetic active radiation (PAR) used in the studies is shown by a red dot. The RAS configuration of studies 1, 2, 3 and 4 is shown in Fig. 4.

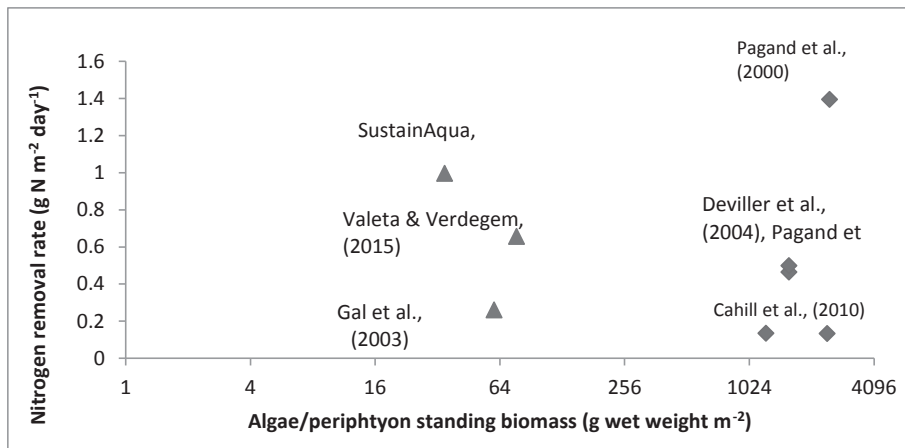


Fig 2 Nitrogen removal rate versus algae/periphyton standing biomass in a recirculating aquaculture system. The red triangles represent microalgae biomass for the study of Gál et al., (2003) and periphyton biomass for the studies of SustainAqua (2009) and Valeta and Verdegem (2015). The periphyton biomass consisted of microorganisms such as phytoplankton, bacteria, fungi, protozoa, range of invertebrates and detritus. The blue diamond symbols represent the macroalgae biomass from the study of Cahill et al., (2010), Deviller et al., (2004) and Pagand et al., (2000).

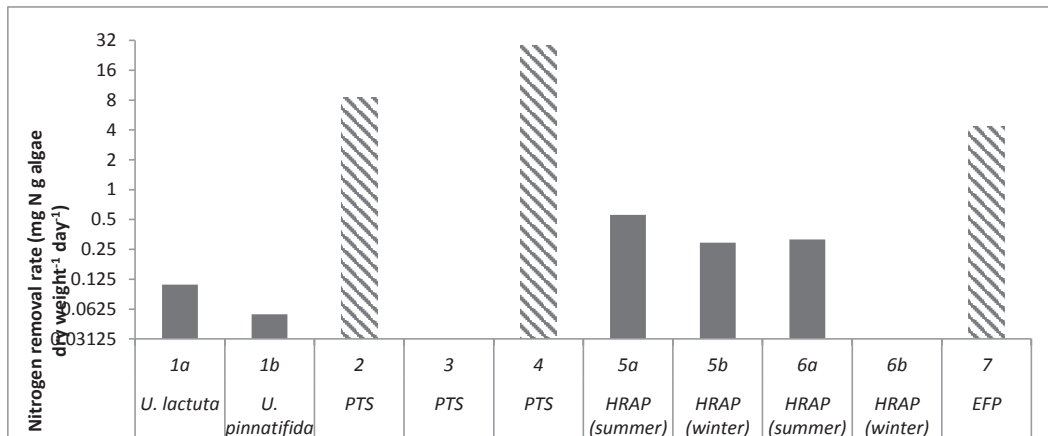


Fig 3 Nitrogen removal rate of algae (mg N g algae dry weight⁻¹ day⁻¹) in a recirculating aquaculture system. The algae reactor in study 1 used mono-algal species (Cahill et al., 2010), *Ulva lactuta* and *Ulva pinnatifida*. Study 2 (Valeta and Verdegem, 2015), study 3 (Huang et al., 2013) and study 4 (SustainAqua, 2009) used a periphyton turf scrubber (PTS), study 5 (Pagand et al., 2000), and study 6 (Deville et al., 2004) used a high rate algal pond (HRAP) and study 7 (Gál et al., 2003) used an extensive fish pond (EFP) as methods to integrate algae in the RAS. The red bars with a diagonal pattern represent microalgae and the blue bars represent macroalgae. Information for algae for study 3 and study 6b cannot be estimated.

In algae reactors in a RAS, multi species algae were observed instead of mono-species (Supp. Table 2). Multi species algae are not a problem as long as harmful algae do not dominate. Harmful algae are normally from the group of dinoflagellates, diatoms, raphidophytes, and cyanobacteria which can produce a diverse array of toxins (Anderson et al., 2012; Blackburn, 2013). They can dominate a system when the culture conditions are favorable. In mono-species cultures, growth factors are more easily controlled. For instance, in the study of Cahill et al., (2010), a single species of alga was used in the algae reactor and the culture conditions were set according to the algae requirements. The use of a mono-species culture for a specific function in a RAS, for example for nitrate removal, would be beneficial if the algae perform well under the RAS conditions.

2.4.2 Nitrogen loading rates and waste composition – determining factors

From the literature, one of the most striking differences between the studies is the nitrogen loading rate (Fig. 1). Studies which indicated a low loading rate ($0.11 \text{ g N m}^{-2} \text{ day}^{-1}$) had a 100% removal rate (Cahill et al., 2010). However, other studies which indicated nitrogen loading rates above $0.8 \text{ g N m}^{-2} \text{ day}^{-1}$ had a nitrogen removal rate of between 17 to 27% except for two cases which received a high light intensity ($690 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and a low light intensity ($46 \mu\text{mol m}^{-2} \text{ s}^{-1}$), having a 90% and 5% nitrogen removal rate, respectively. The nitrogen loading rate affects the nitrogen removal rate because different algal species have different nitrogen requirements, different affinities towards different nitrogen species, and different sensitivities towards the ammonia and/or nitrate concentration in the culture medium (Cromar and Fallowfield, 1997). Before the effects of nitrogen loading rates are discussed, factors that determine the nitrogen loading rates will be elaborated first.

In a RAS, the nitrogen loading rates tend to be dependent on the types of culture, stocking density and the RAS configuration (Fig. 4). Metabolism, nutrient requirement, and husbandry of fish, crustaceans and mollusks are different from each other, and therefore different nutrient loading rates have to be applied (Butterworth, 2010; Nunes et al., 2014; Tacon, 1987).

Meanwhile, the stocking density determines the loading rate for an algae reactor. An indoor RAS is more intensive than an outdoor RAS (Supp. Table 1). For example, the indoor RAS of Valeta

and Verdegem (2015) maintained tilapia fish at densities ranging between 30 to 70 kg m⁻³, producing a nitrogen loading rate into the algae reactor of 3.79 g nitrogen m⁻² day⁻¹. In Pagand et al., (2000) and Deviller et al., (2004), an indoor RAS contained sea bass and the maximum stocking density used were 100 kg m⁻³ and 80 kg m⁻³ respectively. Even though the algae reactor in these studies only received between 6% to 10% input from the fish culture tank, the nitrogen loading was high (Fig. 1). However, for SustainAqua (2009) even though the stocking density in the carp pond was 15 kg m⁻³, the high stocking density was due to the RAS configuration used which will be discussed in the following section.

RAS configuration

Based on the studies of a RAS which included an algae reactor (Supp. Table 1), three different RAS configurations can be organized to enhance the effectiveness of ammonia removal (Fig. 4). In these configurations, only units supplying input to the algae reactor are considered. The first configuration comprises a fish culture unit and an algae reactor. The second configuration connects three components, a fish culture unit, a solid removal unit and an algae reactor, and the third configuration is the same as the second configuration except that a nitrification unit is integrated before the algae reactor. The first RAS configuration uses an algae reactor as the only means to remove nitrogen. Since there is no nitrification unit installed, the algae reactor must be designed for a complete removal of the nitrogen excreted by the fish (Cahill et al., 2010; Gál et al., 2003; Valeta and Verdegem, 2015). The waste composition, i.e. carbon to nitrogen (C/N) ratio of the waste entering the algae reactor was expected to be high under this set-up because particulate waste entered the algae reactor.

In the second configuration, the algae reactors served as a post-treatment unit since approximately 70 to 80% of the particulate waste was removed in the RAS (Deviller et al., 2004; Pagand et al., 2000; SustainAqua, 2009). The solid removal process was performed to support the biofilter which requires a low C/N ratio (preferably between 0 to 1 (Zhu and Chen, 2001)), therefore, the algae reactor would receive the same water composition as the biofilter under the second configuration. With the solid removal process, the N/P ratio of the waste entering the algae reactor would also be affected

because particulate P would be detained in the solid removal unit. However, the amount of water channeled from the solid removal unit can be controlled. For example, in the study of SustainAqua (2009), 100% of the water was channeled into the algae pond. In the meanwhile, for Deviller et al., (2004) about 10% of water was channeled into the algae pond. In the study of Pagand et al., (2000), the influent of the algae reactor was supplied from the supernatant of the solid removal unit.

For the third configuration, an algae reactor is located after the nitrification reactor. The nitrification reactor reduced the ammonia concentration and increased the nitrate concentration allowing the algae to function specifically for the removal of nitrate-N. As reported in Huang et al., (2013) who used this configuration, the nitrate level is significantly lower in the RAS with algae than in the control RAS without algae. The second and the third configuration allow the flexibility to control the nitrogen loading and size of the algae reactor, including the flow rates and hydraulic retention time (HRT), thus influencing the nitrogen removal rate by the algae.

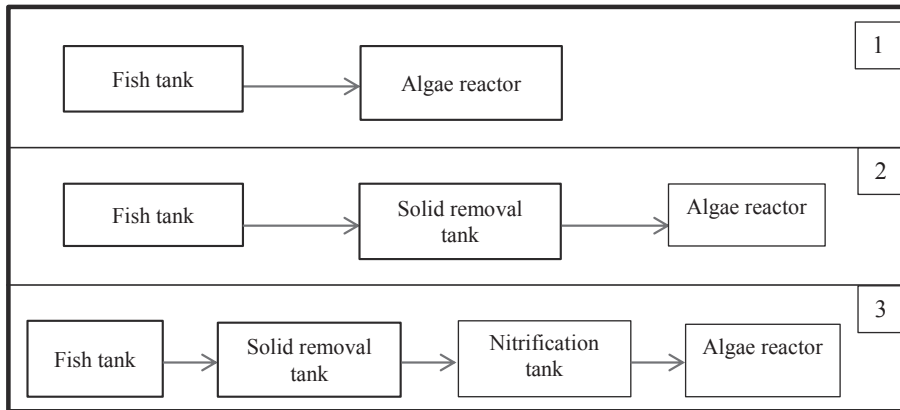


Fig 4 Recirculating aquaculture system configurations with algae reactor.

2.4.3 Nitrogen loading rate and waste composition – effects on nitrogen removal rate

Effects of ammonium loading

Generally, an ammonium concentration below 1.4 mg L^{-1} would not affect the growth of microalgae (Collos and Harrison, 2014). However, some microalgae have less tolerance to ammonia. For example, for a marine phytoplankton, *Nephroselmis pyriformis*, unionized ammonia-nitrogen at 0.0328 mg L^{-1} and ammonium-nitrogen at 3.14 mg L^{-1} was found to be toxic to this microalga (Källqvist and Svenson, 2003). Meanwhile, Collos and Harrison (2014) reviewed 45 fresh water and 68 marine microalgae species and concluded that ammonium was found toxic to microalgae species at 546, 182, 32, 50, 35, 16 mg L^{-1} for Chlorophyceae, Cyanophyceae, Prymnesiophyceae, Diatomophyceae, Raphidophyceae, and Dinophyceae, respectively and the ammonium concentration was optimum for the growth of the microalgae at 106, 35, 19, 5, 3.6 1.4 mg L^{-1} , respectively. In these studies, the ammonia toxicity was mainly observed when the pH was > 9 and ammonium toxicity occurred when the pH was < 8 .

Therefore, if an algae reactor receives as high as 376 to 381 $\text{mg L}^{-1}\text{day}^{-1}$ total ammonia such as in the study of an indoor RAS (Valeta and Verdegem, 2015), the growth of microalgae would be negatively affected depending on the species of microalgae present in the RAS. This could lead to low nitrogen removal by the algae. Collos and Harrison (2014) suggested *Nannochloropsis* sp. as a suitable candidate for aquaculture systems since this species can tolerate ammonium levels at 12 mg L^{-1} (Hii et al., 2011). Further, *Chlorella vulgaris*, which is a common species in aquaculture ponds was reported to tolerate ammonium at 280 mg L^{-1} (Tam and Wong, 1996).

Preference of nitrogen species

The preference of algae for the reduced form of nitrogen (ammonium, urea, dissolved free amino acids and adenine) or the oxidized form of nitrogen (nitrate) could affect the nitrogen removal rate by algae (Dortch, 1990; Yuan et al., 2012). Most algae prefer ammonium as the nitrogen source because less energy is needed compared to other forms of inorganic nitrogen such as nitrate (Dortch, 1990; Hii et al., 2011). Only when ammonium was not detected was nitrate uptake positive, and correlated with the

phytoplankton cell size (Yuan et al., 2012). The common view of the nitrogen cycle assumes that bacteria decompose organic nitrogen and algae use inorganic nitrogen. In reality, there is some overlap as both bacteria and algae use organic and inorganic nitrogen (Allen et al., 2002; Bronk et al., 2007; Kirchman, 1994). When inorganic nitrogen is limited, algae are capable of using urea as a nitrogen source (Bradley et al., 2010). For example, *Prochlorococcus* spp. was found to assimilate organic nitrogen in a low nutrient environment (Zubkov et al., 2003). Yuan (2012) found that after ammonium, algae would use organic N (including urea and amino acids) rather than nitrate. *Nannochloropsis oculata* and *Stigeoclonium nanum* prefer ammonia more than nitrate in contrast to *Chlorella vulgaris* that prefers nitrate (Mohamed Ramli et al., 2017; Podevin et al., 2015).

While most green algae and cyanobacteria prefer ammonium to nitrate, diatoms and dinoflagellates prefer nitrate over ammonium (Domingues et al., 2011; Dortch, 1990). In the Gulf of Riga, only diatoms were able to use the oxidized form of nitrogen (nitrate) while other phytoplankton such as cryptophytes, dinoflagellates and filamentous cyanobacteria were able to use reduced forms of nitrogen (Berg et al., 2003). There is mounting evidence that supports this finding as reported in Glibert et al., (2014), Glibert et al., (2014a) and the references therein. For instance, the occurrence of harmful algal bloom was encouraged under an elevated N/P condition with a high concentration of ammonium or urea. This finding has led many researchers to strategise that the effluent entering the San Francisco Bay Delta should have a high nitrate concentration through nitrification in order to encourage the diatoms which are more beneficial for fish and higher trophic level consumers (Glibert et al., 2014). In a RAS, where the nitrate concentration can become too high, the use of diatoms should be encouraged for nitrate removal in the RAS.

Effects of waste composition (C/N and N/P ratio)

Waste composition may influence the nitrogen assimilation (nitrogen removal) by the algae (Glibert, 2012). In aquaculture systems, waste composition influences the contributions of heterotrophic, autotrophic, or phototrophic processes to waste removal (Avnimelech, 1999; Ebeling et al., 2006). A carbon to nitrogen (C/N) ratio of more than 10 will encourage heterotrophic processes while a C/N ratio

between 6 to 7 will encourage photosynthetic process by microalgae (Ebeling et al., 2006; Hargreaves, 2006). When decomposition of microalgae is high, the C/N ratio in the water will increase, which favors heterotrophic processes. Sometimes, even though a high microalgae abundance is observed, heterotrophic processes dominate the removal of N which has been observed in an intensive system receiving a high feed load (Rakocy et al., 2004).

The nitrogen to phosphorus ratio (N/P) affects the algae composition (Glibert, 2012; Heisler et al., 2008). In turn, the algae composition affects the nitrogen removal in an ecosystem. For example in a community where cyanobacteria dominate then ammonium removal is high, whereas in a community where diatoms dominate it is observed that nitrate removal is high (Glibert et al., 2014). A specific example of the N/P ratio affecting algae growth was reported for *Tisochrysis lutea* and *Nannochloropsis oculata*; and a N/P ratio of 20 improved their growth while a N/P ratio of 120 reduced their growth (W.Rasdi and Qin, 2014). The improved or reduced algae growth under a certain nutrient composition will have a direct effect on the algae composition. In addition to the waste composition (N/P ratio), the nutrient concentration, especially nitrogen, phosphorus and silica influence the microalgae community structure in ponds (Yusoff and McNabb, 1989; Yusoff and McNabb, 1997). For instance, a recent study showed that the effect of the N/P ratio is dependent on the nutrient concentration for *Microcystis aeruginosa*. When the initial nitrogen concentration was 10 mg L⁻¹, an N/P ratio of 16 was the optimum for their growth, but when the initial P was 1 mg L⁻¹, a N/P ratio of 40 was found to be optimum (Liu et al., 2011).

In aquaculture ponds, the microalgae community composition is highly dynamic, thus an algae reactor connected to an aquaculture pond should experience similar dynamics. Shaari et al., (2011) reported that before shrimp were introduced into a culture pond, cyanobacteria dominated. After the shrimp had been introduced, diatoms dominated (Shaari et al., 2011). In contrast, Yusoff et al., (2002) found that diatoms were dominant at the early and middle stage of shrimp culture. Towards the end of the culture period, cyanobacteria were found to be dominating (Yusoff et al., 2002). The study of Yusoff was supported by the study of Casé et al. (2008) which also found a similar trend where diatoms were replaced by Cyanobacteria towards the end of the shrimp culture (Casé et al., 2008).

2.4.4 Outdoor versus indoor algae reactor (light and temperature)

From the RAS studies, the major differences between outdoor and indoor algae culture are the options to control light and temperature. Light is an important parameter that affects algae growth which correlates with the assimilation of nitrogen by algae. The saturation irradiance observed for many algae species was between 100 to 400 $\mu\text{mol photons per m}^2 \text{ per second}$ (Necchi Jr, 2004). However, light availability in the water is subject to water turbidity, therefore, even though sufficient light was provided, the light availability for algae might be restricted (Anthony Kenneth et al., 2004; Tait et al., 2014).

During summer when light irradiance is high, an outdoor culture system which received sunlight had a higher nitrogen removal rate than an indoor algae reactor (Fig. 1). On the other hand, the algae cultures are exposed to fluctuations in sunlight irradiance due to the day/night cycles and changes in weather conditions and seasons. A wide range of irradiance was reported between 46 to 1700 $\mu\text{mol photons per m}^2 \text{ per second}$ (Fig. 1 and Table 3). Quick changes in irradiance pose a high risk of culture collapse (Blanken et al., 2013; Brune et al., 2003).

Valeta and Verdegem (2015) applied artificial light with an intensity of 120 $\mu\text{mol photons per m}^2 \text{ per second}$. When artificial light is supplied, no fluctuation of light intensity occurs. Microalgae can use all the photons in the photosynthetic active radiation (PAR) which have a wavelength between 400 to 700 nm (Blanken et al., 2013). However, red light (660 nm) is the optimum light for the photosynthesis (Chen et al., 2010; Cuaresma et al., 2010). Therefore, by using artificial light for example LED light, the specific wavelength required can be supplied (Schulze et al., 2014). However, it is well accepted that the costs of artificial light for culturing algae is high. Blanken et al., (2013) reported that the cost of artificial light is 25.3 \$ per kg dry-weight biomass (during the time when the paper was published, 1.34 US dollars was equal to 1 €). From the point of biofuel production, this value would make the cost of algae production 25 times more expensive than using sunlight. This is due to the biofuel production which requires a cost under 1.3 \$ per kg dry-weight biomass (Slade and Bauen, 2013; Wijffels et al., 2010). Therefore, the lighting cost could be an issue and impede integration of algae in a RAS.

Temperature is important because it influences enzymatic reactions which occur during photosynthesis. With a 10°C temperature increase, the enzymatic reactions are doubled (Goldman Joel and Carpenter Edward, 1974), thus doubling the nutrient uptake by the algae. In an outdoor culture where temperature cannot be controlled, minimizing the temperature fluctuation is a challenge, especially in areas that experience drastic temperature fluctuations (Supp. Table 1). During winter, the water temperature can be low which results in a low nitrogen removal rate as observed in Pagand et al., (2000) and Deviller et al., (2004). Again, the advantage of an indoor reactor is that temperature can be controlled enabling a stable nitrogen removal all year round.

2.4.5 Effects of the algae cultivation method

There are two algae culture methods used in a RAS: namely suspended or attached. From the comparison given (Fig. 1) the method of cultivation did not seem to influence the nitrogen removal rate because of the interacting effects of other factors such as light and CO₂. Nonetheless, each method requires specific management, for example reactor preparation or mixing which have a direct impact on algae growth, thus the nitrogen removal rate by the algae. For the suspended culture, the preparation of the reactor is relatively simple with a simple pond or a tank as sufficient. The high rate algal pond (HRAP) term is used to describe the specific characteristic of the pond which is shallow (normally at 0.5 m depth) and intensively mixed (Benemann et al., 1977). Mixing through paddle wheels or aeration is provided to circulate the water in order to expose the algae cells to sunlight, the distribution of nutrients and to promote gas exchange (Brune et al., 2003). Reports on the use of suspended algae in an indoor algae reactor have not been found.

The attached culture method refers to an algal turf scrubber or periphyton turf scrubber (PTS), which used substrates to support the growth of algae mats (Azim et al., 2005). In ponds, vertical poles, e.g. bamboo, fixed at the bottom are often used as a substrate (Azim et al., 2002; Richard et al., 2010). The substrates provide additional surface area for algae growth (Asaduzzaman et al., 2010; Asaduzzaman et al., 2010; Rahman et al., 2008). Air lifts or paddle wheels are also used to keep the water column mixed. The pond depth is also shallow, ranging between 0.5 to 1.0 m. This is unlike the

suspension pond where microalgae can have equal exposure to light through proper mixing. The bottom section of the poles receives less light than close to the surface. Even so, the presence of substrates contributes to a large portion of autotrophic productivity by the periphyton community. Guiral et al., (1993) reported that a pond with a periphyton community indicated $7.9 \text{ g C m}^{-2} \text{ day}^{-1}$ productivity where this value was 4.5 times higher than a pond with a phytoplankton community. Also reported in Azim et al., (2002), periphyton counted for 50% of the total primary productivity in a fish pond. In an indoor RAS, a flat wire mesh can be used as substrate and horizontally laid to provide an optimum surface area for the algae mats (Valeta and Verdegem, 2015). For mixing, a tipping bucket which is located at the top section of the substrate is filled and emptied continuously to create waves over the substrate in order to move nutrients across the substrate and facilitate gas exchange.

The major disadvantage for these two methods is that to capture sufficient light to control the ammonia, a larger surface area is needed (Fig. 2). However, in many RAS, the surface area is of great concern. Since the surface area problem is not limited to the application of algae in a RAS, a recent innovation was made for the algae cultivation method where a solid-state biofilm method was applied (Naumann et al., 2013). The basic principle of this method is that algae were cultivated on vertically orientated twin layer modules which consisted of two ultrathin layers. The first layer is a macroporous layer where the algae culture medium passes through by the force of gravity, and the second layer is a microporous layer where the microalgae biofilm is attached (Naumann et al., 2013). The vertical arrangement of the biofilm substrates allows a more efficient use of the surface area and exposure to light (Cuaresma et al., 2010). Blanken et al. (2014) used a very similar approach by applying a microalgae biofilm on a rotating Algadisk, which was vertically positioned and placed in a liquid container. The disk rotated between the air (light) and water (dark) phase, and nutrients are supplied to the microalgae biofilm during the latter phase. When *Chlorella Sorokiniana* was cultured using the Algadisk method, an algae productivity of $20.1 \pm 0.7 \text{ gram per m}^2 \text{ disk surface per day}$ was observed. This productivity would be equal to the removal of $1 \text{ g N m}^{-2} \text{ day}^{-1}$ (using the estimation method used in Gál et al., (2003)) which is higher when compared to the nitrogen removal in the study of Valeta and Verdegem (2015) which had a removal rate of $0.66 \text{ g N m}^{-2} \text{ day}^{-1}$ when using an indoor PTS. The Algadisk concept is better than the PTS because of the optimum use of the surface area.

2.4.6 Effects of CO₂, O₂ and pH

Unlike light and temperature, the pH, carbon dioxide and oxygen values are directly affected by the rate of photosynthesis and respiration in a RAS. In a RAS, the pH could become the least of the problems for algae because the pH in a RAS is kept close to neutral for the fish culture. Normally in a RAS relying on nitrification, the pH is kept above 6 by supplying bicarbonate to compensate for the loss in alkalinity due to nitrification. As shown in Table 2, in all studies the pH was maintained between 6.5 and 8.4. By employing photosynthesis in a RAS, the annual amount of bicarbonate addition was reduced, in spite of low light irradiance during winter (Deviller et al., 2004). In the study by Pagand et al., (2000), the treated water had a higher pH level than the untreated water. The measurement was taken at midday when photosynthesis was at the highest rate. However, no pH was reported during dark hours, therefore the effect of pH on the algae during dark hours was unknown. Nonetheless, for a RAS set-up, the fish tank is separated from the algae tank and the pH in the fish tank is controlled. Therefore, the fluctuation of pH in the algae reactor has minimal effect on the fish.

A RAS is a highly aerated system to allow sufficient oxygen for fish and bacterial respiration. Diel oxygen fluctuations caused by photosynthesis as reported from pond systems are not encountered in an indoor RAS. The oxygen produced by algae could create super-saturation which could negatively affect algae growth. Chisti (2007) proposed that oxygen super saturation at 400% should be avoided, and is a point of attention when including an algae reactor in a RAS.

In addition, due to the highly aerated environment in a RAS, CO₂ insufficiency can become a serious problem for algae. Fish require oxygen which can be produced by the algae and in return, the CO₂ produced by fish respiration can be absorbed by the algae. Yet, there are very few quantitative data applied in RAS which demonstrate this synergism. The requirements of CO₂ by the algae and of oxygen by the fish should be a complementary process when algae are integrated in a RAS. The mass transfer of O₂ and CO₂ should be monitored to provide solid proof for the supposed mutual benefit and to develop management criteria which guarantee a suitable optimization of this synergetic effect.

2.4.7 Effects of hydraulic retention time

Generally, flow rates through fish tanks in a RAS are set to supply enough O₂ for the fish. Flow rates are also important to guarantee that solid and non-solid wastes (CO₂, total ammonia, dissolved organic carbon) are quickly transferred out of the culture tanks. This means that in general, short hydraulic retention times (HRT) prevail in the fish tanks of a RAS. For a culture tank less than 1 m³, a HRT of 10 minutes is quite normal but for culture tanks of more than 1 m³, an HRT of 30 minutes or more is needed (Timmons and Ebeling, 2007). In addition, the type of the solid removal system used in a RAS sets different requirements for the HRT for proper solid waste removal. Normally the longest HRT applied in solid waste removal systems or settling basins is 15 to 30 minutes (Liao and Mayo, 1974). Further, fluidized bed sand biofilters which use fine sand particles require a longer HRT than other bio-filtration systems. However, fluidized bed sand biofilters are not commonly used as most RAS are operated under a short HRT in the culture tanks. In contrast, algae reactors require a longer HRT for the algae to grow.

The HRT of an algae reactor influences the nutrients, CO₂ and O₂ transfer and therefore affects the algae growth rate (Inoue and Uchida, 2016). The applied HRT in the algae reactor will affect the gradients of nutrients, pH, CO₂, and O₂ along the reactor. A HRT that is too short will not ensure complete nutrient removal by the algae, whereas a HRT that is too long may cause starvation of the algae cells (Anbalagan et al., 2016; Larsdotter, 2006). The HRT of an algae reactor should not exceed the time required to maintain the growth rates of algae in the photobioreactor (Larsdotter, 2006). A HRT less than 0.5 days causes a washout of algae cells and a HRT of 2 to 3 days is recommended to obtain maximum biomass yield under 12 to 25 °C and 190 to 450 µmol m⁻² s⁻¹ (Takabe et al., 2016). In general, a relatively short HRT is normally used in algae reactors which might explain the low nitrogen removal rates achieved (Table 3). The HRT for the algae reactor will affect the size of the reactor. The longer the HRT, the larger the algal reactor required. Nonetheless, even for a short HRT, the size of the algae reactors used were similar or twice the size of the RAS (Table 3). The size of the algae reactor is expected to be one of the main factors influencing the farmers' choice of which type of algal reactor to install in their RAS.

2.5 Cost of algae production

Culturing microalgae using aquaculture wastewater has been found to be efficient (Guo et al., 2013; Venkatesan et al., 2006; Yusoff et al., 2001). In this way, the cost of nutrients and water for the algae can be eliminated. It was reported that the cost to produce microalgae using wastewater from a fish farm in a tubular photo bioreactor was 36€ kg⁻¹ dry weight¹ (Michels, 2015).

The current interest concerns how integration of microalgae in a RAS could affect the RAS total production cost. From Timmons et al., (2002), the cost of producing tilapia was 2.06 € kg⁻¹ (1.76 \$US kg⁻¹, 1 € = 1.17 US\$)². It was assumed that the tilapia was fed at 2.5% body weight per day, with the feed containing 32% crude protein. Therefore, for a 100 kg m⁻³ production, 2.5 kg feed would be given per day. This would produce 62 g ammonia-N day⁻¹, using the same assumptions as in Section 1. Considering that the nitrogen content in microalgae dry matter is 6% (Equation 1, Section 3), then 1033 g microalgae biomass is required to take up 62 g ammonia-N per day. For simplification, 1000 g (1 kg) microalgae dry weight is taken as the final value.

At a production cost of 2.06 € kg⁻¹, 206 € is needed to produce 100 kg tilapia. One kg microalgae is needed to assimilate all the ammonia-N, and the cost of microalgae production was 36€/kg dry weight. Therefore, the cost addition by microalgae is about 17.5% of the cost for producing tilapia. However, if artificial light is used the algae production cost increases by 23€ kg⁻¹, raising the cost of tilapia production in the RAS by 29%.

From the aspect of water use, based on a productivity of 0.3 g L⁻¹ day⁻¹ achieved by Michels (2015), then 1000 g algae dry weight would require 3333 liters (3.3 m³) of photo bio-reactor. Therefore, it can be summarized that 3.3 m³ of microalgae culture is needed to remove the ammonia-N produced by a 1 m³ culture tank in a RAS.

The advantageous effect of algae integration on the cost is dependent on the value of the microalgae. If the culture of a high value microalgae species can be realized in a RAS, the production

¹ For this estimation, microalgae were cultured in a tubular photo bioreactor (PBR) with the total area of 1000 m². Sunlight and minimum cost of temperature were used. The average microalgae productivity was 0.3 g L⁻¹ day⁻¹ at average biomass concentration of 0.7 g L⁻¹ and PAR at 11.8 mole m⁻² day⁻¹.

² The tilapia was produced in a RAS facility producing 590,000 kg tilapia per year. The stocking density applied was 100 kg m⁻³ (Timmons et al., 2002).

of microalgae will increase the total revenue of the RAS. In terms of water volume, adding three times the volume of the fish culture tanks to culture algae in a RAS raises system and production costs. Therefore, the percentage of nitrogen immobilized in the algal biomass might be reduced to the level that is economically acceptable. Nonetheless, technological advancement in algae cultivation is moving towards a higher algae productivity and cheaper cost. The same development is also occurring in a RAS. If cost reductions can be realized in algae systems and in RAS systems, then a cost-effective integration of an algae reactor in a RAS might become feasible.

2.6 Conclusion

Even though the role of microalgae is very significant for aquaculture, microalgae are generally studied under the domain of biotechnology, biofuel technology and waste water technology. Therefore, there is a huge gap between the application in aquaculture and technological advancements made for microalgae in the field of biotechnology. Nowadays, the application of microalgae in aquaculture mainly focuses on outdoor ponds. Less attention is given to the application of microalgae in a RAS. Algae should be part of a RAS so that the sustainability and health of a RAS will be improved. In order to improve nitrogen removal by algae in a RAS, the algae reactor performance has to be improved. From this review, the options to integrate an algal reactor in a RAS require a different approach than for biodiesel production or waste water treatment. The RAS configuration affects nutrient loading, nutrient composition, and nitrogen species availability in the algae reactor. Therefore, future research should focus on algae species selection and algae cultivation methods that match the conditions provided by the RAS. Finally, although currently cost might hinder the application of algae integration in a RAS, it is believed that future technological advancements in algae cultivation methods will make algae integration become more economically feasible.

Supporting information

Supp. Table 1 Rate of nitrogen removal by an algae reactor in a recirculating aquaculture system (RAS). The system configurations are detailed in Table 2.

System type	Cultured animal (stocking density, kg m ⁻³)	Feeding	Nitrogen loading rate, g m ⁻² day ⁻¹	Nitrogen removal rate, g m ⁻² day ⁻¹	NH ₄ -N, mg L ⁻¹	NO ₂ -N, mg L ⁻¹	NO ₃ -N, mg L ⁻¹	Standing crop, g wet weight m ⁻² (g wet weight m ⁻³)	Reference
Microalgae (indoor)									
RAS + Periphyton turf scrubber, (PTS) which comprise microalgae and other micro- organisms	Tilapia (28 - 70)	n.a	3.76 to 3.81 g TAN	0.302 to 0.656	1.58 to 3.21	0.55 to 1.28	80.5 to 217.3	77.14 ³	(Valeta and Verdegem , 2015)
RAS + PTS	Rainbow mussels, <i>Villosa iris</i> (245 animal m ⁻² , 17.3 mm length)	<i>Nannochlorop</i> <i>sis</i> (0.02 ml L ⁻¹) and mixture of <i>Isochrysis</i> sp., <i>Pavlova</i> sp., <i>Thalassiosira</i> <i> weissflogii</i> , and <i>Tetraselmis</i> sp. (0.007 ml L ⁻¹)	n.a	0.01	0.026	0.008	0.104	n.a	(Huang et al., 2013)

³ 1.08± 0.32 kg week⁻¹/7 day/2 m²

Microalgae (outdoor)									
RAS + outdoor PTS	Fish pond - common carp (15)	Commercial feeding in fish pond (40% CP)	3.8	1	negligible	negligible	negligible	34.72 (34.72)	(SustainAqua, 2009)
Combined Intensive - pond system	Periphyton pond - tilapia (0.5)								
	Extensive pond - 90% common carp and 10% Chinese carp (0.014 to 0.15)	Commercial feed (0.042 to 0.06 g N day ⁻¹)	0.87	0.26	0.9 to 6.02 (nitrogen)	n.a	n.a	60 ⁴	(Gál et al., 2003)
Intensive pond - common carp, African catfish and tilapia (0.07 to 3.9)									
Macroalgae (indoor RAS)									
RAS + Algae tank (<i>Ulva lactuca</i>)	<i>Halimotis iris</i> (4.45)	Commercial feeding (0.75% body weight) CP not mentioned, but 35% used from reference	0.1134 ⁵	*0.135	0.03±0.01	n.a	0.03±0.01	*1230 (4098) ⁶	(Cahill et al., 2010)
RAS + Algae tank (<i>Ulva pinnatifida</i>)	<i>Halimotis iris</i> (0.004)	Commercial feed (0.75% body weight)	*0.1134	*0.135	0.03±0.01	n.a	0.02±0.00	*2430 (8111.25) ⁷	(Cahill et al., 2010)

⁴ 2.4 to 2.6 g C m⁻² day⁻¹ (primary production)

⁵ when assuming that the depth of algae tank is 0.3 m (also for other information which is labelled with an *)

⁶ 21.64 g m⁻² day⁻¹ growth rate

⁷ 34.04 g m⁻² day⁻¹ growth rate

RAS + outdoor high rate algal pond, HRAP - Mix of microalgae and macroalgae species (<i>Ulva</i> sp., <i>Enteromorpha</i> sp. <i>Ectocarpus</i> sp.)	<i>Dicentrarchus</i> <i>labrax</i> L. (100)	Commercial feed (n.a) estimate CP=40%	1.6	0.9 (winter) 2.79 (summer)	0.05	n.a	0.06	2500 ⁸	(Pagand et al., 2000)
RAS + HRAP - Mix of microalgae and macroalgae species (<i>Ulva</i> , <i>Enteromorpha</i> and <i>Cladophora</i>)	<i>Dicentrarchus</i> <i>labrax</i> L. (10±2; initial density, 82±22; final density)	Commercial feed, 44 to 52% protein (1.5% body weight)	2	1.0 (0.5 ± 0.2 g N m ⁻² day ⁻¹)- summer (0.09 g N m ⁻² day ⁻¹) winter	0.34±0.12	0.13±0.05	14.5±5.1	455 to 2727 ⁹ (909- 5454)	(Deviller et al., 2004; Metaxa et al., 2006)

⁸ 3.3 kg m⁻² year⁻¹ of algal dry weight were harvested

⁹ HRAP was restocked between 0.1 and 0.6 g of dry weight per litre (g DW L⁻¹) weekly in summer and autumn and biweekly in winter and spring

Supp. Table 2 System configuration of a recirculating aquaculture system (RAS) integrated with algae. The rates of nitrogen removal of algae reactors are shown in Table 1.

Reference	RAS configuration	System flow rate, L min ⁻¹	Plant tank flow rate, L min ⁻¹	Hydraulic retention time, HRT, (day)	Algae tank surface area, m ² (volume, m ³)	Algae reactor/ fish tank or pond ratio	Study duration, days	T, °C	pH	Animal tank's dissolved oxygen, mg L ⁻¹	Light intensity for plant (photoperiod, L:D), $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$
Microalgae											
RAS - Periphyton turf scrubber, (PTS) (Valeta and Verdegem, 2015)	Fish tank - PTS - sump	6.7	6.7	0.002	2 m ² (0.02 m ³)	n.a	49	27 to 28	7.6±0.3	6.15±0.52	120.68 (18:6)
RAS - PTS (Huang et al., 2013)	Mussel trough-sump/biofilter - PTS-	23.3	n.a	n.a	0.36 m ² (n.a)	n.a	91	22.1±1.6	8.72±0.3	8.4±1.6	Day- natural light intensity mentioned) Night- 1034
RAS - PTS pond (SustainAqua, 2009)	Fish pond-sedimentation pond - periphyton pond	900	434	1.6	1000 m ² (1000 m ³)	3:1	180	n.a	n.a	n.a	n.a
Combined Intensive-extensive RAS (Gál et al., 2003)	Outdoor pond system	23148	60	-	20000 m ² (200000 m ³)	13.5:1	1095				n.a
Macroalgae											
<i>Ulva lactuca</i> (Cahill et al., 2010)	Fish tank - alga tank	10	10	(0.003)	(0.04 m ³)	1:1	14	9.04±0.22	8.4	n.a	207 to 782
<i>Ulva pinnatifida</i>	Fish tank - alga tank	10	10	(0.003)	(0.04 m ³)	1:1	14	9.04±0.22	8.4	n.a	207 to 782

Chapter 3

Removal of ammonia and nitrate in recirculating aquaculture systems by *Stigeoclonium nanum*, a fresh water filamentous epiphyte, immobilized in alginate beads

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Abstract

Incorporation of microalgae in recirculating aquaculture systems (RAS) would absorb the inorganic nitrogen and phosphorus, thus potentially contributing to water purification. Immobilization or entrapment of microalgae cells in spherical gels is a potential method to incorporate microalgae in the RAS. Filamentous microalgae are presumed to suit the immobilization technique because the gels can serve as substrates for the microalgae to attach. In the first experiment of this study, growth and nitrogen uptake of *Stigeoclonium nanum*, a filamentous microalga, was compared when cultured using an immobilization technique or in a normal suspension. In the second experiment, immobilized *S. nanum* was cultured in 4 media with different total ammonia nitrogen (TAN) and nitrate-nitrogen ($\text{NO}_3\text{-N}$) concentrations. The results showed a significantly higher algal growth and TAN removal by *S. nanum* immobilized in alginate than for *S. nanum* in free suspension culture. When both TAN and $\text{NO}_3\text{-N}$ were added to the culture medium, the uptake of TAN by immobilized *S. nanum* was significantly more efficient than $\text{NO}_3\text{-N}$ uptake. Our results indicated that *S. nanum* was able to grow in an immobilized medium, exhibiting a higher growth and TAN uptake than when the algae were in free suspension. *Stigeoclonium nanum* preferred ammonium over nitrate, which is suitable for the RAS that requires removal of the total ammonia which is produced by fish and by organic decomposition in the system.

3.1 Introduction

A recirculating aquaculture system (RAS) is a more sustainable aquaculture practice than a flow through system considering that the waste discharge into the environment can be better controlled, the volume of water used per kg fish produced can be optimized, the biosecurity can be increased and reliance on disinfectants can be reduced (Martins et al., 2010; Piedrahita, 2003; Verdegem et al., 2006). Water purification in RAS works by removing solid and metabolic wastes which originate from uneaten feed, fish fecal and non-fecal metabolic losses. Total ammonia nitrogen (TAN: ammonia, NH_3 + ammonium, NH_4^+) is a toxic nitrogenous metabolic waste material. It is removed by nitrification, during which ammonia is converted to nitrite and subsequently to nitrate. Therefore, nitrate accumulation becomes a common problem in RAS. Since nitrate is a fish growth inhibiting substances (Davidson et al., 2014; vanRijn et al., 2006) nitrate must be removed to maintain optimal water quality in RAS. The most common method of nitrate removal is by partial water exchange, however, this method is not sustainable because nitrate discharge will pollute the environment (Martins et al., 2009; vanRijn et al., 2006).

Because microalgae are capable of absorbing ammonium and nitrate, they are used widely in wastewater treatment. (Abdel-Raouf et al., 2012). Dortch (1990) explained that ammonium would be directly assimilated into amino acids, and thus would be taken up by algae more efficiently and with more energy savings than nitrate. More energy is needed for nitrate reduction to nitrite and subsequently to ammonium compared to direct uptake of ammonium by algae (Needoba et al., 2004; Perez-Garcia et al., 2011). However, preference for ammonium or nitrate might also occur as a result of genetic and environmental conditions such as light, carbon and heavy metal presence (Podevin et al., 2015; Raven et al., 1992).

Water quality in RAS is mainly controlled by heterotrophic and autotrophic bacteria (Ebeling et al., 2006) and the application of microalgae in RAS is limited. One example is the use of an algal turf scrubber (ATS), relying on a periphytic biofilm community including bacteria, microalgae, fungi and protozoans, to maintain water quality in RAS (Valeta and Verdegem, 2015). However, an ATS requires large surface area to perform efficiently. Therefore, there is a need to find another approach which could increase the efficiency in terms of nutrient uptake and space utilization. Thus, introducing microalgae

into the RAS by immobilizing the cells in spherical gels could be a suitable way to improve water quality and fish production. The use of small gels containing algae would increase the surface area for absorption and increase the uptake rate. Furthermore, this immobilization technique will reduce the risks of contamination of the biofilter by microalgae and reduce risk of clogging pipes in RAS.

Immobilization or entrapment of microalgae cells in spherical gels is used in the wastewater industry to ease the harvesting method (de la Noüe and Proulx, 1988; Travieso et al., 1992). To date, about 30 species of microalgae have been studied using this immobilization technique for removing nitrogen and phosphorus in wastewater (de-Bashan and Bashan, 2010). Of the studied species, 75% were green microalgae, e.g. *Chlorella*, *Scenedesmus*, and *Botryococcus*, 20% were cyanobacteria, e.g. *Anabaena* and *Spirulina* and the remaining 5% were brown microalgae, e.g. diatoms and euglenoid microalgae. Most of the species tested were planktonic microalgae. Reports on the use of epiphytic microalgae are few, not only in immobilized technique studies but also in wastewater treatment. According to de Paula Silva *et al.* (2008), one reason to explain why epiphytic microalgae were less studied might be because their economic values were unclear. However, the application of epiphytes such as *Cladophora coelothrix* Kützinger and *Chaetomorpha indica* Kützinger in Northern Queensland, Australia, proved that epiphytic microalgae were effective in removing inorganic nitrogen and phosphate from aquaculture waste (de Paula Silva et al., 2008). In the case of the RAS, epiphytic microalgae on substrates posed lesser risks of clogging and could be easily removed from the systems *in-situ*.

Previous studies reported that the epiphytic green microalgae *Stigeoclonium* sp. can be an indicator of highly contaminated water because this species has a high tolerance to heavy metals (Pawlik-Skowronska, 2001). This implies that *Stigeoclonium* sp. can be potentially used to remove nutrients from the wastewater. Moreover, this species also has a high lipid content, making it a potential food or biofuel source (Praveenkumar et al., 2012).

Motivated by these benefits, we explored the suitability of culturing *Stigeoclonium nanum* immobilized in alginate beads and its inorganic nitrogen uptake. First we compared the growth and ammonium uptake by *S. nanum* in free suspension to that of *S. nanum* immobilized in alginate. Then we analyzed the rates of ammonium and nitrate uptake in immobilized *S. nanum* beads.

3.2 Material and methods

3.2.1 Microalgae culture maintenance

Stigeoclonium nanum was isolated from a tilapia grow-out tank in the Aquatic Animal Health Unit, Faculty of Veterinary Medicine, and Universiti Putra Malaysia. The pure culture was maintained in Bold's basal medium, maintained in 24 hours light ($55\text{--}60\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$) at $26 \pm 0.5\ ^\circ\text{C}$ and a pH of 8.0 ± 0.5 . Low light conditions are suitable for this epiphytic species, which is thus a candidate for RAS operated under relatively low light conditions. In nature, *S. nanum* has an affinity for low light and is commonly found on substrates in highly shaded forest streams (Steinman, 1992).

For immobilization of *S. nanum*, a pure culture of *S. nanum* was inoculated in 3% sodium alginate solution. Round beads approximately 3 mm in diameter were produced in 2% calcium chloride solidification solution as an ionic cross-linking agent. One ml of sodium alginate solution produced 30 ± 2.8 beads weighing 0.952 ± 0.03 g. Before the beads were used, they were acclimatized in the culture water (before the addition of ammonium and nitrate) at the experimental light and temperature for 3 hours.

3.2.2 Experimental design and procedures

Two experiments were carried out to illustrate the effects of immobilized microalgae in alginates in a RAS system. The first experiment compared growth and total ammonia nitrogen (TAN) uptake by free-living and immobilized microalgae. The negative control for the free-living treatment contained only culture water while the negative control for immobilized microalgae contained alginate beads without microalgae.

In the second experiment, a 2×2 factorial design was used to determine the uptake of TAN and nitrate-nitrogen ($\text{NO}_3\text{-N}$). The treatments were; 1) TAN concentration = $0\ \text{mg l}^{-1}$, $\text{NO}_3\text{-N}$ concentration = $0\ \text{mg l}^{-1}$ (T0N0); 2) TAN = $5\ \text{mg l}^{-1}$, $\text{NO}_3\text{-N}$ = $0\ \text{mg l}^{-1}$ (T5N0); 3) TAN = $0\ \text{mg l}^{-1}$, $\text{NO}_3\text{-N}$ = $10\ \text{mg l}^{-1}$ (T0N10); and 4) TAN = $5\ \text{mg l}^{-1}$, $\text{NO}_3\text{-N}$ = $10\ \text{mg l}^{-1}$ (T5N10). Experiments were conducted with 3 replicates each.

The experiments were carried out under 55-60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light at 26 ± 0.5 °C and a pH of 8.0 ± 0.5 under 24 hours light per day. The cultures were aerated by continuous bubbling of sterile air. The culture medium was lake water that was filtered and autoclaved before use.

3.2.3 Experiment 1

An initial concentration of 0.5 g l⁻¹ *S. nanum* (wet weight) was inoculated in 1200 ml medium as free-living *S. nanum*. In the immobilized beads treatment, 200 g microalgae beads were used which also contained 0.5 g l⁻¹ *S. nanum*.

A microalgae culture medium containing 2.0 mg l⁻¹ of TAN was prepared using an ammonium chloride stock solution (3.819 g NH₄Cl in 1 l of ultrapure water; 1 ml = 1 mg N). The TAN level was determined on alternate days for 20 d. Every time the TAN level reached 0 mg l⁻¹, the stock solution was added to raise the concentration to 2.0 mg TAN l⁻¹. Beads (6 g) from the immobilized microalgae treatments and 10 ml from the free-living treatments were sampled every 4 d to determine chlorophyll-a content and microalgal biomass.

3.2.4 Experiment 2

In experiment 2, the same experimental conditions as in experiment 1 were applied. The TAN stock solution was prepared as in experiment 1. NO₃-N stock solution was prepared using potassium nitrate (0.7218 g KNO₃ in 1 l of ultrapure water; 1 ml = 100 $\mu\text{g NO}_3\text{-N}$). Concentrations of TAN and NO₃-N in culture water were measured daily. The experiment lasted for 6 d.

3.2.5 Algal growth rate and total ammonia nitrogen and nitrate measurement

For microalgal biomass (g l⁻¹ dry weight) determination in the bead treatment, 3 g of beads were solubilized by immersing them in 10 ml of 0.5 mol trisodium citrate solutions (pH 6.5). Microalgal cells were then filtered on prewashed GF/F Whatmann filter paper and dried overnight at 60 °C. For

free-living microalgae, 5 ml of culture medium were filtered. The mass difference between prewashed filter paper and filter paper with oven-dried microalgae was recorded as the biomass (g) of the microalgae. The specific growth rate (day^{-1}) was calculated from the exponential growth phase of the microalgae $(\ln W_1 - \ln W_0)/\Delta t$; where W_0 is biomass of microalgae at the beginning of time interval, W_1 is the biomass at the end of the time interval, and Δt is the length of the time interval ($T_1 - T_0$). The growths of free-living and immobilized microalgae were fitted with a logistic growth model using the non-linear regression function in SPSS. The formula for the logistic growth model is

$$P = \frac{K}{1 + Ae^{-rT}}; P \quad (1)$$

Where P is the population of microalgae; K is the carrying capacity; A is a constant; r is the intrinsic growth rate and T is time in days.

Chlorophyll-a was determined following the standard method for the examination of water and wastewater (APHA, 1999). Beads (3 g) were solubilized in 0.5 molar trisodium citrate at room temperature. Microalgal cells in the solubilized beads were then retained on GF/F Whatmann filter paper using a filtration unit attached to a vacuum pump. After filtration, chlorophyll-a pigment was extracted by mechanical disruption in 10 ml of a 90% acetone solution using a tissue grinder until it was converted to the slurry. The solution was allowed to stand overnight at 4 °C. The clarified extract was then left until it reached room temperature. Then, 3 ml of extract were transferred to a 1 ml cuvette and absorbance was read at wavelength of 750 and 664 nm (before acidification) and 750 and 665 nm (after acidification) with a spectrophotometer (UV-Vis Spectrophotometer, UV-1700 series, Shimadzu). Chlorophyll-a is expressed in $\mu\text{g l}^{-1}$.

The pigments were calculated using the equation:

$$\text{Chlorophyll } a (\mu\text{g l}^{-1}) = \frac{26.7 (664_b - 665_a) \times V1}{V2 \times L} \quad (2)$$

where 26.7 is the absorbance coefficient used for chl a at 664 nm (11.00) multiplied by the ratio expressing the correction for acidification (2.43); $V1$ is the volume of extract (ml), $V2$ is the volume of

sample (l), L is the light path length or width of the cuvette (cm), and $664b$, $665a$ are the absorbance of 90% acetone extract before and after acidification, respectively.

The TAN and $\text{NO}_3\text{-N}$ concentrations (mg l^{-1}) were measured by ion chromatography. All chromatographic analyses were performed at room temperature using a Metrohm model 882 Compact IC Plus (Metrohm, Herisau, Switzerland) with suppressor module. Data were collected using a data acquisition system interfaced to a computer running MagIC Net 1.1 software (Metrohm).

In this study, we defined removal of TAN and $\text{NO}_3\text{-N}$ by microalgae as their disappearance from the culture medium (Dortch, 1982).

3.2.6 Statistical analysis

A one-way ANOVA was used in the first trial, with culture method (presence/absence of alginate beads) as the main factor and sampling day as the repeated measure. In the second experiment, addition/no addition of TAN and NO_3 were the main factors, and sampling day was included as repeated measure factor. Statistical software SPSS (IBM SPSS Statistics, version 20) was used for the analyses.

3.3 Result

3.3.1 Experiment 1

Algal growth in free-living and immobilized microalgae beads (Stigeoclonium nanum)

A significant difference in algal biomass and chlorophyll-a content were observed between free-living and immobilized microalgae throughout the experiment ($P < 0.001$) (Table 1). The specific growth rate of the immobilized microalgae was significantly higher than that of the free-living microalgae ($P < 0.05$). The growth curve (Fig. 1) showed that the lag phases for immobilized and free living microalgae lasted until Day 4. Growth rates were exponential from Day 4 through Day 12 for immobilized microalgae and from Day 4 through Day 8 for free living microalgae. The logistic growth model could explain 73% of the growth for immobilized microalgae and 71.4% for free living microalgae. The chlorophyll-a content in immobilized microalgae was 10 times greater than in free-living microalgae (Fig. 2). Chlorophyll-a content in immobilized microalgae increased until day 20 from $140 \mu\text{g l}^{-1}$ to $5900 \mu\text{g l}^{-1}$, whereas chlorophyll-a content in free-living microalgae increased from $.200 \mu\text{g l}^{-1}$ to $620 \mu\text{g l}^{-1}$.

Table 1 Mean \pm SE of biomass, chlorophyll *a* content, and specific growth rate (SGR) of free-living and immobilized microalgae *Stigeoclonium nanum* in Expt 1. An initial concentration of 0.5 g l^{-1} *S. nanum* (wet weight) was inoculated in 1200 ml medium as free-living algae. In the immobilized beads treatment, 200 g microalgae beads were used which also contained 0.5 g l^{-1} *S. nanum*. Sampling occurred every 4 d for 20 d. Asterisks indicate significant differences between the methods; *** $p < 0.001$, * $p < 0.05$. DW: dry weight

Culture method	Biomass (g l^{-1} dry weight)	Chlorophyll-a ($\mu\text{g l}^{-1}$)	Specific growth rate (day^{-1})
Free-living microalgae	0.10 ± 0.01	300 ± 35	0.23 ± 0.06
Immobilized microalgae	0.23 ± 0.01	3240 ± 43	0.40 ± 0.06
Significant difference	***	***	

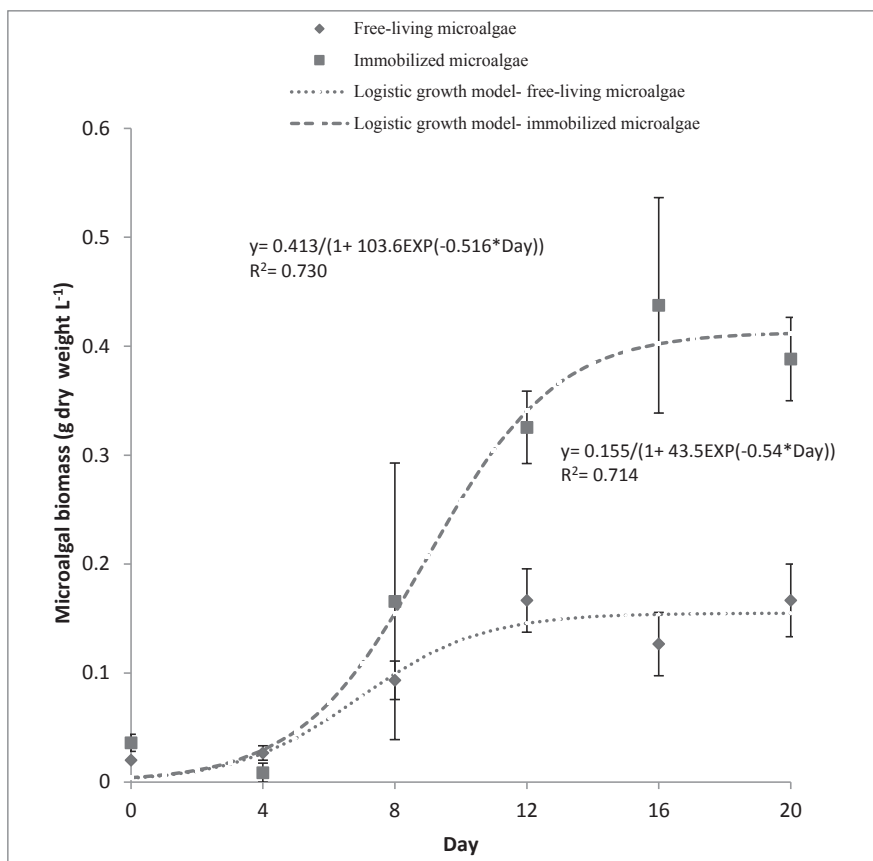


Fig 1 Biomass (g dry weight l⁻¹, mean ± SD) of free-living and immobilized microalgae *Stigeoclonium nanum*. Dotted lines indicate the logistic growth regression curves obtained in this study.

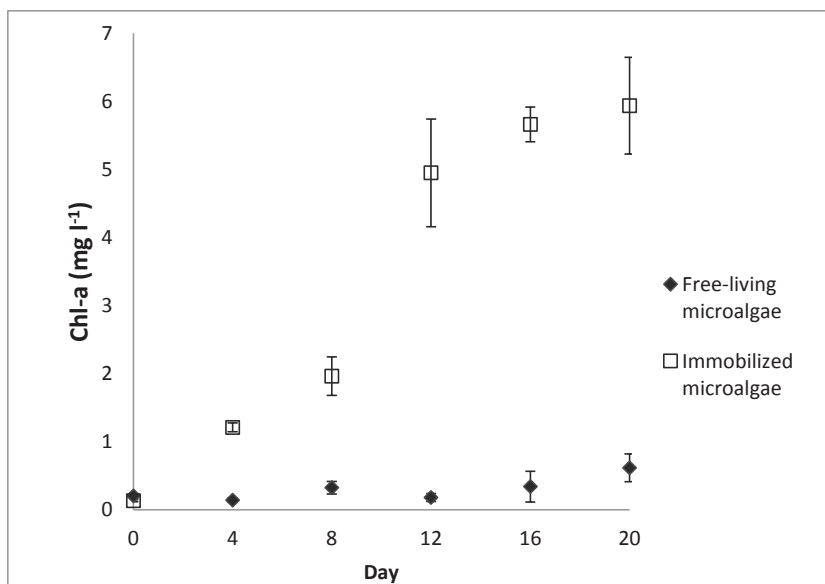


Fig 2 Chlorophyll-a ($\mu\text{g l}^{-1}$) content (mean \pm SD) in free-living and immobilized microalgae *Stigeoclonium nanum*.

TAN removal by free-living and immobilized microalgae

The slope of the graph in Fig. 3 represents the TAN removal rate ($\text{mg l}^{-1} \text{ day}^{-1}$). A higher TAN removal rate was achieved by immobilized microalgae ($0.68 \text{ mg l}^{-1} \text{ day}^{-1}$) than by free-living microalgae ($0.38 \text{ mg l}^{-1} \text{ day}^{-1}$; $P < 0.05$)

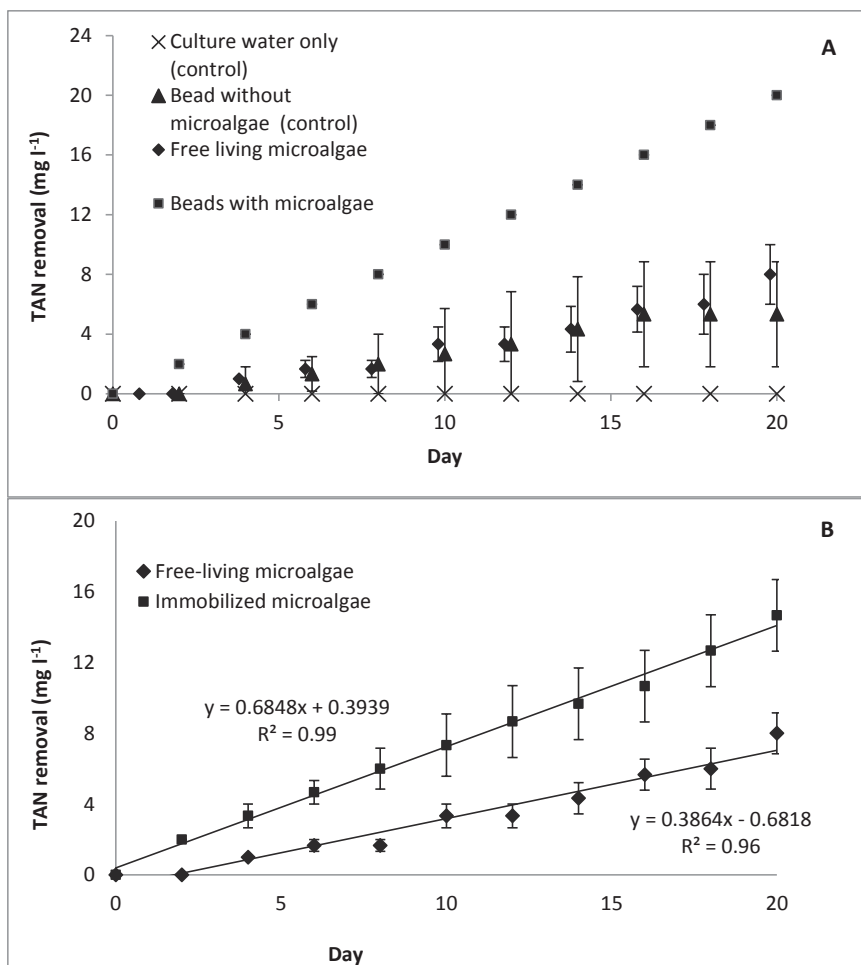


Fig 3 (A) Mean (\pm SD) values of cumulative total ammonia nitrogen (TAN) removal (mg l^{-1}) in culture water only (control), in beads without microalgae *Stigeoclonium nanum* (control), in free-living microalgae and in beads with immobilized microalgae during the experiment; (B) Mean (\pm SD) values of cumulative TAN removal (mg l^{-1}) in free living and immobilized microalgae (= beads with microalgae – beads without microalgae)

3.3.2 Experiment 2

The $\text{NO}_3\text{-N}$ removal rate in treatment T0N10 was $0.53 \text{ mg NO}_3\text{-N l}^{-1} \text{ day}^{-1}$ ($R^2 = 0.98$) and negligible in treatment T5N10 (Fig. 4A). The removal rate which was shown by the negative slope was $0.51 \text{ mg TAN l}^{-1} \text{ day}^{-1}$ ($R^2 = 0.86$) in treatment T5N0 and $0.67 \text{ mg TAN l}^{-1} \text{ day}^{-1}$ ($R^2 = 0.92$) in treatment T5N10 (Fig. 4B). T5N10 had higher TAN removal at the end of the study (83%) when compared to T5N0 (70%). The interaction between TAN and $\text{NO}_3\text{-N}$ factor was significant (Table 2).

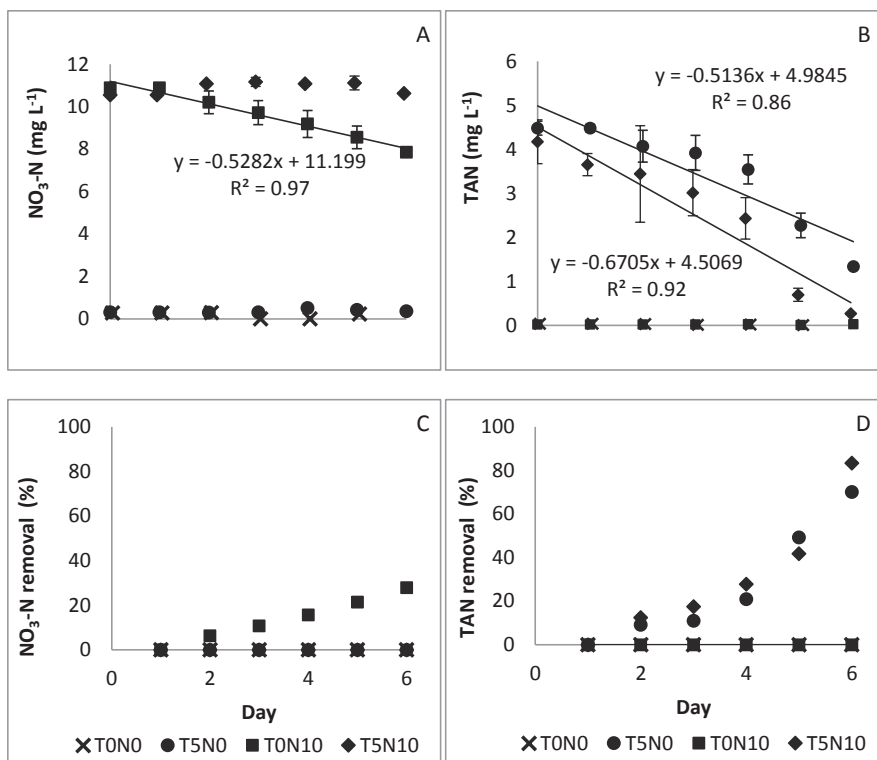


Fig 4 Mean (\pm SD) values of **(A)** nitrate-N (NO₃-N) and **(B)** total ammonia nitrogen (TAN) concentration (mg l⁻¹) in the culture water of immobilized *Stigeoclonium nanum*, **(C)** nitrate-N (NO₃-N) and **(D)** TAN expressed in cumulative percentage removal (%). The treatments were T0N0: both TAN and NO₃-N concentration = 0 mg l⁻¹; T5N0: TAN = 5 mg l⁻¹, NO₃-N = 0 mg l⁻¹; T0N10: TAN = 0 mg l⁻¹, NO₃-N = 10 mg l⁻¹; and T5N10: TAN = 5 mg l⁻¹, NO₃-N = 10 mg l⁻¹

Table 2 Two-way repeated measure ANOVA of total ammonia nitrogen (TAN), and nitrate (NO₃-N) concentration (mg L⁻¹) in the culture water of immobilized *Stigeoclonium nanum*, comparing between factors TAN, NO₃, and day. Values shown are p-values

Parameters	TAN	NO ₃	TAN x NO ₃	Day	Day x TAN	Day x NO ₃	Day x TAN x NO ₃
TAN	<0.0001	0.024	0.026	<0.0001	<0.0001	0.982	0.978
NO ₃ -N	0.002	<0.0001	0.010	0.002	0.003	0.003	0.055

3.4 Discussion

3.4.1 Experiment 1

Algal growth in free-living and immobilized microalgal beads

We found that immobilized *S. nanum* had 2.3 times greater biomass and 10 times greater chlorophyll-a content than the free-living *S. nanum*. At the beginning of this study, *S. nanum* was expected to grow well in low light, but growth was similarly low as in other microalgal species under the same light conditions (Imaizumi et al., 2014). For comparison, Imaizumi et al. (2014) reported that *Chlorella zofingiensis* showed a high growth rate of 0.7 day^{-1} at $1000 \mu\text{mol photon m}^{-2}\text{s}^{-1}$, but the growth rate decreased to 0.4 day^{-1} at $75 \mu\text{mol photon m}^{-2}\text{s}^{-1}$. That study reported the maximum production of *C. zofingiensis* cultured under non-limiting nutrient and carbon dioxide conditions. The growth rate of *S. nanum* in our study was comparable to that of *C. zofingiensis* in Imaizumi et al. (2014) under low light conditions. Microalgal production is a critical parameter for the uptake of ammonium, so it may be that if the beads were cultured in high light intensity, the growth rate would increase, and ammonium uptake would also increase.

This finding was similar to other studies which reported that immobilization did not negatively affect growth of the microalgae *Synechococcus elongatus* (Aguilar-May and Sánchez-Saavedra, 2009), *Chaetoceros gracilis*, *Thalassiosira pseudonana*, *Phaeodactylum tricornutum* (Moreno-Garrido et al., 2005), and *Dunaliella salina* (Thakur and Kumar, 1999). One study with *Chlorella vulgaris* immobilized in carrageenan showed a two times higher chlorophyll synthesis rate when compared to free-living microalgae (Lau et al., 1998). In addition to better growth, immobilized *Botryococcus braunii* and *B. protuberans* produced 8% more chlorophyll, 15% more carotenoids, 15% higher dry weight, and 7% more lipid during stationary growth phases in comparison to free-living cells. In addition, photosynthesis in both species was enhanced and senescence was significantly delayed under immobilized conditions (Singh, 2003). In other studies, chlorophyll content of harvested immobilized *S. elongatus* was 50% higher than that of free cells (Aguilar-May and Sánchez-Saavedra, 2009).

Scenedesmus sp. immobilized in chitosan was found to have 2.6 higher growths than the free cell cultures (Fierro et al., 2008).

Not all microalgae grow well under immobilized conditions, for example, *Skeletonema costatum* (Moreno-Garrido et al., 2005), *Heterocapsa* sp. and dinoflagellates (Moreno-Garrido, 2008). Immobilization changes the nature of algal growth. When microalgae are confined in a limited space, interactions occurring between the immobilization matrix and the cell wall affected algal metabolism (Moreno-Garrido, 2008). Characteristics of alginate such as alginate chemistry, mechanical and chemical stability, pore size, and pore distribution influenced algal growth (Thu et al., 1996). Alginate consists of a family of copolymers which contain 1–4-linked β -D-mannuronic acid and α -L-guluronic acid in different proportions and sequences (Martinsen et al., 1989). High content of guluronic acid contributes to high gel strength, volume stability and large pore size which permits high permeability. These characteristics are advantageous for immobilization of living cells (Martinsen et al., 1989; Thu et al., 1996). Additionally, alginate did not cause extreme physical – chemical changes during the immobilization process which is an advantage of using alginate and makes it one of the most used polymers for cell immobilization (Moreno-Garrido, 2008). The carrying capacity (K) and intrinsic growth rate (r) calculated with the logistic growth model were higher for immobilized microalgae than for free-living microalgae. Carrying capacity for microalgal growth is normally determined by nutrient content and environmental factors in the culture, such as light and carbon dioxide. In this experiment, in which nutrient and environmental factors were kept the same between treatments, the higher carrying capacity was due to immobilization in alginate. Some microalgae attached on the flask wall during the early growth phase of *S. nanum* in free suspension. With increasing biomass, detached microalgae formed floating mats at the surface. In the immobilized microalgae treatment, the beads must have acted as a substrate for the microalgae to grow and contributed to the carrying capacity in this treatment. This might be the explanation why *S. nanum* was able to grow well in immobilization beads. At the end of the experiment, the microalgae protruded out of the beads, overgrowing the bead's surface, but did not switch to free-living conditions. This situation could be beneficial to ease the harvesting even in those beads in which microalgae grew out from the spherical beads.

Ammonium removal by free-living microalgae and immobilized microalgae

In immobilized microalgae culture, ammonium removal is defined as the adsorption of the nutrient from the external medium into the alginate and the uptake of the nutrient from the alginate into the microalgal cells (Tam and Wong, 2000). Ammonium removal in the culture media might also be caused by nitrification or by ammonia volatilization. Therefore, in this study, a control treatment which contained only culture water was used to account for nitrification and volatilization in the water column. The blank bead control treatment was used to account for nitrification, volatilization of ammonia and adsorption of ammonium by the alginate. After the control treatment has taken into account, this study showed that microalgal cells in beads removed 46% more ammonium than microalgae cells in free-living culture. This result was in accordance with the higher growth that was achieved in the microalgae beads. Similarly, Lau et al. (1997) suggested that ammonium consumption was dependent on the metabolic activity of the algal cells even in an immobilized state. In that study, Lau et al. (1997) compared the growth of *Chlorella vulgaris* in free suspension, immobilized in alginate and carrageenan. Higher metabolic activity was indicated by the higher chlorophyll which was correlated with the higher uptake of nitrogen and phosphate in the immobilized beads than in the free living *C. vulgaris*.

In our study, immobilized *S. nanum* consumed 19.54 ± 0 mg ammonium per 1200 ml flask on the final day of the trial. Free floating *S. nanum* only used 40% (7.84 ± 2.61 mg) and empty beads used 35% (7.02 ± 2.34 mg) of the total amount that immobilized *S. nanum* had used. This trend was similar to the result of a previous study where blank chitosan beads were responsible for removing up to 20% nitrate and 60% phosphate from the culture medium (Fierro et al., 2008). Uptake by the gel matrices could be explained by the fact that polyanionicity of the polysaccharide gels could bind with ammonium in a saturable and mass balance manner (Lau et al., 1997).

In a review by de-Bashan & Bashan (2010), higher ammonium uptake was observed in most immobilized microalgae than suspended microalgae. When *C. vulgaris* immobilized in carrageenan and alginate were used to treat primary domestic wastewater, over 95% of $\text{NH}_4^+\text{-N}$ was removed in three days. However, only 50% of $\text{NH}_4^+\text{-N}$ was removed by suspended microalgae during the same time period (Lau et al., 1997).

De la Noüe & Proulx (1988) found that chitosan-*Phormidium* sp. aggregates were capable of removing 95% of inorganic nitrogen from a secondary effluent within 4-6 hours. Meanwhile, ammonium uptake by immobilized *Dunaliella salina* was 17 mg l⁻¹ h⁻¹ if compared to free-living *D. salina* which only had 14.5 mg l⁻¹ h⁻¹ (Thakur and Kumar, 1999).

3.4.1 Experiment 2

Selective removal of ammonium and nitrate ions can be defined as the preference of microalgae for ammonium ions and inhibition of nitrate uptake in the presence of the former (Dortch, 1990). The latter study concluded that the uptake competition between nitrate and ammonium ion is complex and influenced by environmental conditions.

Our study showed that nitrate removal occurred in treatment T0N10 but not in treatment T5N10, indicating that immobilized *S. nanum* preferred ammonium above nitrate as the nitrogen source. The significant interaction found in this study might indicate that the presence of ammonium influenced the removal of nitrate. Past studies reported that some microalgae preferred ammonium above nitrate as the nitrogen source (Domingues et al., 2011; Dortch, 1990; Parker et al., 2012; Raven et al., 1992). Dortch (1990) listed microalgae which prefer ammonium, including *Chlamydomonas pulsatilla*, *Phaeodactylum tricornutum* and *Chaetoceros gracilis*. A more recent study also showed that *Nannochloropsis* sp. prefers ammonia above nitrate (Hii et al., 2011). In contrast to this, another study found that *C. vulgaris* preferred nitrate above ammonium (Podevin et al., 2015). However, we found no specific report on *S. nanum*. Domingues *et al.* (2011) reported that preference of ammonium was mainly observed in green microalgae and cyanobacteria but not in diatoms and dinoflagellates.

When both ammonium and nitrate are present, ammonium will be used first by the microalgae and inhibit nitrate uptake (Cordoba et al., 1986; Dortch, 1990; Hii et al., 2011; Ohmori et al., 1977; Serra et al., 1978). A possible explanation is that when ammonium enters the cell at a high rate, strong membrane depolarization occurs which blocks the anion/H⁺ co-transport (Flynn, 1991). On the other hand, during ammonium assimilation, glutamine synthetase (GS), an enzyme which is involved in the

ammonium metabolism in microalgae cells, is active. GS competes with the nitrate uptake systems for adenosine triphosphate. This competition may cause inhibition of nitrate uptake (Ohmori et al., 1977).

Knowing the removal rate of ammonium and nitrate is important to be able to predict the time needed to remove these compounds from the RAS. In this study, the removal rate, as predicted by linear regression in Fig. 4B showed that ammonium removal rate was higher in cultures where both ammonium and nitrate were present than in a culture where only ammonium was present. Therefore, a higher removal percentage of ammonium was achieved when both ammonium and nitrate were present. Raven et al., (1992) mentioned a situation where a higher growth rate was achieved when both ammonium and nitrate were available compared to when only ammonium or nitrate was available, however, this situation did not occur frequently. Therefore, in our study, we speculated that the higher growth rate could link to higher ammonium uptake when both nutrients were available. However, a difference in growth was not observed in our study (data not shown) probably due to a short experimental period. Until further research is done, this finding remains inconclusive.

Finally, our results suggest that immobilized *S. nanum* is a suitable candidate to be incorporated in a system in which ammonia is produced daily, as in aquaculture systems; however, if *S. nanum* is incorporated for nitrate removal in RAS, an *S. nanum* reactor should be placed after the nitrification reactor when all ammonia has been converted to nitrate, in order to reduce the inhibition of nitrate uptake by the ammonium. Furthermore, the flow into an *S. nanum* reactor should be regulated independently from the nitrifying reactor to allow for a higher retention time and thus a more efficient nitrate uptake by the *S. nanum*. Future studies should investigate how the stability of the beads is influenced by aquaculture water conditions. Stability in this case is related to the time before disruption of the alginate. The information is important to predict the life span of the beads. In this way, the time to harvest and beads replacement will be known, allowing for continuous nitrogen removal by the immobilized microalgae in RAS.

Chapter 4

Effects of *Stigeoclonium nanum*, a fresh water periphytic microalga on water quality in a small-scale recirculating aquaculture system

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Abstract

Recirculating aquaculture systems (RAS) are becoming important for aquaculture due to land and water supply limitations, and due to their low environmental impact. Bacteria are important in RAS as their role in nutrient recycling has been the main mechanism for waste removal in these systems. Besides bacteria, the presence of microalgae can benefit the water quality through the absorption of inorganic nitrogen (ammonium and nitrate) and phosphorus from the water. However, reports on the inclusion of microalgae in RAS are very scarce. The objective of this study was to determine the effect of microalgae on water quality (total ammonia nitrogen, nitrite, nitrate, and phosphate) and bacterial composition in a fresh water small-scale RAS. A periphytic microalga, *Stigeoclonium nanum* was used in this study. A rapid fingerprint analysis, denaturing gradient gel electrophoresis (DGGE) was used to determine the bacterial community composition in the water. The results showed that ammonia concentrations were not significantly different ($P>0.05$) between RAS with microalgae (RAS+A) and RAS without microalgae (RAS-A). However, nitrite, nitrate and phosphate were significantly lower in the RAS+A than the RAS-A ($P<0.05$). Pielou's evenness and Shannon diversity index of bacterial community between the treatments were not different ($P>0.05$), however, the bacterial composition between the treatments was significantly different ($P<0.05$).

4.1 Introduction

Recirculating aquaculture systems (RAS) are becoming more important due to land and water limitation for aquaculture activities and the ability of the system to minimize environmental impact (Badiola et al., 2012). Bacteria play the major role in nutrient recycling which is the main mechanism of waste removal in RAS. Besides, the bacterial community in the culture tank is influenced by the fish gut bacterial community (Cahill, 1990; Giatsis et al., 2015). Literature reports several types of microorganisms present in the RAS with the heterotrophic and autotrophic bacteria as the most studied microorganisms (Blancheton et al., 2013; Leonard et al., 2000; Michaud et al., 2006; Michaud et al., 2009). In RAS, a good bacterial community can be defined as a community which helps to maintain a good water quality and reduces the risk of disease outbreaks (Zhou et al., 2009). A beneficial bacterial community normally contains a broad range of harmless or beneficial bacteria species, and a few potentially harmful opportunistic bacteria species (Zhou et al., 2009). However, studies found that the bacterial community in the RAS changes rapidly in a stochastic manner (Giatsis et al., 2015; Verschuere et al., 2000), making it difficult to control microbial community composition in RAS (Leonard et al., 2000; Michaud et al., 2006; Michaud et al., 2009; Schreier et al., 2010).

Besides bacteria, the presence of microalgae can benefit the water quality through the absorption of inorganic nitrogen (ammonium and nitrate) and phosphorus from the system (Ebeling et al., 2006; Martínez-Córdova et al., 2014). Some culture systems in which microalgae are incorporated are known as green water systems (Neori, 2011), periphyton-based aquaculture systems (Asaduzzaman et al., 2008; van Dam et al., 2002) and partitioned aquaculture systems (PAS) (Eversole et al., 2008). In aquatic systems, microalgae interact with the co-existing bacteria in numerous ways. The interactions can be either positive or negative which result in either stimulation or inhibition of co-occurring algae and bacteria (Cole, 1982; Desbois et al., 2009; Joint et al., 2007; Natrah et al., 2011; Schumacher et al., 2003; Vardi et al., 2006; Volk and Furkert, 2006). Microalgae and bacteria interactions are categorized into nutrient exchange, signal transduction and gene transfer and have been reviewed extensively in more recent publications (Cooper and Smith, 2015; Kouzuma and Watanabe, 2015; Natrah et al., 2014).

Some of the interactions such as improved system hygiene benefited the aquaculture system and larval survival (Liao et al., 2001; Salvesen et al., 1999) and helped to lessen pathogenic bacteria in the culture water (Banerjee et al., 2010; Tendencia and dela Peña, 2003).

Based from the above evidences, this study hypothesized that microalgae would influence the water quality and bacterial community in the RAS. However, whether the effect of the influence is good or not is yet to be determined. Therefore, the objective of this study was to study the effect of microalgae in RAS on water quality and bacterial community.

4.2 Materials and methods

4.2.1 RAS experimental setup

This experiment consisted of two small-scale triplicated experimental treatments, RAS with microalgae (RAS+A) and RAS without microalgae (RAS-A) as the control treatment. The RAS set-up (Fig. 1) as described in a previous study was used (Mohamed Ramli et al., 2018). RAS-A had the same configuration as RAS+A except that no microalgae were grown in the microalgae tanks. The flow rates applied in the RAS was 6 L min⁻¹ except for the microalgae tanks which received half of the water flow (3 L min⁻¹). About 15% of the water was discharged weekly from the bottom of the solid waste collector (hydro-cyclone) to remove accumulated solids because decomposing solids might raise the total ammonia nitrogen (TAN) concentration in RAS (Burford et al., 2003). During solid removal, the water flow from the fish tank was directed to the moving bed reactor, bypassing the hydro-cyclone.

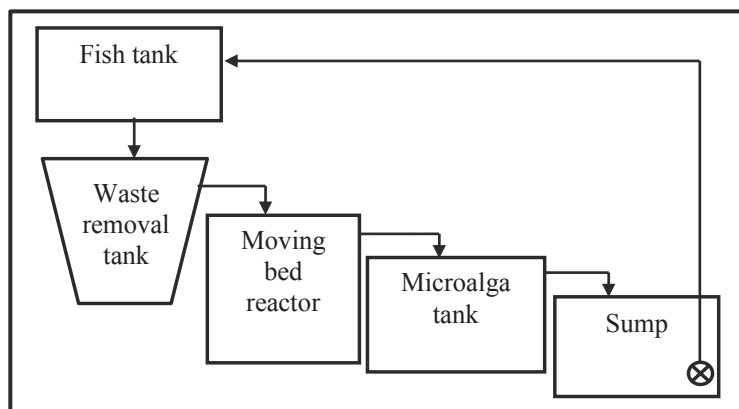


Fig 1 Conceptual experimental set-up of recirculating aquaculture system (RAS) with microalgae tanks (RAS+A) - a fish tank (65 L), a hydro-cyclone for solid waste removal (effective volume 42 L), a moving bed reactor for nitrification (effective volume 14 L), two microalgae tanks (15.6 cm depth X 30 cm wide X 30 cm length) (effective volume 14 L each) and a sump (112 L).

To avoid clogging of pipes and biofilters, the non-planktonic periphytic microalgal species *Stigeoclonium nanum* was used. *S. nanum* was isolated from the university's aquaculture experimental facility. The microalgae tanks were maintained under 24 hours light conditions of $55\text{--}60\ \mu\text{mol photons m}^{-2}\text{ sec}^{-1}$ and were continuously aerated. In nature, *S. nanum* is commonly found on substrates in shaded areas, thus has an affinity for low light conditions (Steinman, 1992). Six hundred grams of alginate beads which contained $1.5\text{ g wet weight } (0.64\ \mu\text{g chlorophyll-a g}^{-1}\text{ dry weight})$ of *S. nanum* were introduced in the system 21 days before the measurements started. When the microalgae were introduced, the RAS already had fish for two weeks. The alginate beads functioned as a confinement substrate for the microalgae to enable them adapt to the RAS environment. Our previous study indicated that *S. nanum* had a positive growth when immobilized in alginate beads (Mohamed Ramli et al., 2017). When the beads dissolved, the microalgae continue to grow and attached on the algae tank walls, while some of them floated in the tank. At the start of the measurement (d0), the initial microalgae biomass (chlorophyll-a) was $86.1 \pm 2.4\ \mu\text{g L}^{-1}$ of microalgae tank and was remained continuously at $181.4 \pm 48.6\ \mu\text{g L}^{-1}$ (approximately equals to 15 mg m^{-2} chlorophyll-a) of microalgae tank by maintaining the same area covered by the microalgae through scrapping the old cells from the wall of the tank. The chlorophyll-a was measured weekly to monitor microalgae growth according to APHA (1999) (Fig. 2). For this purpose, microalgae were sampled randomly from the microalgae tanks.

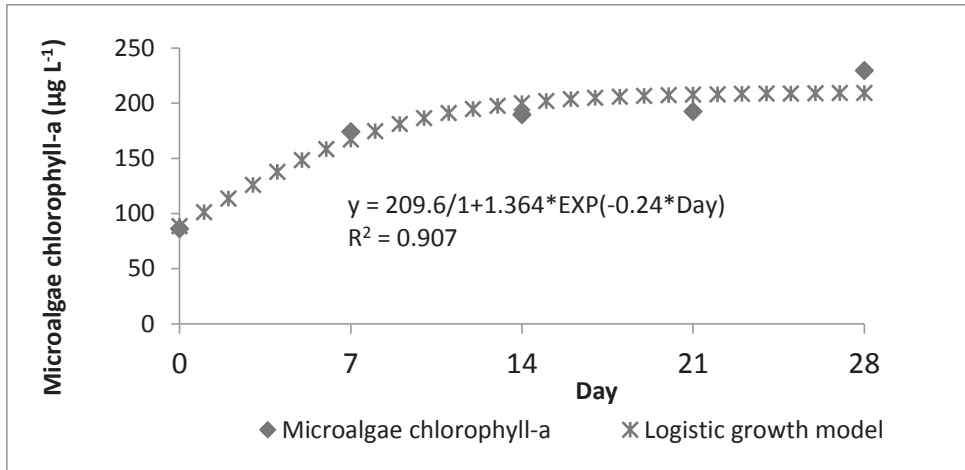


Fig 2 Microalgae biomass (as indicated by microalgae chlorophyll-a) in a microalgae tank of a recirculating aquaculture system. The curve follows the standard logistic growth model, $y = \frac{K}{1 + Ae^{-rT}}$; y = Population of microalgae; K = carrying capacity (209.6); A = constant (1.364); r = intrinsic growth rate (0.24); T = day. ($R^2 = 0.907$).

4.2.2 Experimental animals and diets

A red tilapia strain of Nile tilapia (*Oreochromis niloticus*) was used. The fish were bought from a commercial fish farm at Puchong, Selangor (Atlantys Hatcheries Sdn. Bhd.). Prior to the experiment, fish were acclimatized in the hatchery at the same conditions as those in the experiment. Each RAS was stocked with 70 fish with an initial wet weight of 20 ± 9.8 g (total biomass of 1351 ± 4.8 g for each RAS). Fish were manually fed 1.8% of body weight, twice daily (crude protein, 43%; fat, 6%; and moisture, 12%- Starfeed 9971, Star Feedmills Sdn. Bhd., Malaysia). The specific growth rate (SGR), feed conversion ratio (FCR), and fish survival were monitored during the experiment.

4.2.3 Water quality measurements

During the experiment, temperature, pH, dissolved oxygen (DO), oxidation-reduction potential (ORP), electrical conductivity (EC), total dissolved solid (TDS) and salinity were measured daily (Aquaread, 2000, UK). For total ammonia nitrogen (TAN), $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, and phosphate-P ($\text{PO}_4\text{-P}$) analyses,

water samples were collected on day 0, 7, 14, 21, and 28 (final) from the fish tank, the nitrification tank and microalgae tank.

Total ammonia nitrogen (TAN) was analyzed using phenate method (APHA, 1999). The $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and $\text{PO}_4\text{-P}$ (orthophosphate) concentrations (mg L^{-1}) were analyzed using ion chromatography. All chromatographic analyses were performed at room temperature using a Metrohm model 882 Compact IC Plus (Metrohm, Herisau, Switzerland) with suppressor module. The machine was equipped with an anion column model Metrosep A Supp 5-250 analytical column (250×4.6 mm) and a guard column model Metrosep A Supp 5 guard. The injection volume was 20 μL . Data were collected using a Metrohm 761 data acquisition system interfaced to a computer running MagIC Net 1.1 software (Metrohm).

4.2.4 Analysis of the microbial communities

To determine the water bacterial composition in different system compartments, water samples from the fish, nitrification and microalgae tanks were collected at the start of the experiment (d0) and at the final day of the experiment (d28). One liter of water was filtered using membrane water filters (isopore polycarbonate membrane filter, 0.22 μm pore size, Merck, New Jersey, USA) and stored at -80°C until further use.

Procedure for DNA extraction and PCR were conducted following methods described in our previous study (Mohamed Ramli et al., 2018). Macherey Nagel genomic DNA extraction kit (Nucleospin® Soil, Düren, Germany) was used for DNA extraction. DNA purity was visualized using 0.8 % agarose gels using a nucleic acid gel stain (GelRed™ Nucleic acid gel stain, Biotium, California, USA) and quantified using NanoDrop spectrophotometer (Thermo Scientific NanoDrop, NanoDrop Technologies, Wilmington, DE, USA). DNA was stored at -20°C until analysis.

Target fragments of bacterial 16S ribosomal RNA gene were amplified from the extracted DNA by PCR using the following cycle conditions. Pre-denaturation at 95°C for 2 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, hybridization at 53°C for 40 s and elongation at 72°C for

1 min and then a final elongation at 72 °C for 10 min. Then, samples were cooled to 4 °C. PCR for DGGE was performed by using primer 968-GC-F (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC -3') and L1401-R 5'- GCG TGT GTA CAA GAC CC -3' (Postma et al., 2000). Equal concentration of the extracted DNA samples were used for PCR.

The 25 µl of PCR reaction mixture (Bioline, London, UK) consisted of 2.5 µl of 1x NH₄ reaction buffer, 1.5 µl of 3 mM MgCl₂ solution, 0.5 µl of 0.2 mM dNTPs, 0.5 µl of 0.2 µM forward and reverse primers, 0.13 µl of 0.625 unit BIOTAQ polymerase (Bioline, London, UK), 1 µl of DNA template and 18.38 µl of ultra-pure water. PCR quality was visualized using 1.5% agarose gel with nucleic acid gel stain (GelRed™ Nucleic acid gel stain, Biotium, California, USA).

DGGE analysis of PCR amplicons was performed as described previously (Muyzer and Smalla, 1998) using DGGE 2001 system (CBS Scientific, USA). Polyacrylamide gels consisted of 8% (vol/vol) polyacrylamide (37.5: 1 acrylamide-bisacrylamide) containing a denaturing gradient of 30% to 60% urea. The gels were poured from the top by using a gradient maker (GM-40 gradient maker, CBS Scientific, USA) and pumping the solution at a speed of 4.5 ml min⁻¹. Electrophoresis was performed for 16 h at 85 V in a 0.5x Tris-acetate-EDTA (TAE) buffer at a constant temperature of 60 °C. Subsequently, gels were stained with AgNO₃ (Sanguinetti et al., 1994) and visualized using GS 800 Calibrated densitometer (Bio-Rad Laboratories, USA). We used a marker (DGGE marker III, 10 fragments, Code no- 311-06923, Wako, Japan) as standard reference for enabling intra and inter gel comparison as suggested elsewhere (Joossens et al., 2011; Muyzer and Smalla, 1998; Thompson, 2014; Tourlomousis et al., 2010).

4.2.5 Data handling and statistical analysis

For daily water quality analysis, repeated measure one-way analysis of variance (ANOVA) was used to compare between treatments (RAS+A and RAS-A). For weekly water quality analysis, repeated measure two-way ANOVA with factors treatment (RAS+A and RAS-A) and location (fish, microalgae

and nitrification tank) was used. Statistical software SPSS (IBM SPSS Statistics, version 20) was used for the t-test and ANOVA analyses.

DGGE patterns were analyzed using Bionumerics software 7.0 (Applied Maths, St-Martens-Latem, Belgium) following the method described in (Giatsis et al., 2014). The patterns were normalized and individual bands were marked automatically and by visual inspection. After that, band matching analysis (0.5% optimization, 1% position tolerance) was executed. In this analysis, all common bands found across different profiles were categorized under the same class. Each class was referred to as one operational taxonomic unit (OTU) or species (S). As a measure of relative abundance, relative intensity of each band within individual DGGE profiles was used. From relative abundance data, Shannon diversity (H'), and Pielou's evenness (J') (Hughes and Bohannon, 2008) were calculated.

Next, relative abundance data was square root transformed and beta-diversity analysis was performed based on Bray Curtis similarity. PERMANOVA was used to compare the three possible factors in the experimental design: "treatment" (two levels; RAS+A and RAS-A; fixed), "location" (three levels; fish, microalgae and nitrification; fixed) and "day" (two levels; 0 and 28; fixed). The pseudo- F statistic was used to test the general null hypothesis of no relationship with P-value to give significance level of the tests. Sample ordination was visualized using Principle Coordinates Analysis (PCoA). Statistical analyses (Bray Curtis similarity, PCoA, and PERMANOVA) were performed using the multivariate statistical software package Primer- Permanova V7 (Primer-E Ltd, Plymouth, UK).

4.3 Results

4.3.1 Fish growth performance and water quality

Initial and final fish biomass, FCR and SGR were not affected by the presence or absence of microalgae in the RAS ($P > 0.05$) (Table 1). Temperature, ORP, pH, DO, EC, TDS and salinity values were not significantly different between RAS+A and RAS-A (Table 2). Significantly higher $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, and $\text{PO}_4\text{-P}$ concentrations were observed in the control (RAS-A) than those in RAS+A (Fig 3 and Table S1) while no differences of TAN, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, and $\text{PO}_4\text{-P}$ were observed among locations ($P > 0.05$) (Table S1). TAN, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, and $\text{PO}_4\text{-P}$ concentrations were different between days (Table S1).

Table 1 Means and standard deviation (sd.) of initial and final fish biomass, feed conversion ratio (FCR) and specific growth rate (SGR) in recirculating aquaculture systems (RAS) with microalgae (RAS+A) and RAS without microalgae (RAS-A).

Parameter	RAS+A	RAS-A	P-value
Initial fish biomass (g)	1355 (4.3)	1348 (2.7)	0.086
Final fish biomass (g)	2291 (111)	2237 (37)	0.466
Survival (%)	84 (0.8)	84 (4)	0.905
FCR (g g^{-1})	1.5 (0.2)	1.5 (0.1)	0.912
SGR (%body weight day^{-1})	1.3 (0.1)	1.3 (0.03)	0.967

Table 2 Means and standard deviation (sd.) of physical water parameters in recirculating aquaculture systems (RAS) with microalgae (RAS+A) and RAS without microalgae (RAS-A). Effect of treatment was analyzed using the repeated measure analysis of variance (ANOVA).

	Treatment		P-value
	RAS-A (sd.)	RAS+A (sd.)	
Temperature ($^{\circ}\text{C}$)	27 (0.4)	27 (0.4)	0.501
Oxidation-reduction potential (mV)	144 (22)	147 (30)	0.572
pH	6.8 (0.3)	7.0 (0.3)	0.122
Dissolved oxygen (mg L^{-1})	8.2 (0.3)	8.3 (0.4)	0.612
Electrical conductivity ($\mu\text{S cm}^{-1}$)	2025 (581)	1999 (553)	0.453
Total dissolved solid (mg L^{-1})	1315 (376)	1298 (359)	0.451
Salinity (ppt)	1.0 (0.3)	1.0 (0.3)	0.829

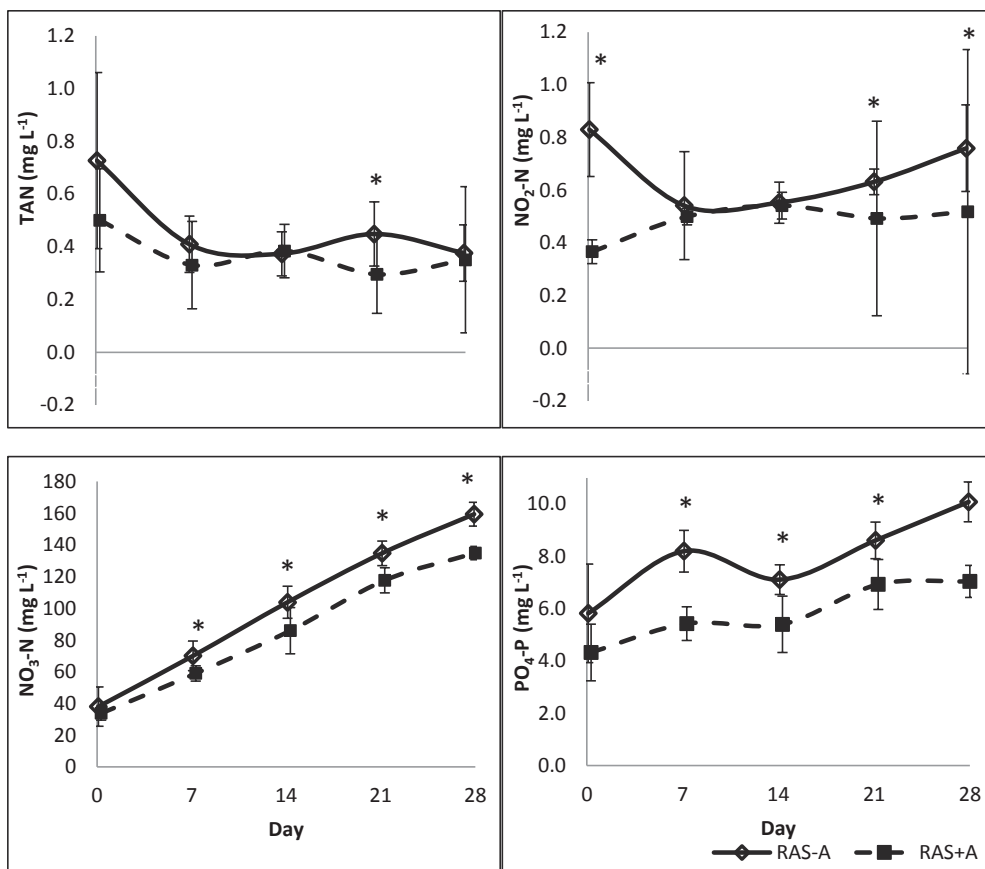


Fig 3 Mean and standard deviation of total ammonia nitrogen (TAN), nitrite-N (NO₂-N), nitrate-N (NO₃-N), and phosphate-P (PO₄-P) concentration in the recirculating aquaculture system (RAS) with microalgae (RAS+A) and RAS without microalgae (RAS-A). Points marked with an asterisk indicate significant difference between treatments on that specific day.

4.3.2 Bacterial community in RAS

Diversity indices

Based on DGGE data, Pielou's evenness and Shannon diversity index were not significantly different between treatments and between sampling days but significant differences were observed between locations (Table 3). Post-hoc tests showed that bacterial evenness and diversity were not different between microalgae and nitrification tank, but both tanks were different from the fish tank.

Table 3 Means and standard deviation (sd.) of Pielou's evenness (J') and Shannon diversity (H') in recirculating aquaculture system (RAS) with (RAS+A) and without microalgae (RAS-A) in three locations (fish (F), microalgae (A) and nitrification (N) tanks) are given. Means which are marked with different letters indicate significant difference (data were analysis using ANOVA repeated measure analysis with treatment and location as main factors).

Parameters	Treatment				Location				P-value			
	RAS+A	RAS-A	F	N	A	Treatment	Location	Treatment X Location	Day	Day X Treatment	Day X Location	Day X Treatment X Location
J'	0.93 (0.05)	0.91 (0.1)	0.87 (0.1) ^a	0.93 (0.03) _b	0.96 (0.02) ^b	0.462	0.011	0.115	0.476	0.079	0.632	0.065
H'	2.1 (0.5)	2.1 (0.6)	1.6 (0.6) ^a	2.5 (0.4) ^b	2.2 (0.2) ^b	0.998	0.002	0.624	0.1	0.107	0.004	0.146

Analysis of beta-diversity

Different DGGE patterns were observed between treatments (RAS+A vs. RAS-A) (Fig 4). Based on Bray Curtis similarity of square root transformed relative abundance of bacterial community derived from DGGE output, the bacterial community in the system was significantly different between treatments and locations but no difference was observed between days (Table 4). Significant interactions were observed between the treatment and location factors. Pair-wise comparisons of the main factors are shown in Table 5. The bacterial community was different between treatments on d0 but not on d28. Between treatments, a significant difference of bacterial community was observed for fish and nitrification tank but not for the microalgae tank (Table 5). Fig 5 shows the ordination of bacterial community in microalgae, fish and nitrification tank. Treatments were clustered separately for the fish and nitrification tank, but not for the microalgae tank.

Table 4 Overall PERMANOVA test based on Bray Curtis similarity of square root transformed relative abundance of bacterial community derived from DGGE output. PERMANOVA compared between factors and interactions: “treatment” (two levels; RAS+A and RAS-A; fixed), “location” (three levels; fish, microalgae and nitrification; fixed) and “day” (two levels; 0 and 28; fixed).

PERMANOVA table of results							
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Treatment(Tr)	1	7536.2	7536.2	3.9109	0.001	998	0.001
Location (Loc)	2	22074	11037	5.7276	0.001	997	0.001
Day	1	2465.8	2465.8	1.2796	0.237	998	0.291
Tr*Loc	2	17536	8767.9	4.5501	0.001	999	0.001
Tr*Day	1	1851.8	1851.8	0.96099	0.473	998	0.493
Loc*Day	2	4382	2191	1.137	0.282	997	0.294
Tr*Loc*Day	2	5906.5	2953.3	1.5326	0.047	997	0.086
Residuals	24	46247	1927				
Total	35	1.08E+05					

Table 5 Pair-Wise comparison between and within RAS+A and RAS-A according to time and location factor based on Bray Curtis similarity of square root transformed relative abundance of bacterial community derived from DGGE output.

Between Treatment – RAS+A vs. RAS-A				
		t	P (perm)	
Factor – Time (Day)	0	1.8648	0.001	
	28	1.2372	0.126	
Factor – Location				
Microalgae tank	Overall	1.228	0.118	
Day	0	1.2602	0.111	
	28	1.0755	0.415	
Fish tank	Overall	2.5588	0.001	
Day	0	2.5786	0.074	
	28	1.4661	0.099	
Nitrification tank	Overall	2.4284	0.001	
Day	0	2.0045	0.109	
	28	1.9407	0.114	
Within Treatment				
	RAS+A		RAS-A	
	t	P (perm)	t	P (perm)
Factor – Location				
Microalgae tank vs. Fish tank				
	1.5349	0.016	2.3616	0.006
Microalgae tank vs. Nitrification tank				
	1.7557	0.001	2.4235	0.003
Fish tank vs. Nitrification tank				
	2.0793	0.001	3.7489	0.002
Factor – Day				
0 vs. 28				
	1.2056	0.157	0.8118	0.685

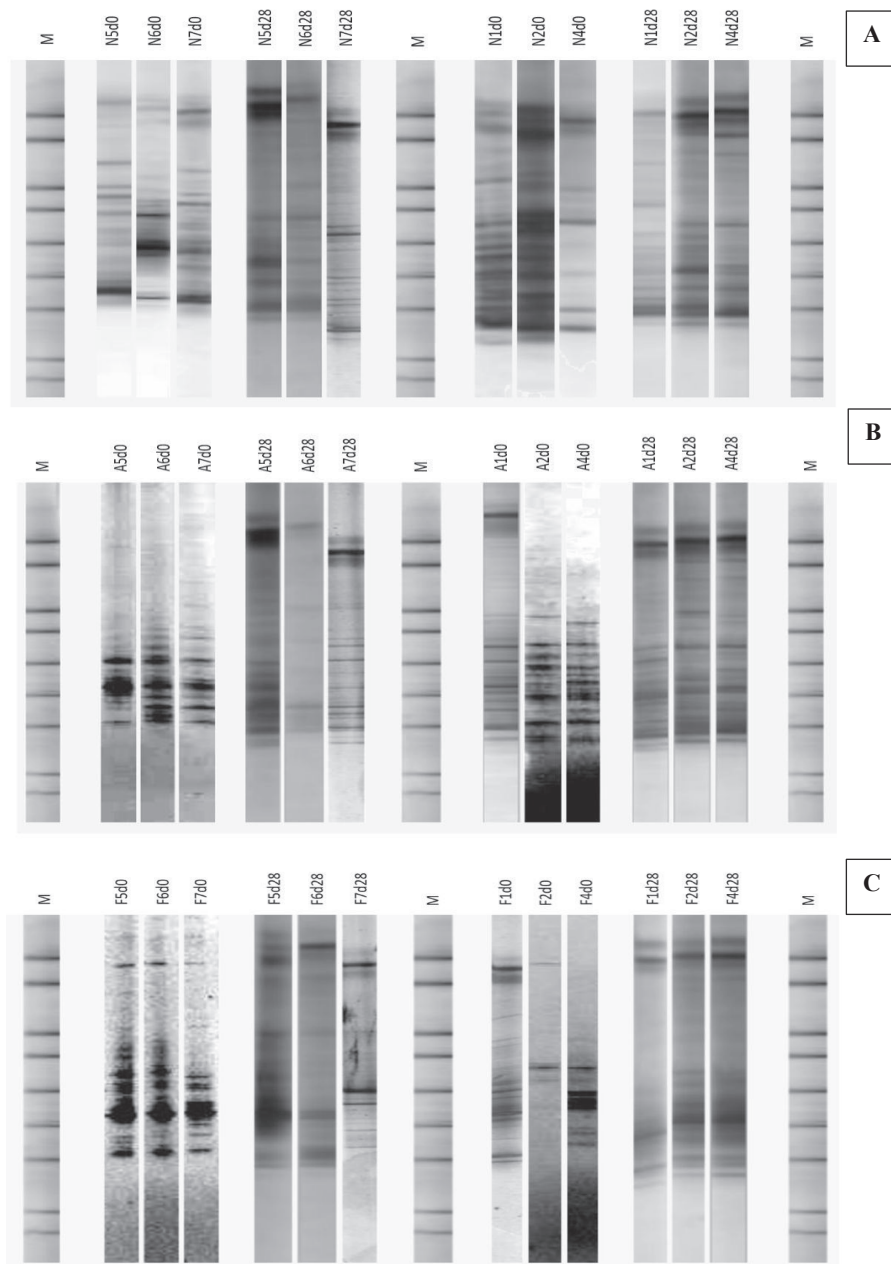


Fig 4 Denaturing gradient gel electrophoresis patterns of bacterial population in recirculating aquaculture system (RAS) with microalgae (replicate number 5, 6, and 7) and RAS without microalgae (replicate number 1, 2, and 4) at the start (d0) and final (d28) day of the experiment. A = Nitrification tank, B = Microalgae tank, C= Fish tank.

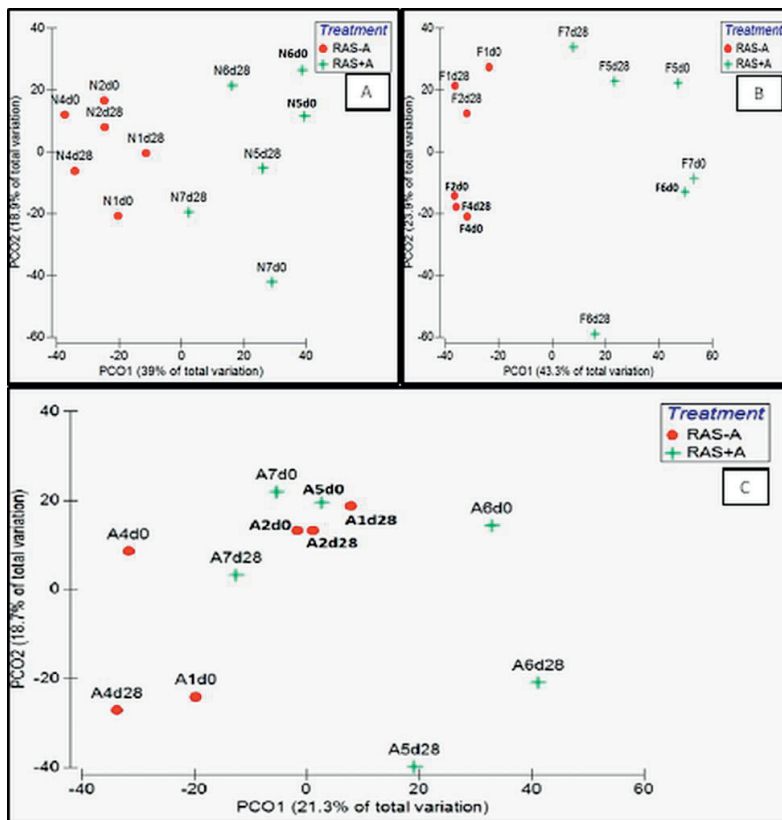


Fig 5 Principle coordinates analysis (PCoA) of the bacterial community at different locations; (A) nitrification tank, (B) fish tank, and (C) microalgae tank in recirculating aquaculture systems with microalgae (RAS+A) and without microalgae (RAS-A). Plots are based on Bray-Curtis distance after square root transformation of relative abundance DGGE data. Data are labelled by location (fish, F; nitrification, N; microalgae, A), RAS number (1, 2, 4 for RAS-A; 5, 6, 7 for RAS+A), and day (d0, and d28).

4.4 Discussion

4.4.1 Water quality

Studies which incorporated algae in aquaculture systems reported lower ammonia, nitrite, and nitrate levels than systems without algae incorporation (Cahill et al., 2010; Khatoon et al., 2007). For example, RAS using algae as bio-filter had significantly lower ammonia and nitrate concentrations than RAS using a bacterial biofilm as bio-filter (Cahill et al., 2010; Valeta and Verdegem, 2015). In our study, total ammonia was not significantly different between the RAS+A and the RAS-A. This could be

explained by the system configuration as ammonia was converted to nitrate in the nitrification bioreactor in both treatments. However, significantly lower nitrite and nitrate levels were observed in the RAS+A than in the RAS-A treatment. These results might indicate that microalgae used ammonia; hence, lower total ammonia was converted to nitrite and nitrate. The hydraulic retention time (HRT) in the microalgae tanks (4.6 mins) was double the HRT in the nitrification tank (2.3 mins), hence, this gave time for microalgae to use ammonia. Nonetheless, the HRT applied for the microalgae tank in this study was high when compared to other study which concluded that HRT less than 0.5 days might cause washout of microalgae cells and HRT of 2-3 days is recommended to obtain maximum biomass yield under 12-25 °C and 190- 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensities (Takabe et al., 2016).

A significantly lower concentration of $\text{NO}_3\text{-N}$ was observed in the RAS+A than the RAS-A and the average difference of $\text{NO}_3\text{-N}$ between RAS+A and RAS-A was $17.6 \pm 5.5 \text{ mg L}^{-1}$. We could estimate how much of the difference was due to uptake by microalgae using microalgae growth rate (Fig. 2). From Fig. 2, the growth rate estimated from the linear part of the curve was $11.2 \mu\text{g chlorophyll-a L}^{-1} \text{ day}^{-1}$. It was assumed that chlorophyll-a content was 1% of microalgae dry solid (APHA, 1999), 50% of dry solid was microalgae carbon content (Chisti, 2007), and carbon to nitrogen ratio of microalgae was 10 (Gál et al., 2003). Therefore, the nitrogen uptake estimated was $0.056 \text{ mg N L}^{-1} \text{ day}^{-1}$. Since this value was very low when compared to the difference of nitrate between RAS+A and RAS-A, we expected that denitrification process might have occurred more often in the RAS+A than RAS-A. Denitrification process is a common process in RAS which occurs under anoxic conditions in specific areas such as inside bacterial biofilms or under sediments, and can cause up to 21% nitrogen loss (Shnel et al., 2002; Van Rijn et al., 2006). The presence of microalgal biofilms increased the anoxic condition in the RAS+A. A study suggested that microalgal biofilms were a suitable place for nitrate respiration (denitrification, dissimilatory nitrate reduction to ammonium, and anaerobic ammonium oxidation) based on findings and identification of genes involve in nitrate respiration in microalgae biofilms (Krohn-Molt et al., 2013).

Besides nitrate, the effluent of RAS has a high level of phosphorus due to the lack of appropriate methods for phosphorus removal (Barak and van Rijn, 2000). Methods for phosphorous removal include chemical precipitation followed by filtration of particulate phosphorus (Timmons et al., 2002),

enhanced biological phosphorus removal (EBPR) by alternation of anaerobic and aerobic processes (Sathasivan, 2008) and phosphorus uptake by microalgae (de-Bashan and Bashan, 2004). A chemical precipitation method is seldom applied due to technical and economic constraints (Barak et al., 2003). The integration of anaerobic processes in RAS is also limited due to high investment costs and the required expertise to monitor the operation (Martins et al., 2010). Therefore, the incorporation of microalgae in RAS to reduce phosphorus levels serves as a better alternative even though some studies reported that harvesting of microalgae might limit their application in waste water treatment (de-Bashan and Bashan, 2010). In our study, we showed that the level of phosphorus in the form of orthophosphate was significantly lower in RAS+A than in RAS-A. Furthermore, the used of periphytic microalgae could potentially reduce the harvesting difficulty as they were easier to be handled than the planktonic species.

In intensive and semi-intensive tilapia pond system, it was reported that TAN and $\text{PO}_4\text{-P}$ concentrations were in the range of 0.03 – 0.37 and 0.04 – 0.85 mg L^{-1} respectively (150 days monitoring, 4 fish m^{-2} stocking density) (Brown et al., 2001). In order to illustrate an efficiency of microalgae in pond system, Brune et al. (2003) reported that a catfish pond which was fed a feeding rate of 143 $\text{kg ha}^{-1} \text{day}^{-1}$ (36% protein) with tilapia as the co-cultured species had a standing algal biomass of 50 mg L^{-1} volatile solid at a growth rate between 10 – 12 $\text{g C m}^{-2} \text{day}^{-1}$. Water quality in the pond was $<1 \text{ mg L}^{-1}$ for NO_2+NO_3 and NH_3 was 1.5 mg L^{-1} (Brune et al., 2003). In this case, the ratio of microalgae protein to feed protein in the pond was estimated to be 2.5. Therefore, in a pond system we can conclude that if microalgae are used for reducing inorganic and organic wastes, the biomass and growth of microalgae should be higher than the feed introduced in the system. The high microalgae biomass was attributed mainly to nutrients, sunlight, efficient pond management (sufficient mixing and harvesting by the co-culture fish) and the large surface area for the algae which at least double the fish pond area (Schneider et al., 2005). Our study was conducted in indoor RAS by which the condition of microalgae culture might be limited by light intensity, surface area, and a short retention time. Nonetheless, the positive effect of *S. nanum* on water quality has been demonstrated. Therefore, for commercial application, efficacy of *S. nanum* can be improved by improving the microalgae culture condition.

4.4.2 Bacterial community in RAS

Microbial community diversity is important to preserve functional stability of an ecosystem (Griffiths et al., 2000). High evenness, e.g., all species are equally present in the community, is important in preserving microbial functional stability in a changing environment (Wittebolle et al., 2009). When evenness is low, the community is dominated by only a few highly abundant species. Those species should be tolerant to the perturbation otherwise the community equilibrium will collapse and so will the functionality.

In our study, we measured the evenness and Shannon diversity based on DGGE results to predict the stability of the system. As these parameters were not different between treatments, we suspected that the stability of the bacterial community in RAS+A and in RAS-A were not significantly different. Even so, this interpretation could only serve as the basic guide line since DGGE is a robust method with some limitations such as the co-migration with the different sequences and limited sensitivity to detect rare community members (Muyzer, 1999). Therefore, a more precise method is needed for a better measurement of community evenness and diversity. Nevertheless, DGGE was found reliable to predict bacterial community of tilapia larvae and culture water in the study of Giatsis et al. (2014) since the DGGE and pyrosequencing of PCR-amplified 16S rRNA gene results in the study did not contradict each other.

Our results showed that the bacterial communities in our RAS systems were significantly different between treatments and locations. In these tanks, the bacterial community could associate either with the fish, the microalgae or biofilm substrates. Different bacterial communities between biofilters and culture water (Bourne et al., 2004; Cytryn et al., 2003; Michaud et al., 2009) reflecting the uniqueness of different RAS compartments (Schreier et al., 2010). In the study of Bourne et al. (2004), different bacterial communities of different locations (water column, tank biofilm and larvae environment) were found within the fish larval rearing system. Fish introduce their own unique bacterial flora (Sugita et al., 2005) which also might explain part of the difference between compartments. Furthermore, a study found that planktonic bacterial communities and biofilm communities were

different (Verhagen et al., 2011) which might explain the differences found between the nitrification, microalgae and fish tanks.

Furthermore, it was shown that presence or absence of microalgae in RAS influenced the composition of bacterial communities. In the future, identification of bacterial species is important to further confirm this finding as microalgae roles are significant in aquaculture systems. More precise estimations of the bacterial community composition, and hence, stability, may be obtained by a high throughput genomic approach such as 16S rRNA metagenomic sequencing.

4.5 Conclusion

Conventional RAS have some problems related to the stability of maintaining good water quality (Badiola et al., 2012). In this study, we showed that a small inclusion of microalgae improved RAS water quality. Since microalgae can be more efficient in removing ammonia, nitrate and phosphate from the water than bacteria, the use of microalgae as additional bio-filter in the RAS can be beneficial. Hence, the stability of RAS will be improved. Microalgae too could influence the bacterial community in the RAS. The result demonstrated the potential use of microalgae to manipulate bacterial communities in the RAS. In the future, a more valuable species of microalgae which has a specific role to prevent harmful bacteria or to promote beneficial bacteria could be incorporated in RAS. A more precise and sophisticated methods used for bacteria identification and functions such as 16s rRNA metagenomic analysis is needed to elucidate the interaction of algae and bacteria in aquaculture.

This study suggested four important unknowns which requires further research if the role of microalgae in influencing the bacterial community in the RAS is to be fully understood; (1) identification and characterization of the functions of the microorganisms and how it is affected by the inclusion of algae should be studied as these factors are more directly important for the maintenance of the aquaculture water quality; (2) the bioactive compounds produced by the microalgae which can promote or inhibit bacterial growth; (3) a carbon compound exists in the aquaculture water which encourages growth of certain bacterial species and; (4) the minimum biomass of microalgae that can make significant impact to the bacterial community in the RAS.

Supporting information

Table S1 Effects of treatment (RAS with microalgae (RAS+A) and RAS without microalgae (RAS-A)), location (microalgae, fish and nitrification tanks) and time (d0 and d28) on the total ammonia nitrogen (TAN), nitrite (NO₂-N), nitrate (NO₃-N) and phosphate (PO₄-P) (mg L⁻¹) in the recirculating aquaculture systems (RAS) based on two-way analysis of variance (ANOVA) repeated measure analysis.

Parameters (mg L ⁻¹)	P-value						
	Treatment	Location	Treatment X Location	Day	Day X Treatment	Day X Location	Day X Treatment X Location
TAN	0.111	0.561	0.438	0.000	0.088	0.001	0.807
NO ₂ -N	0.002	0.805	0.298	0.610	0.047	0.553	0.435
NO ₃ -N	0.001	0.876	0.776	0.000	0.000	0.136	0.495
PO ₄ -P	0.000	0.965	0.991	0.000	0.082	0.916	0.963

Chapter 5

Resistance and resilience of small-scale recirculating aquaculture systems (RAS) with or without algae to pH perturbation

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Abstract

The experimental set-up of this study mimicked recirculating aquaculture systems (RAS) where water quality parameters such as dissolved oxygen, pH, temperature, and turbidity were controlled and wastes produced by fish and feeding were converted to inorganic forms. A key process in the RAS was the conversion of ammonia to nitrite and nitrite to nitrate through nitrification. It was hypothesized that algae inclusion in RAS would improve the ammonia removal from the water; thereby improving RAS water quality and stability. To test this hypothesis, the stability of the microbiota community composition in a freshwater RAS with (RAS+A) or without algae (RAS-A) was challenged by introducing an acute pH drop (from pH 7 to 4 during three hours) to the system. *Stigeoclonium nanum*, a periphytic freshwater microalga was used in this study. No significant effect of the algae presence was found on the resistance to the acute pH drop on ammonia conversion to nitrite and nitrite conversion to nitrate. Also the resilience of the ammonia conversion to the pH drop disruption was not affected by the addition of algae. This could be due to the low biomass of algae achieved in the RAS. However, with regard to the conversion step of nitrite to nitrate, RAS+A was significantly more resilient than RAS-A. In terms of overall bacterial communities, the composition and predictive function of the bacterial communities was significantly different between RAS+A and RAS-A.

5.1 Introduction

Stability of a system can be described as the ability to maintain its functions under changing conditions (Orwin and Wardle, 2004; Wang et al., 2011). In the context of recirculating aquaculture systems (RAS), water quality is an important function which relates to stability. Two properties of stability are system resistance (the ability to withstand a disturbance) and resilience (the speed of recovery of a system to its pre-disturbance state) (Griffiths and Philippot, 2013; Loreau et al., 2001; Pimm, 1984). In RAS, disturbances such as pH, oxygen and temperature changes may occur which will consequently affect stability.

Attramadal et al. (Attramadal et al., 2014) suggested that a stable RAS is linked to its stable bacterial community since bacterial communities play a central role in maintaining water quality (Blancheton et al., 2013; Rurangwa and Verdegem, 2014; Timmons et al., 2002). On top of that, it is known that bacteria interact with other microorganisms in the water (Martínez-Córdova et al., 2014; Natrah et al., 2014) which may affect the stability of the bacterial community. Therefore, in this study, it is hypothesized that microalgae could improve the stability of RAS. The hypothesis was based on the shared dependency on ammonium by microalgae and nitrifying bacteria. Besides, many studies showed that the association of microalgae with bacteria could lead to a more stable system as is demonstrated by the microalgae-bacterial community in wastewater treatment (Amengual-Morro et al., 2012; Ryu et al., 2015; Unnithan et al., 2014). For example, Ryu et al. (Ryu et al., 2015) showed that a microalgae-bacterial community was more stable and efficient in removing ammonium than nitrifying bacteria alone during thiocyanate degradation. Meanwhile, in waste treatment ponds, the existence of the microalgae population is very important for the stability of the symbiotic relationship with aerobic bacteria (Amengual-Morro et al., 2012).

Therefore, the objective of this study was to assess the role of microalgae on the stability of RAS. In this study, we stressed RAS with (RAS+A) and without algae (RAS-A) by lowering the water pH from 7 to 4 for three hours. Resistance and resilience of the RAS towards the pH perturbation was calculated by measuring water quality. Additionally, the bacterial communities of RAS+A and RAS-A

were compared to determine mechanisms that could explain the RAS stability. In this article, for simplification, microalgae are mentioned as algae.

5.2 Materials and methods

5.2.1 Ethics statement

The animal experiment was approved by Institute of Bioscience, Universiti Putra Malaysia research ethics and IACUC committee under the following reference number, UPM/IBS/700-3/1/IFS/6384000(R22.1).

5.2.2 Recirculating aquaculture system

The experiment was conducted at the Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia. In the experiment, eight recirculating aquaculture systems (RAS) were used. The RAS had been in operation for 10 weeks before this experiment was conducted.

The four RAS with algae (RAS+A) consisted of a fish tank (65 L), a hydro-cyclone for fecal solids removal, diameter 30 cm (effective volume: 42 L), a moving bed reactor (30 cm X 30 cm X 30 cm) (effective volume: 14 L) with bio-filter media (Ai.M K1 Biological Filter Media, size 1 cm, Malaysia Fish Harvest), two tanks units with algae (30cmX 30 cmX 30 cm) (14 L each) and a sump (112 L) (Fig 1). The moving bed reactor was conditioned and had been in operation for ten weeks before the experiment started. The four RAS without algae (RAS-A) had the same configuration as RAS+A except that the tank for algae was filled with water only. The flow rate from the fish tank to the sedimentation tank and the moving bed reactor was 6 L min⁻¹. Water from the moving bed reactor flowed into two algae tanks, each receiving half of the water flow (3 L min⁻¹). Water from the algae tanks flowed to the sump from where it was pumped to the fish tank.

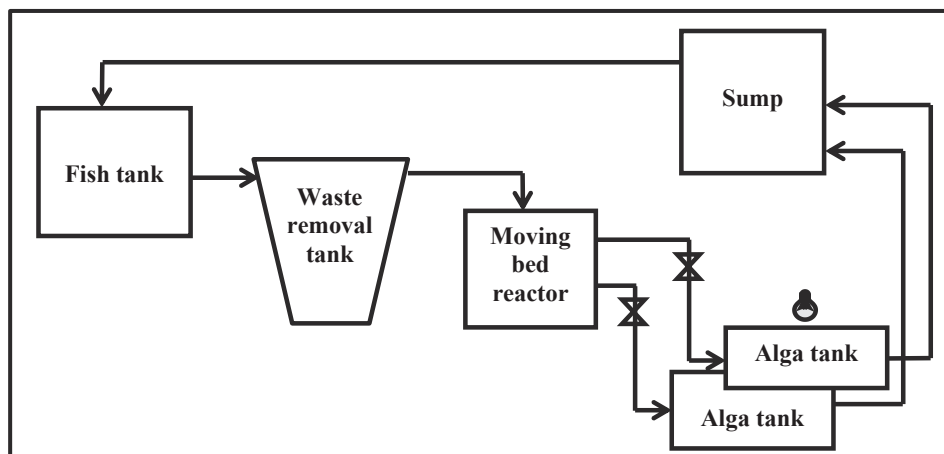


Fig 1 Conceptual experimental set-up of recirculating aquaculture system (RAS) with two algae tanks.

The total system volume for the RAS was 260 L.

A periphytic algae *Stigeoclonium nanum* was incorporated in the RAS. This periphytic algae was chosen instead of planktonic algae so that the density of suspended algae in the RAS could be kept sufficiently low to avoid clogging pipes and bio-filters. The algae was isolated from the university's aquaculture experimental facility. Our previous study indicated that *S. nanum* preferred ammonium than nitrate; therefore, its inclusion in RAS would improve total ammonia nitrogen (TAN) removal (Mohamed Ramli et al., 2017). The algae tanks were maintained in 24 hours light of 55-60 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ and were aerated. RAS water temperature was maintained at 26-28 °C, pH at 6.8-7.0, dissolved oxygen at 7.0-8.0 mg L^{-1} , and conductivity at 2500-3000 $\mu\text{S cm}^{-1}$ (slightly below 1.5 ppt salinity), during the experiment.

5.2.3 Experimental design

The experiment consisted of a period before and after stress. Before the stress (d-1), eight RAS systems were divided over two treatments, RAS with (RAS+A) and RAS without algae (RAS-A). The next day (day 0), two replicates from each treatment were subjected to a stressor (+S) and the other two replicates became the control (no stressor, -S). The stressor that was applied was gradually lowering the pH from

7 to 4 within a period of three to four hours, followed by 3h at pH 4, and thereafter restoring the pH back to 7 within a period of two to three hours. Hence, the whole operation of applying the pH stressor lasted eight – ten hours in total.

5.2.4 Experimental procedure

During the experiment, each RAS had 2200 g red Nile tilapia (*Oreochromis niloticus*). The fish were bought from a commercial fish farm at Puchong, Selangor, Malaysia (Atlantys Hatcheries Sdn. Bhd.). The fish were fed twice a day with a 40% protein diet at 1.8% body weight per day (crude protein 43%; fat 6%; and moisture 12% - Starfeed 9971, Star Feedmills Sdn. Bhd., Malaysia).

Before the stressor was applied to the RAS, the fish were removed from the system and restocked after the pH was raised back to pH 7. During handling, they were anesthetized using 0.4 gL⁻¹ of tricaine methanesulfonate (TMS, Crescent Research Chemicals, Phoenix, Arizona, USA) buffered with 0.8 gL⁻¹ of sodium bicarbonate.

The pH was lowered from 7 to 4 (S1 Fig) by gradually adding 3 ml hydrochloric acid (12 N) at a time. After 3 hours at pH 4, the pH was restored back to 7 gradually by adding 1.0 g sodium bicarbonate (NaHCO₃) at a time. Hydrochloric acid and NaHCO₃ addition were done in the sump. The next morning after the stressor had been applied, TAN increased in some RAS. Therefore, partial water exchange (8-16% from total water volume) was applied to neutralize the effects from lowering the pH and to keep the TAN level <3 mgL⁻¹ (S2 Fig). Water was discharged from the bottom of waste removal tank (hydro-cyclone). During discharge, the hydro-cyclone was disconnected in such a way the other system component maintained functioning. Tap water which was dechlorinated and stored in a reservoir was used to refill the RAS after water discharge. The same water discharge procedure was practiced in all treatments.

During the experiment, temperature, pH, dissolved oxygen, and electrical conductivity levels were monitored daily using a water quality probe (Aquaread, 2000, United Kingdom). TAN level was monitored in the system one day before stress (d-1) until day 20 after stress (d20), with the pH stressor being applied on day 0. Nitrite-N (NO₂-N) and nitrate-N (NO₃-N) were monitored on days -1, 6, 13,

and 20. Analysis of TAN was done using the phenate method (APHA, 1999). Except for day 8 until 11, TAN was measured using API ammonia test kit (Mars Fishcare North America, Inc., USA) due to technical problem with spectrophotometer. $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ concentrations (mgL^{-1}) were analyzed using ion chromatography. Chromatographic analyses were performed using a Metrohm model 882 Compact IC Plus (Metrohm, Herisau, Switzerland) with suppressor module at room temperature. Bacterial community analysis was performed on d-1 and d20. For water quality and bacterial community analysis, one litre water was sampled in the fish tank (Lf), the algae tank (La) and the biofilter (Lb).

The volume of the algal tank was 14 L with 15.6 cm depth. Most of the algae attached on the reactor walls and some were floating in the tank. During the experiment, the chlorophyll-a content of the algae was maintained at 5.8 ± 0.6 mg per RAS ($22.3 \mu\text{g L}^{-1}$) by maintaining the same area covered by the algae by scrapping the old cells that were attached on the tank walls weekly. The outlet of the algal tank was equipped with a strainer to prevent the algae from flowing to the sump. Measurement of chlorophyll-a was done weekly by sampling the area covered by the algae (APHA, 1999).

5.2.5 Microbial analysis (DNA extraction, PCR, and 16 S rRNA metagenomic)

The bacterial composition in the fish, biofilter (moving bed reactor) and algae tanks was determined. The sample was filtered using membrane water filters (isopore polycarbonate membrane filter, 0.22 μm pore size, Merck, New Jersey, USA).

DNA was isolated from the membrane water filters using Macherey Nagel genomic DNA extraction kit (Nucleospin® Soil, Düren, Germany) following instruction by the manufacturer. The membrane was cut into small pieces and 250-350 mg of the membrane was used. The sample was homogenized and lysed in lysis buffer (Buffer SL2) by 15 minutes vortexing using a bead tube (Nucleospin® Bead Tube). After lysis, the sample was incubated in buffer SL3 for 5 minutes at 0 - 4 °C and then centrifuged at 11000 x g for one minute to precipitate the contaminants. After that, supernatant was collected and inhibitors were removed using inhibitor removal column (Nucleospin® Inhibitor Removal Column). The filtrate which contained DNA was bound, washed, dried

and eluted. DNA was quantified using NanoDrop spectrophotometer (Thermo Scientific NanoDrop, NanoDrop Technologies, Wilmington, DE, USA) and visualized using 0.8 % agarose gels using a nucleic acid gel stain (GelRed™ Nucleic acid gel stain, Biotium, California, USA). DNA was stored at -20 °C until analysis.

For 16S rRNA metagenomic analysis for day -1, DNA from four replicates was pooled, resulting in six samples. For day 20, DNA from two replicates was pooled, resulting in 12 samples. 16S rRNA metagenomic analysis was done using Illumina MiSeq according to the protocol described by the manufacturer (Illumina Inc, San Diego, USA). Briefly, the workflow included 16S library preparations, library quantification, normalization and pooling, library denaturing and sample loading, and finally, sequencing and data analyzing.

For the library preparation, two-staged PCR was involved. First, target fragments of Microbial 16S ribosomal RNA gene were amplified from V3 and V4 regions from the extracted DNA by PCR using primers suggested in the protocol (Klindworth et al., 2013). PCR cycle condition was 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and then a final extension at 72 °C for 5 min. Then, samples were cooled to 4 °C. After that, PCR clean-up was run to purify the 16S V3 and V4 amplicon from free primers and primer dimer species using AMPure XP beads. In the second PCR, dual indices and Illumina sequencing adapters were run using Nextera XT Index Kit. PCR cycle condition was 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and then a final extension at 72 °C for 5 min. Then, samples were cooled to 4 °C. Finally, a second PCR clean-up was done to clean-up the library before quantification. Library validation was done using Bioanalyzer DNA 1000 chip to verify the size. After library quantification, normalization, and pooling, the library was denatured and ready to be loaded into the MiSeq system for sequencing.

Open reference operation taxonomic unit (OTU) picking work flow was used to search the reads generated from MiSeq sequencing. Pre-filtration of reads was done in order to discard the sequences which did not represent the targeted marker gene. After that, sequences were clustered using UCLUST v1.2.22 in parallel by a closed-reference OTU picking workflow against the reference database (Greengenes 13_8) at percent identity 97%. The reads that were matched to the reference

sequence at greater than or equal to 97% identity were assigned to the OTU defined by the reference sequences. Next, a random subsample (0.1%) of the sequences that failed to match the reference sequence (0.1% from total sequences) was clustered *de novo*. The cluster centroids for all resulting OTUs were used to define a new reference OTUs. The sequences which were not included in the random subsample went through an additional round of closed-reference OTU picking workflow against the new reference OTUs. Finally the reference OTU and the new references OTUs were combined into a single OTU table.

Functional analysis of OTUs derived from 16S rRNA metagenomic sequencing was performed using PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille et al., 2013). In this analysis references which were clustered *de novo* were removed and only those that have Greengenes OTU identities were further analyzed.

5.2.6 Data processing and statistical analysis

Before statistical analysis, water quality data were checked for normality and equal variances. For water quality, a three-way ANOVA repeated measure analysis with algae (+A and -A), location (La, Lb, and Lf) and stressor (+S and -S) was used. TAN conversion rate was calculated using the formula; $TAN\ conversion\ rate = (TAN\ produced_{day=i-1} - TAN\ measured_{day=i}) \div day$.

TAN produced was calculated based on Ebeling (Ebeling et al., 2006). TAN converted was equal to nitrite produced and used to calculate nitrite conversion rate using the same formula for calculating TAN conversion rate.

Resistance and resilience which were based on the TAN and nitrite conversion rate were calculated following Orwin and Wardle (Orwin and Wardle, 2004). The results were compared between stressed RAS+A and RAS-A using a one-way ANOVA repeated measure analysis.

From the result of Illumina sequencing, Chao1 richness was calculated. To allow fair comparison between samples, random number of sequences for each sample was selected to count based on the minimum reads (315,930 reads) and used for calculation. For d-1, algae and location factors were

compared and for d20, algae, location and stressor factors were compared. ANOVA test on main factor design was performed using Statistical software SPSS (IBM SPSS Statistics, version 20).

From Illumina sequencing, relative abundance of OTUs was square root transformed and the similarity analyses between samples were performed using Bray-Curtis similarity. Then, Principle Component Analysis (PCO) was performed to represent the samples in a low dimensional space in a way that relative distances of all points represent the relative dissimilarities of the samples as measured by the Bray Curtis index.

To examine the significant differences between treatments, permutation based multivariate ANOVA (PERMANOVA) was used. P-value which derived from Monte Carlo algorithm was used when the possible number of permutations was 60 and below. Samples from d-1 were analyzed using two factors; “algae” (two levels; +A and -A; fixed) and “location” (three levels; Lf, Lb and La; fixed). Samples from d20 were analyzed using three factors; “algae” (two levels; +A and -A; fixed), “location” (three levels; Lf, Lb and La; fixed) and “stressor” (two levels; +S and -S; fixed). Similarity percentage analysis (SIMPER) was used to show which OTUs contributed to the difference of bacterial community between algae factor. Cluster of orthologous genes (COG) which were derived from PICRUSt analysis were analyzed using the same procedure for analyzing the OTUs.

Statistical analyses (Bray-Curtis similarity, PCoA, PERMANOVA, and SIMPER) were performed using the multivariate statistical software package Primer V6 Permanova+ (Primer-E Ltd, Plymouth, UK).

5.3 Results and discussion

5.3.1 Water quality

Some of the general benefits of algae inclusion in an aquatic system are; 1) to reduce the pH fluctuations due to extraction of carbon dioxide during photosynthesis; 2) to reduce TAN, NO₂-N and NO₃-N concentration in the water by algae assimilation and; 3) to regulate dissolve oxygen in the water (Brenner and Aharon, 2013). Low biomass of *S. nanum* observed in this experiment was probably due to the low light used in the study. However, effects of the low biomass were still observed on NO₃-N level, on the resilience after the pH perturbation, and on the bacterial community of the RAS.

This experiment was a part of a larger experiment which studied the effect of algae inclusion under normal condition and under stressed condition (this study). Before the stress test was conducted as reported in this study, the RAS was operated under a normal condition for 10 weeks (3 weeks of RAS conditioning, 3 weeks of algae adaptation, and 4 weeks of experiment under normal condition comparing between RAS+A and RAS-A). During the experiment under a normal condition (without a stressor), TAN and NO₂-N concentration below 1 mgL⁻¹ were observed in both treatments. Meanwhile, NO₃-N build-up was observed in both treatments though significantly lower NO₃-N was observed in RAS+A than RAS-A (data not shown).

Therefore, the stress test was conducted to see the effects of algae inclusion on the RAS resistance and resilience towards the pH stressor. TAN, NO₂-N and NO₃-N were measured at three different points, fish tank, nitrification tank, and algae tank. The values were used to estimate the production of TAN in the fish tank, and to evaluate the performance of the nitrification and algae tanks on their role on converting or assimilating TAN, NO₂-N or NO₃-N. However, the results showed that there were no significant differences of TAN, NO₂-N, and NO₃-N between the sampling locations (S1 Table). This might be due to high flow rate (6 L min⁻¹ for nitrification tank and 3 L min⁻¹ for algae tank), thus low retention time in the tanks caused only small changes of TAN, NO₂-N and NO₃-N in the tanks. Therefore, this study presented an average of TAN, NO₂-N and NO₃-N from the three sampling locations

TAN concentrations increased in all systems immediately after the stressor was applied (Fig 2a). Water discharge was performed to control the level of TAN in stressed systems. However, the same water discharge procedure must also be done to control treatment (non-stressed RAS). Water discharge might cause bacterial wash-out and affected nitrifying bacteria. This might be the reason of TAN increased in control treatment after day 7. Unfortunately, from day 8 until day 11, instead of using phenate method, TAN was analyzed using API ammonia test kit due to technical problem with spectrophotometer. The kit could detect a maximum TAN level of 8 mg L⁻¹. From the color indicator, the ranges of water quality in all treatments were more than 4, but below 8 mg L⁻¹. Even though there were differences between treatments from day 1 onwards when the phenate method was used, the test kit was not sensitive enough to detect the differences. This was the reason of the same TAN level on day 8 until 11 as shown in Fig 2a. Significant differences ($p < 0.05$) of TAN concentrations were explained by the factors algae, stressor and day, but not by sample location (S1 Table). In RAS, ammonium may be removed via three processes; conversion to nitrite and subsequently to nitrate through nitrification, immobilization in bacterial and archaeal biomass, and uptake by algae (Ebeling et al., 2006). Since the experiment did not distinguish which process had caused the reduction of TAN in the RAS, apparent TAN conversion is the term used to describe the process. Apparent TAN conversion rate (mg L⁻¹ day⁻¹) (Fig 2b) was significantly affected by the factor stressor (S2 Table). Meanwhile, significant differences ($p < 0.05$) of nitrite concentrations were explained by the factors algae, stressor and day, but not by sample location (S1 Table). Nitrite concentration was below 1 mg L⁻¹ in all treatments on d-1. In RAS-A+S, nitrite increased after the stressor was applied and on d20 after stress, its concentration was 6 ± 3 mg L⁻¹ (Fig 3a). However, for RAS+A+S, nitrite was below 1 mg L⁻¹ during the experiment except on day 13 when the level was 3 ± 2 mg L⁻¹. For RAS+A-S, NO₂-N was below 1 mg L⁻¹ throughout the experiment as a result of reduced TAN oxidation and dilution. Apparent nitrite conversion rate (Fig 3b) was significantly affected by the factor algae (S2 Table). Nitrate levels decreased in all systems on day 6 and 13 after the stress application (Fig 3c). Significant difference ($p < 0.05$) of nitrate was found between the factors algae, stressor, and day (S1 Table).

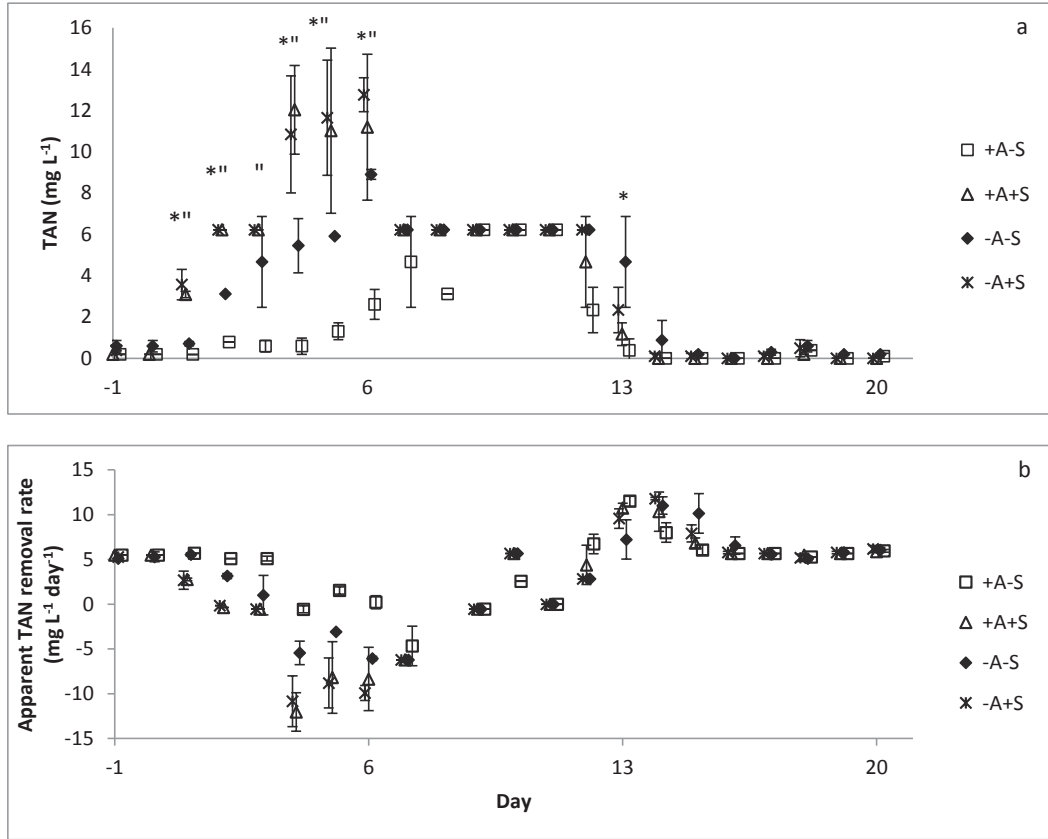


Fig 2 Means and standard deviation (SD) of total ammonia nitrogen (TAN) (mg L⁻¹) in recirculating aquaculture systems (RAS). (a) (TAN) concentration (mg L⁻¹). Points which are labelled with asterisk * show significant differences between algae and no-algae treatments and points which are labelled with asterisk “ show significant differences between stressed and non-stressed treatments on each day, $p < 0.05$. (b) TAN conversion rate (mg L⁻¹ day⁻¹).

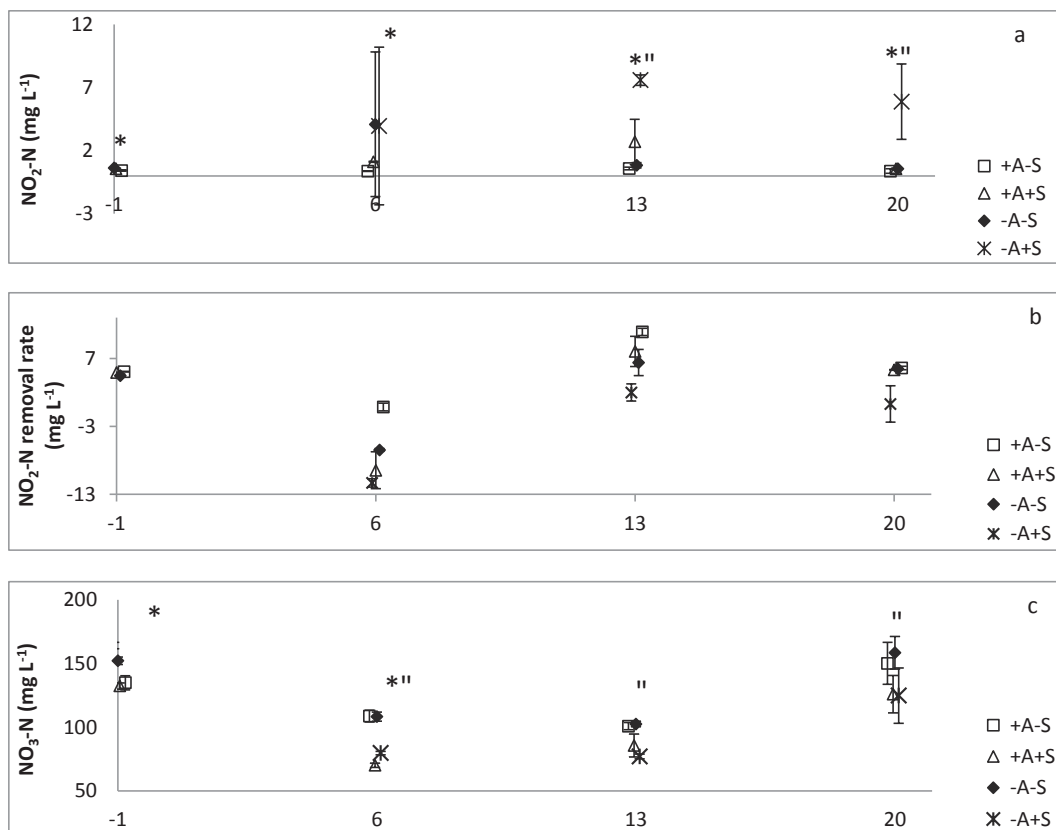


Fig 3 Means and standard deviation (SD) of nitrite-N (NO₂-N) and nitrate (NO₃-N) (mg L⁻¹) in recirculating aquaculture systems (RAS). (a) NO₂-N (mg L⁻¹). (b) NO₂-N conversion rate (mg L⁻¹ day⁻¹). (c) NO₃-N (mg L⁻¹). Points which are labelled with asterisk * show significant differences between algae and no-algae treatments and points which are labelled with asterisk “ show significant differences between stressed and non-stressed treatments on each day, $p < 0.05$.

These results showed that lowering the pH in RAS from pH 7 to pH 4 and maintaining it for three hours disrupted the function of the bacterial communities in the RAS+A and RAS-A as indicated by the deteriorated water quality following the stress application. Similarly, a study on bacterial communities in lakes and rivers found that a low pH was unfavorable for bacterial growth (Bååth and Kritzberg, 2015) and in soilless cultivation media, low pH resulted in a significant decrease of ammonia oxidation rates and ammonia oxidizing bacteria community diversity (Cytryn et al., 2012).

Resistance towards the acute pH drop for RAS+A and RAS-A was not significantly different neither for apparent TAN nor for nitrite conversion (Table 1). The same result was found for the

resilience for TAN conversion. However, the resilience for nitrite conversion was significantly higher for the RAS+A than for RAS-A. Therefore, we concluded that the efficiency of ammonium conversion was not different in both treatments. However, since the nitrite and nitrate concentrations were significantly lower in RAS+A than in RAS-A this might indicate that algae could have absorbed some ammonium, thus less ammonium was available for nitrification, subsequently less ammonium was converted to nitrite and nitrate. Additionally the resilience for nitrite conversion was significantly higher in the RAS+A than RAS-A, indicating that algae had a positive effect on RAS water quality.

Table 1 Resistance and resilience to an acute pH drop for total ammonia nitrogen (TAN) and nitrite-N conversion rate.

TAN conversion rate				Nitrite conversion rate			
Resistance		Resilience		Resistance		Resilience	
+A	-A	+A	-A	+A	-A	+A	-A
-0.28a	-0.30a	0.89a	0.80a	-0.27a	-0.32a	0.79a	0.57b

Means between recirculating aquaculture system with (+A) and without (-A) algae followed by different letter are statistically different by t-test ($P < 0.05$).

In this experiment, TAN production was expected to be similar in all systems which equal to 1475 mg TAN per day (40g feed per day X 40% protein X 0.092) which was equivalent to 5.67 mgL⁻¹ TAN per day. This estimation was based on Timmons et al., (2002). The assimilation of ammonium by algae is normally estimated using the photosynthetic rate. However, since such data were not available the assimilation rate might be estimated using the stoichiometric relationship of phototrophic algal metabolism (Ebeling et al., 2006). Algae chlorophyll-a content in this study was 5.8 mg per RAS+A (22.3 µg L⁻¹). Considering that chlorophyll-a content was 1% from the algae dry weight, a total biomass of 580 mg dry weight algae was estimated to be present in the system. Every gram of ammonium nitrogen assimilated by algae will yield 15.58 g algal biomass (Ebeling et al., 2006). Therefore, 580 mg algal biomass in the experiment might have assimilated 37 mg ammonium which was approximately 2.5% from the TAN produced by the RAS. When the microbial community was stressed uptake of ammonium by algae might stabilize the system and contribute to lower nitrite in RAS+A than RAS-A.

Effects of the algae on pH were mainly observed during the pH lowering from 7 to 4 where significantly more (P -value < 0.05) hydrochloric acid was added to RAS+A (85.5 ± 12.02 ml) than RAS-

A (26.5 ± 0.71 ml). The presence of algae in RAS+A and uptake of CO₂ during the photosynthesis could have contributed to the observed stability of pH in the RAS+A treatment. No pH diurnal effect was observed later throughout the study most probably because of water exchange which was conducted to control the level of TAN in the RAS. pH in RAS-A was 6.81 ± 0.26 and in RAS+A was 6.87 ± 0.29 .

5.3.2 Overall bacterial diversity

Miseq Illumina 16S rRNA gene fragments were used to profile the bacterial communities in RAS. Trimming and quality filtering of the raw reads generated 9,419,626 high-quality reads. Removal of chimeric sequences reduced the number to 9,080,633 reads for downstream analysis. Finally, 8,000,540 sequences were clustered into 5561 OTUs at a similarity threshold of 97% into the bacteria domains. The minimum read count per sample was 315,930 and the maximum was 580,980. Rarefaction curves showed levelling off in all bacterial communities for all samples at maximum sequence depth of 315,930 (S3 Fig).

Overall, 26 bacterial phyla were detected from which Proteobacteria (alpha, beta and gamma) covered 42% of the total sequences. The second most abundant phylum was Actinobacteria (21% of the total) which was dominated by the class Actinobacteria. The third most abundant phylum was Verrucomicrobia (10.6% of the total) which was dominated by the class Verrucomicrobiae. Other major phyla were Bacteroidetes (8.6%, represented by the classes Bacteroidia, Flavobacteriia and Cytophagia), Fusobacteria (6.1%, represented by the only class Fusobacteria), Planctomycetes (5.0%, mainly represented by the class Planctomycetia), Chloroflexi (2.5 %), Nitrospirae (1.1 %), Acidobacteria (0.5 %) and Firmicutes (0.5 %).

5.3.2 Bacterial community structure in RAS with and without algae

Day -1 (before stressor)

Bacterial communities from RAS+A were clustered at the lower half of y-axis and RAS-A were clustered at the upper half of y-axis (Fig 4a). No difference was found between RAS+A and RAS-A

(Pseudo-F = 3.9; P-value = 0.056; Unique permutations = 60), but a significant difference was found between fish, algae and nitrification tanks (Pseudo-F = 5.6; P-value= 0.03).

When predicted functions based on COG categories of bacterial community on d-1 were plotted, a separation can be seen (Fig 4c). A significant difference was found between RAS+A and RAS-A (Pseudo-F = 7.2; P-value = 0.049; Unique permutations = 60) and a significant difference was found between fish, algae and nitrification tanks (Pseudo-F = 7.1; P-value= 0.045). The results from d-1 showed that algae affected bacterial community in the RAS. Summary of COG categories was plotted in S4 Fig. “Organismal systems” and “human disease” which were less relevant to environmental samples (Staley et al., 2014) were omitted in the diagram.

Day 20 (after stressor)

The results from this study strongly suggested that algae influenced the bacterial composition and functions in the RAS as the effect of algae was also observed on day 20 after stress. The ordination of the bacterial communities showed that bacterial communities from RAS+A were separated from the bacterial communities of RAS-A (Fig 4b). When bacterial communities were compared between factors algae, location and stressor, the results showed that there were significant differences ($P < 0.05$) of bacterial communities for all factors (S3 Table). A separation can also be seen when predicted functions based on COG categories of bacterial community were plotted (Fig 4d). A significant difference of predicted functions was found between RAS+A and RAS-A (S3 Table).

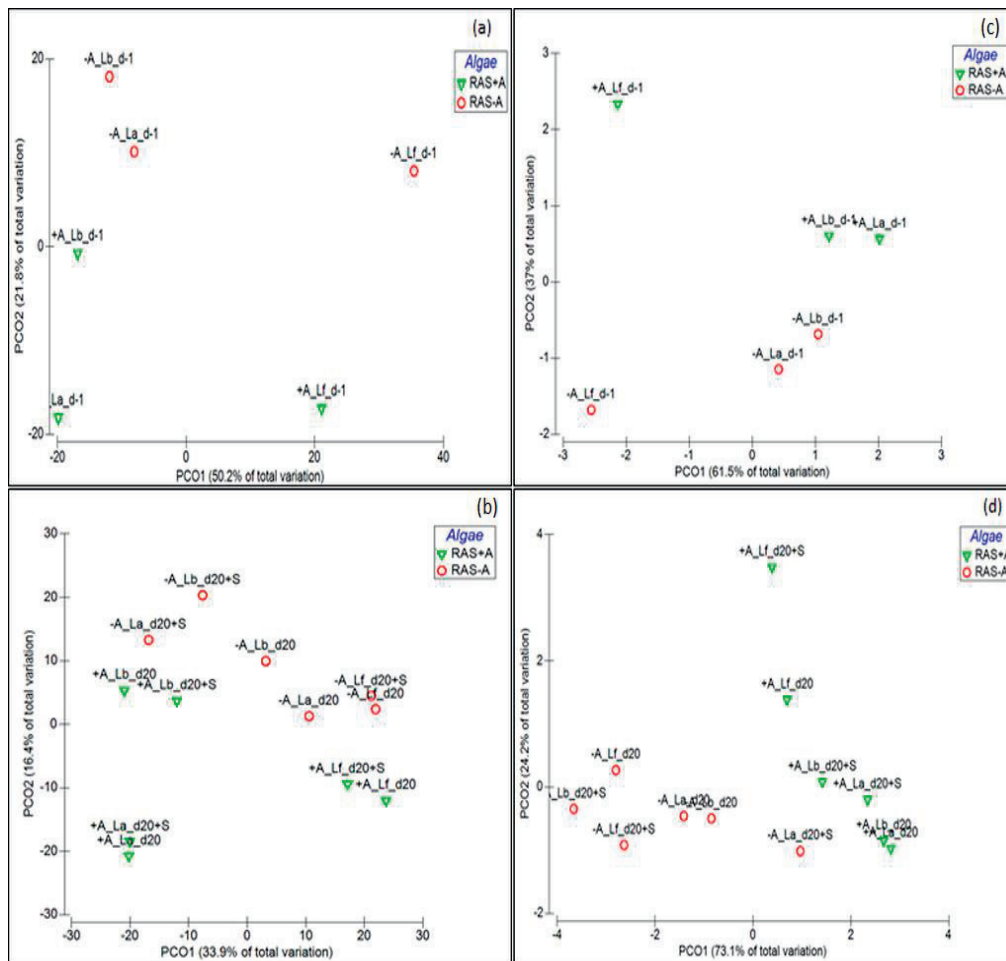


Fig 4 Bacterial communities in recirculating aquaculture systems (RAS) based on Bray-Curtis distance of relative abundance of operational taxonomic unit (OTU) data. (a) a day before stress (d-1). (b) 20 days after stress (d20); Functional categories based on Bray-Curtis distance of relative abundance of cluster of orthologous genes (COG) data (c) a day before stress (d-1). (d) 20 days after stress (d20). Samples are labelled with factors “algae”- with algae (+A), without algae (-A); “location”- fish (Lf), algae (La) and bio-filter (Lb) tanks; “day”- a day before stress (d-1).

Discriminant OTUs – Algae effect

SIMPER analysis showed that for d-1, the dissimilarity between RAS+A and RAS-A was 34% (Bray Curtis dissimilarity index). SIMPER listed 379 OTUs (6.8% of total OTUs) which represented 50% from the total 34% dissimilarity. In total, 5561 OTUs were obtained in this experiment. Here, only 12 OTUs were listed which contributed to the top 10% from the total 34% dissimilarity due to the algae factor (Fig 5a). The dissimilarity between RAS+A and RAS-A on d20 was 44%. SIMPER results listed

15 OTU that contributed to the top 10% from the total dissimilarity between the treatments (Fig 5b). *Mycobacterium* sp. and *Novosphingobium* sp. were the two groups that were consistently higher in RAS-A than RAS+A on d-1 and d20 after stress. Meanwhile, Microbacteriaceae, Xanthomonadaceae, and Verrucomicrobiaceae were found consistently higher in RAS+A than RAS-A on d-1 and d20 after stress.

Based on SIMPER analysis of COG categories, dissimilarity between RAS+A and RAS-A was 3.73 %. Functional category “xenobiotic biodegradation and metabolism” was the highest discriminant (14%) from the total dissimilarity (Fig 6).

From the results, most of the discriminant bacteria that contributed to the differences between RAS+A and RAS-A were heterotrophic bacteria. This could mean that the different bacterial composition might be caused by the dynamics of organic nutrients in the system (Pomeroy et al., 2007; Vadstein et al., 2012). This is very plausible since xenobiotic degradation and metabolism was the highest discriminant function between RAS+A and RAS-A. Xenobiotic compounds are generally known as chemicals that are not natural to the environment and are regarded as environmental pollutants (Narwal and Gupta, 2017). In the RAS-A, *Mycobacterium* sp. from the phylum Actinobacteria was more abundant than in RAS+A. This species is ubiquitous and has the ability to degrade polycyclic aromatic hydrocarbons (PAHs) which are environmental pollutants in all aquatic environments including tap water (Dandie et al., 2004). Therefore, this species is regarded as a potential bioremediation agent (Dandie et al., 2004). Furthermore, *Mycobacterium* is also versatile in using any carbon sources. *Novosphingobium* sp. which was also higher in the RAS-A is a genus within the alpha subclass of Proteobacteria. This genus is Gram-negative, non-sporulating, strictly aerobic, chemo-organotrophic and able to reduce nitrate (Takeuchi et al., 2001). This species is known to be metabolically versatile, often associated with biodegradation of aromatic compounds which is the reason the species is often regarded as a bioremediation agent (D'Argenio et al., 2014; Dworkin et al., 2006; Gan et al., 2013). Though some studies showed that algae had the ability to degrade xenobiotic compounds which might be the reason why these bacteria were less in the RAS+A, such conclusion cannot be made until a further test was conducted on the algae.

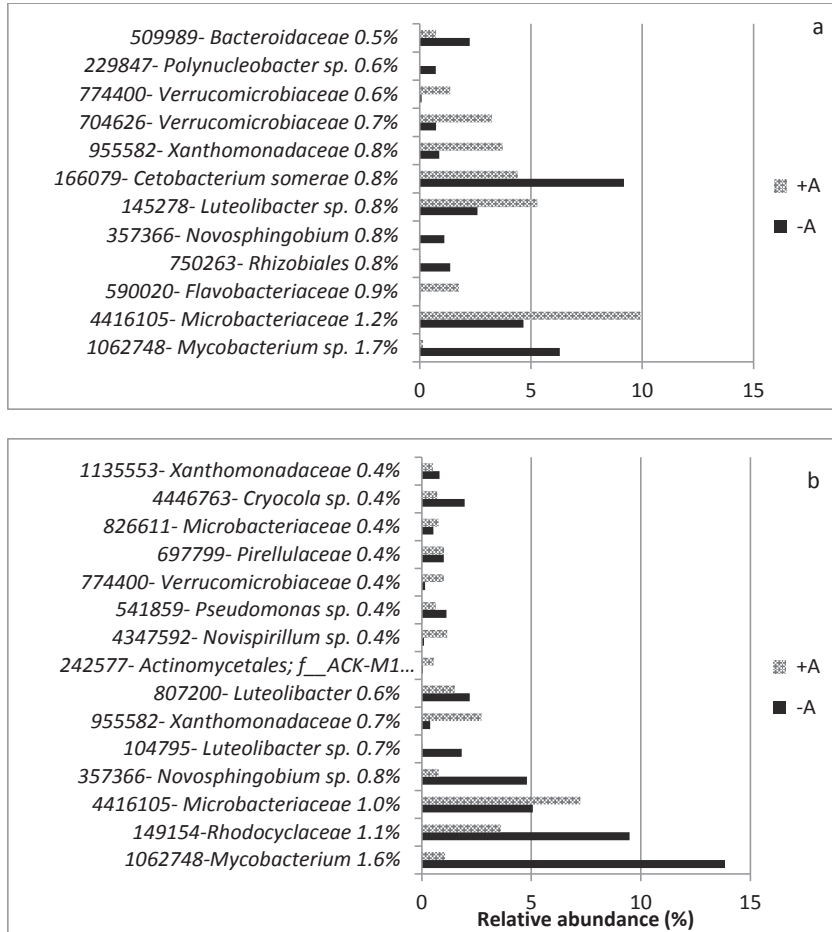


Fig 5 Operational taxonomy unit (OTU) dissimilarity between recirculating aquaculture system (RAS) with (+A) and without (-A) algae. (a) a day before stress (d-1). (b) 20 days after stress (d20). The graphs show abundances of the top 10% OTU that contributed to the total dissimilarity as given by SIMPER analysis. A number of percentage (%) written next to the identity of OTU denoted the % of contribution to the dissimilarity between the RAS.

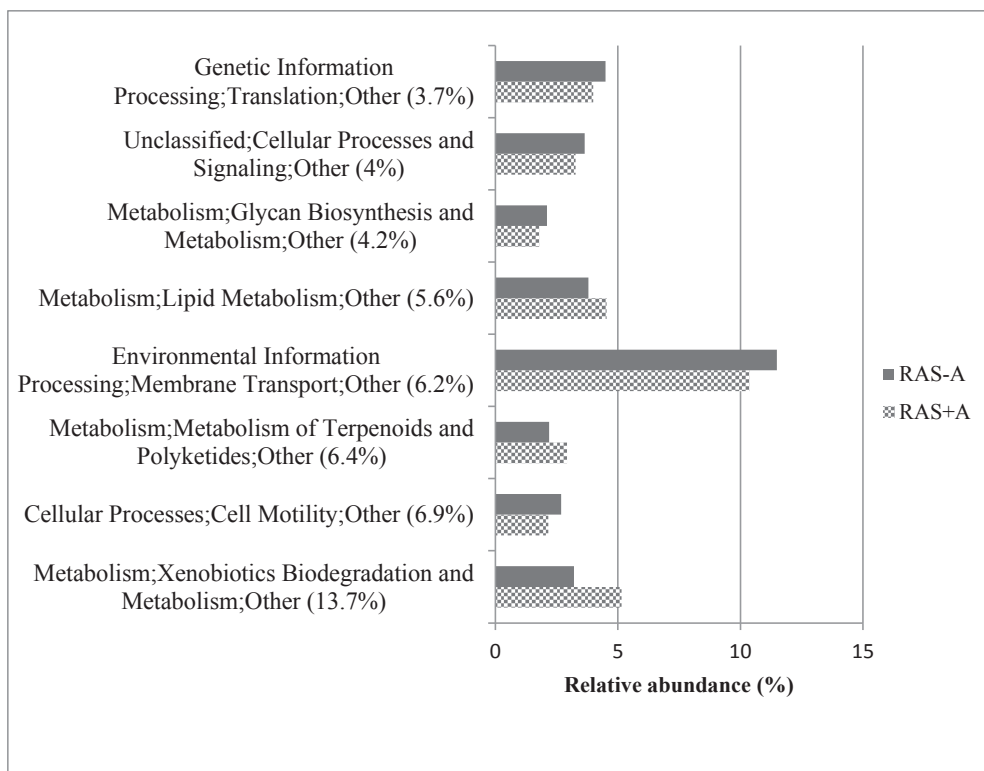


Fig 6 Predicted functions dissimilarity between recirculating aquaculture system (RAS) with (+A) and without (-A) algae. The predicted functions were based on cluster of orthologous genes (COG). The graph shows abundances of the top 50% COG that contributed to the total dissimilarity as given by SIMPER analysis. A number of percentage (%) written next to the functions denoted the % of contribution to the dissimilarity between the RAS.

In the meanwhile, bacteria that were more dominant in the RAS+A had the ability to degrade organic nutrients originated by microalgae. For example, the family Verrucomicrobiaceae which was more abundant in RAS+A than in RAS-A, is member of the phylum Verrucomicrobia (Yoon et al., 2007). Some genera which were found under this family were Gram-negative, facultative anaerobic, non-motile and able to degrade algal metabolites as discovered for *Prostheco bacter algae* (Lee et al., 2014). The other important group that was found more abundant in RAS+A was *Luteolibacter* sp. also a member of the family Verrucomicrobiaceae (Yoon et al., 2008). In the study of Park et al. (Park et al., 2013), *Luteolibacter yonseiensis*, a Gram-negative aerobic and heterotrophic bacterium was isolated from activated sludge using algal metabolites. This could mean that *Luteolibacter* sp. and some other

members under the family Verrucomicrobiaceae which were found in our study might be able to degrade algal metabolites in the RAS+A. Flavobacteriaceae was also higher in the RAS+A than RAS-A. This family is from the phylum Bacteroidetes which are normally regarded as specialists in the degradation of high-molecular weight organic matter which might be the reason why it is normally in association with algae (Krohn-Molt et al., 2013). It was also reported that Flavobacteria-Sphingobacteria group of the Bacteroidetes phylum were among the main bacteria group that were associated with diatoms (Grossart et al., 2005). Meanwhile, *Flavobacterium algicola* has been reported as having the ability to degrade fucoidan, a type of polysaccharide which originate from brown macroalgae (Miyashita et al., 2010). Summarizing, our data showed that the presence of algae stimulates bacterial species which metabolize organic compound released by the algae.

Discriminant OTUs - Stress effect

On d20, the dissimilarity between stressed and non-stressed RAS, as given by Bray-Curtis index, was 43%. SIMPER listed 20 OTUs that contributed to the top 10% from the total dissimilarity between the +S and -S (S5 Fig). *C39* sp., *Novosphingobium* sp., Xanthomonadaceae, Verrucomicrobiaceae, *Pseudomonas* sp., and *Cryocola* sp., were among the most discriminant in the non-stressed systems and Microbacteriaceae, *Mycobacterium* sp., *Luteolibacter* sp., Aeromonadaceae, Pirellulaceae, and *Nitrospira* sp. were among the most discriminant group in the stressed system. Twenty days after the stressor was applied, even though the bacterial communities between +S and -S were different, PICRUST showed that there were no significant difference functions between +S and -S systems (S3 Table). The bacteria species those were more abundant in +S than in -S systems indicated that the stressor influenced the abundance of bioremedial species which contributed to maintaining system functionality. In addition, stressful system (+S system) usually provides room for tolerant species, such as the members of the genus *Mycobacterium* which are known to be tolerant to low pH (Cotter and Hill, 2003).

Nitrifying bacteria

This study found Nitrosomonadaceae and *Nitrospira* as bacteria involved in autotrophic nitrification, whilst for heterotrophic nitrification and denitrification *Rhodococcus* (Chen et al., 2012), *Chryseobacterium* (Kundu et al., 2014), *Bacillus* (Yang et al., 2011), *Acinetobacter* (Zhao et al., 2010), and *Pseudomonas* (Zhang et al., 2011) were the groups of bacteria involved (S6 Fig). Presence of Nitrosomonadaceae was almost negligible in all RAS (relative abundance < 0.05%). More changes of these bacteria occurred in RAS-A than RAS+A. However, the relative abundance of these bacteria was not significantly different between RAS-A and RAS+A (Pseudo-F = 0.8436; P-value = 0.475; Unique permutations = 974). These bacteria count about 3.5 to 10% from the total bacterial abundance and their presence was not affected by algae. Bacteria which were affected by the algae were mostly from the heterotrophic group. It was expected that the algae concentration was too low to reduce TAN availability for nitrification to measure effects. Therefore, in the future an experiment which will allow a higher immobilization of ammonium by algal biomass should be conducted to be able to measure algae effect on nitrifiers.

5.4 Conclusion

The study showed that RAS with and without algae had the same resistance and resilience in restoring to pre-stressor maintenance of low ammonium levels after an acute pH perturbation. Algae supported RAS in keeping the nitrite concentration low before and after the perturbation. In this regard, this research concluded that RAS+A had a better stability than RAS-A. Algae influenced the bacterial community composition in the RAS causing more algal-associated bacteria species to be found in the RAS+A. This suggests strongly that algae can be used to manipulate the bacterial community in RAS.

Supporting information

S1 Table Three way repeated measure analysis of variance of total ammonia nitrogen (TAN), nitrite (NO₂-N), and nitrate (NO₃-N) concentration (mg L⁻¹) in recirculating aquaculture systems. The results compare between factors algae (with algae (+A) and without algae (-A)), location (fish, algae and nitrification), stressor (with stressor (+S) and without stressor (-S)) and day (-1, 6, 13 and 20).

Parameters (mg L ⁻¹)	P-value								
	Algae	Location	Stressor	Algae X Stressor	Day	Day X Algae	Day X Stressor	Day X Algae X Stressor	Day X Algae X Location
TAN	<0.001	0.960	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	1.000
NO ₂ -N	0.001	0.112	0.003	0.048	<0.001	0.001	<0.001	0.002	<0.001
NO ₃ -N	0.001	0.710	<0.001	0.456	<0.001	<0.001	<0.001	0.109	0.876

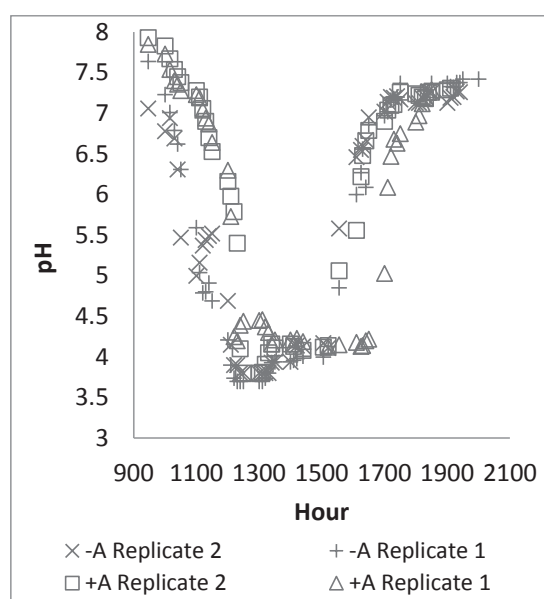
S2 Table Repeated measure analysis of variance of apparent total ammonia nitrogen (TAN) conversion rate (mg L⁻¹ day⁻¹) and apparent nitrite (NO₂-N) conversion rate (mg L⁻¹ day⁻¹) in recirculating aquaculture systems. The results compare between factors algae (with algae (+A) and without algae (-A)), and stressor (with stressor (+S) and without stressor (-S)) in recirculating aquaculture systems.

P-value							
Parameters (mg L ⁻¹ day ⁻¹)	Algae	Stressor	Algae X Stressor	Day	Day X Algae	Day X Stressor	Day X Algae X Stressor
Apparent TAN removal rate	0.062	0.001	0.061	<0.001	<0.001	<0.001	<0.001
Apparent NO ₂ - N removal rate	<0.001	0.861	0.014	0.092	0.658	0.009	0.019

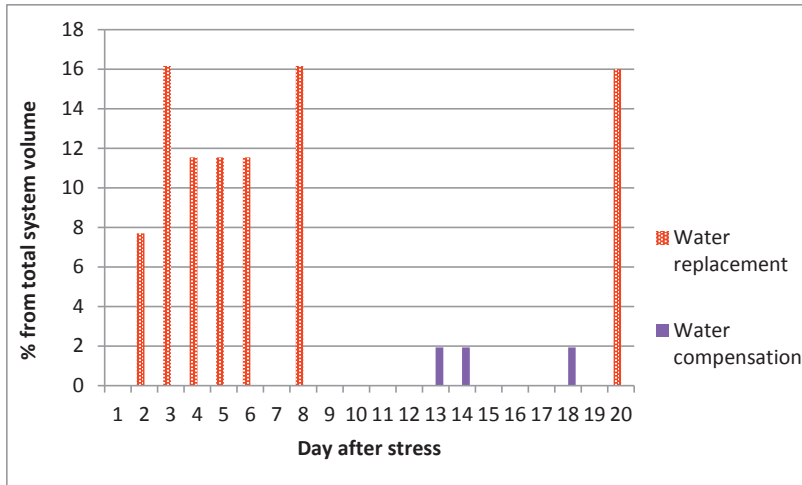
S3 Table Microbiota differences based on operational taxonomy units (OTU) and cluster of orthologous genes (COG).

Source	df	OTU based analysis		COG based analysis	
		P(perm)	Unique perms	P(perm)	Unique perms
Algae	1	0.002	999	0.012	998
Location	2	0.001	997	0.110	998
Stressor	1	0.005	998	0.664	999
Algae x Location	2	0.179	998	0.366	998
Algae x Stressor	1	0.01	996	0.430	998
Location x Stressor	2	0.37	999	0.567	999
Res	2				
Total	11				

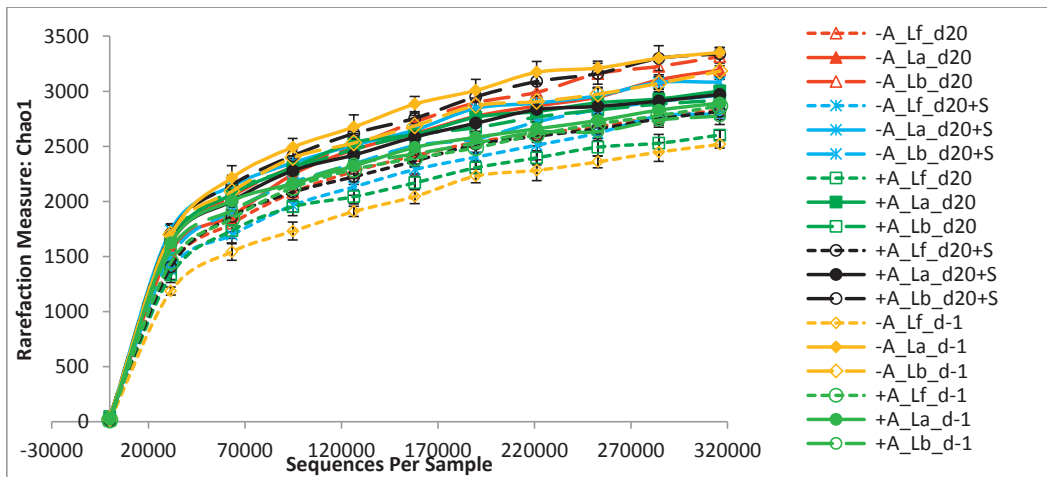
RAS were compared between algae treatments (with algae, +A and without algae, -A), location (fish tank, bio-filters and algae tank) and stressor (when a stressor was applied, +S and when no stressor was applied, -S).



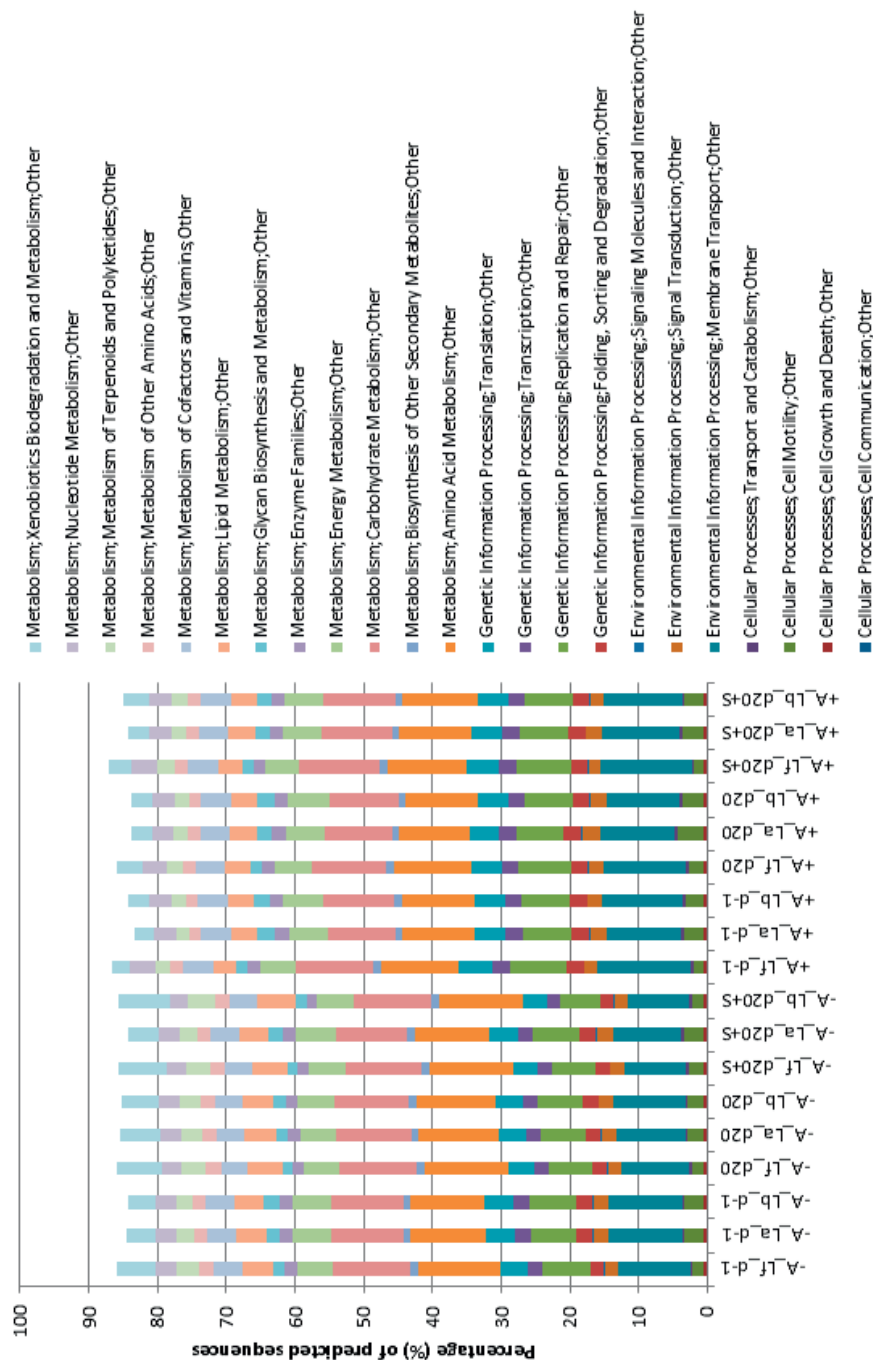
S1 Fig pH changes in recirculating aquaculture system (RAS) with algae (+A) and without algae (-A).



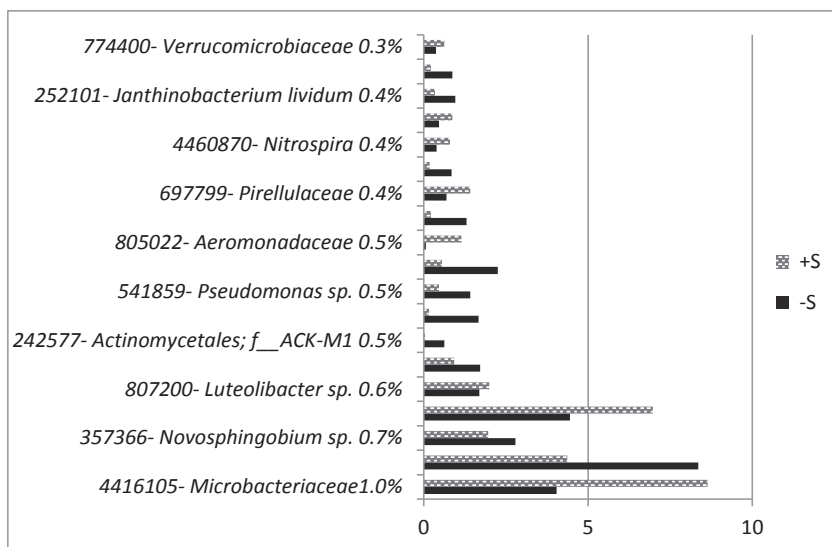
S2 Fig Percentage (%) of daily water replacement from recirculating aquaculture system on days after pH drop was applied.



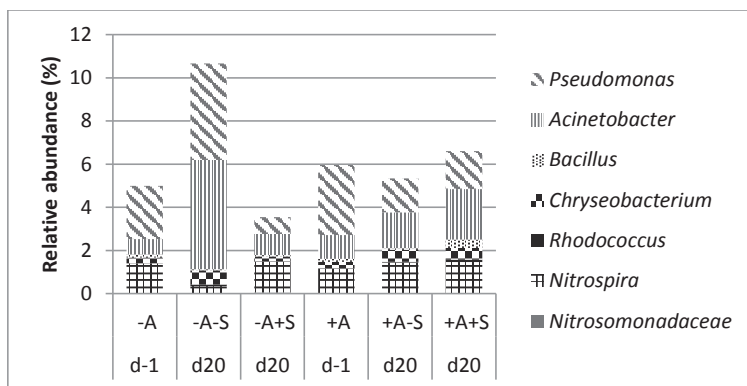
S3 Fig Curves based on Chao1 (richness analysis) at a sequencing depth of 315930. Samples are labelled with factors “algae”- with algae (+A), without algae (-A); “location”- fish (Lf), algae (La) and bio-filter (Lb) tanks; “day”- a day before stress (d-1), 20 days after stress (d20) and “stressor”- stressed (+S) and not stressed (-S).



S4 Fig Percentages of predicted sequences by cluster of orthologous genes (COG). Samples are labelled with factors “algae”- with algae (+A), without algae (-A); “location”- fish (Lf) and bio-filter (La) tanks; and stressor- stressed (+S) and not stressed (-S).



S5 Fig Operational taxonomy unit (OTU) dissimilarity of bacterial community between stressed (+S) and non-stressed (-S) recirculating aquaculture system (RAS). The graph shows the top 10% OTU which contributed to the total dissimilarity as given by SIMPER analysis. A number of percentage (%) written next to the identity of OTU denoted the % of contribution to the dissimilarity between +S and -S.



S6 Fig Relative abundance of nitrifying bacteria. Bacteria which were able to perform autotrophic nitrification (Nitrosomonadaceae, *Nitrospira*) or heterotrophic nitrification and denitrification (*Rhodococcus*, *Chryseobacterium*, *Bacillus*, *Acinetobacter*, and *Pseudomonas*) identified in the recirculating aquaculture systems with (+A) and without algae (-A) a day before stress (d-1) and 20 days after stress (d20) which were stressed (+S) and not stressed (-S).

Chapter 6

General discussion

6.1 Outline

The general aim of this study is to find the effects of microalgae inclusion in a recirculating aquaculture systems (RAS) on water quality and system stability. In this chapter, a brief summary of the thesis findings will be given (Section 6.1). The discussion is grouped along the following issues: effects of microalgae on water quality (Section 6.1.1); effects of microalgae on bacterial composition (Section 6.1.2); effects of microalgae on RAS stability (Section 6.1.3); algae selection and growth (Section 6.1.4); research implications (Section 6.2); limitations and suggestions (Section 6.3); and main conclusions (Section 6.4).

6.2 Roles of microalgae in recirculating aquaculture systems: thesis findings

In this study, the water quality and stability (resistance and resilience as a result of a perturbation) between a RAS with algae (RAS+A) and a RAS without algae (RAS-A) were compared. The addition of microalgae in a RAS did not only result in improved water quality, but made the system more stable and resilient, even when subjected to disturbances (Fig. 1). *Stigeoclonium nanum*, a periphytic microalga preferred ammonium more than nitrate when both nutrients were available. When integrated in a RAS under normal conditions, *S. nanum* improved water quality. When the RAS was perturbed by an acute pH drop (from pH 7 to 4 over three hours), *S. nanum* improved the RAS stability by recovering faster than the RAS-A in maintaining a low nitrite concentration. *Stigeoclonium nanum* influenced the bacterial community composition by increasing bacterial populations that were able to degrade algal metabolites in the RAS+A. Meanwhile, the lesser abundance of bacteria involved in xenobiotic degradation in the RAS +A compared to the RAS-A might indicate that the *S. nanum* also degraded also xenobiotic compounds in the RAS.

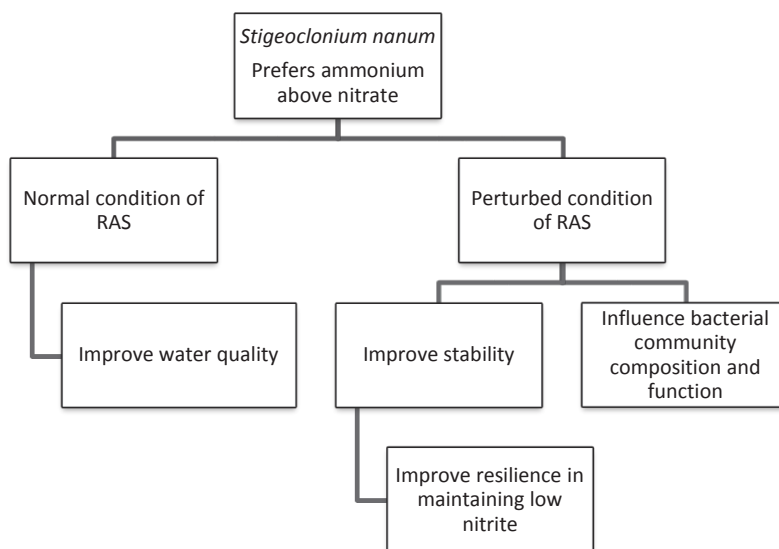


Fig 1 The effects *Stigeoclonium nanum*, a periphytic microalga when integrated in a recirculating aquaculture system (RAS) under normal and perturbed conditions.

6.2.1 Effect of microalgae on water quality

To date, the integration of a microalgae reactor in an aquaculture recirculation system has not been very successful (**Chapter 2**). This is mainly due to the difficulty in managing the algae and high surface area needed for the photosynthesis process (Borowitzka, 1997; van Rijn, 2013). With the surface area ratio of an algae reactor to the fish culture unit being between 1:1 to 2:1, the nitrogen removal rates were between 6 to 25% in the algae reactor receiving nitrogen loading rates between 1 to 4 g N m⁻² day⁻¹. If the depth of the algae reactor is assumed as 0.5 m on average, the nitrogen loading rate would be between 2 to 20 g N m⁻³ day⁻¹. Thus, the nitrogen removal rate would be between 0.12 to 5 g N m⁻³ day⁻¹. On the other hand, denitrification reactors were reported to remove between 70 to 2500 g N m⁻³ (denitrification reactor volume) day⁻¹ (Christianson et al., 2015; Gutierrez-Wing et al., 2012; Klas et al., 2006; Meriac et al., 2014; Suhr et al., 2014; Tsukuda et al., 2015; Visvanathan et al., 2008; Yogeve et al., 2017). Obviously, the nitrogen removal rate of the algae reactor would be negligible if compared with the denitrification reactors. To optimize the benefits of the inclusion of microalgae in a RAS, this analysis suggested that success depends largely on the configuration of a RAS that influences the

nitrogen loading rate, the nitrogen species (ammonium versus nitrate), the cultivation methods (suspended versus attached), and on the prevailing environmental conditions (light, temperature, pH, oxygen, and carbon dioxide). A microalgae reactor may not totally replace nitrification and denitrification reactors, yet, microalgae should be used to assimilate part of the nitrogen in a RAS to increase the efficiency of nutrient use.

The mechanism of water quality improvement by algae can be described by using the concept of resource partitioning. The co-existence of microalgae and bacteria in an aquaculture system indicates that they can be mutualistic and, through ecological resource partitioning, they help to stabilize the ecosystem. Meanwhile, a RAS is an eutrophic system, which from an ecological macro-scale point of view, represents a segment of an ecological niche (Coutinho et al., 2015). Microalgae and bacteria share the role of ammonia removal in a RAS. The mechanism of ammonia removal is different between bacteria and microalgae. Through nitrification, ammonia is converted to nitrite and then to nitrate. On the other hand, the microalgae assimilate ammonia. When algae assimilate ammonia, a reduced amount of ammonia is available for nitrification, thus reducing the nitrite and nitrate concentrations in the RAS. Therefore, the addition of microalgae in a bacterial-based system, such as a RAS, can be regarded as a niche partitioning through the integration of different nitrogen removal mechanisms and habitat creation i.e., the addition of an algae reactor to support algae growth (Fig. 2). Niche partitioning, by incorporating different species within a community, is known to improve system functioning (Cardinale, 2011). The author proposed to broaden this concept to different organisms that share the same general function in a system. Interestingly, a RAS is already a partitioned system. The main compartments (partitions) are the fish tank, the solid removal tank and the bio-filter tank, which each differ in each of their functions and set-up. The effect of this partitioning is clearly demonstrated in the bacterial composition in these different parts in a RAS. As was shown in this study, the bacterial compositions between fish tank, algae tank and bio-filter are significantly different (Fig. 3). The nature of a RAS assists the implementation of microalgae addition in the RAS where a niche is created in each partition.

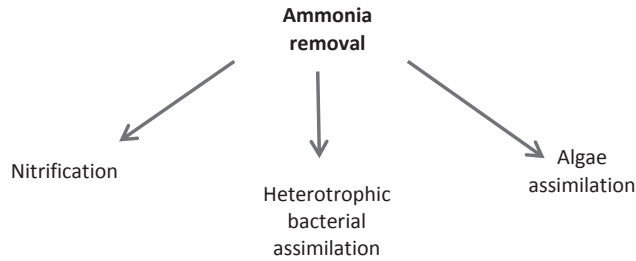


Fig 2 Ammonia removal processes in a recirculating aquaculture system with algae.

Zhu and Chen (1999) showed that ammonia removal through nitrification is concentration dependent. This report agrees with the finding of Cahill et al. (2010) who found that the ammonia removal rate using nitrification decreased at an ammonia-N concentration of 0.11 mg L^{-1} and below. As a result, an ammonia-N concentration close to 0.00 mg L^{-1} could never be achieved by relying on nitrification alone. Therefore Cahill et al. (2010) used *Ulva lactuca* and *Ulva pinnatifida* in a RAS and found that an ammonia-N level at 0.00 mg L^{-1} was possible through the use of these macroalgae. This indicates that algae can be used to complement the ammonia removal process in a RAS. This application is not limited to ammonia removal as a RAS contains other inorganic nutrients (nitrite, nitrate, and phosphate), organic carbons, heavy metals and other metabolites that can be used by microalgae. With the right approach, microalgae can improve the function of a RAS.

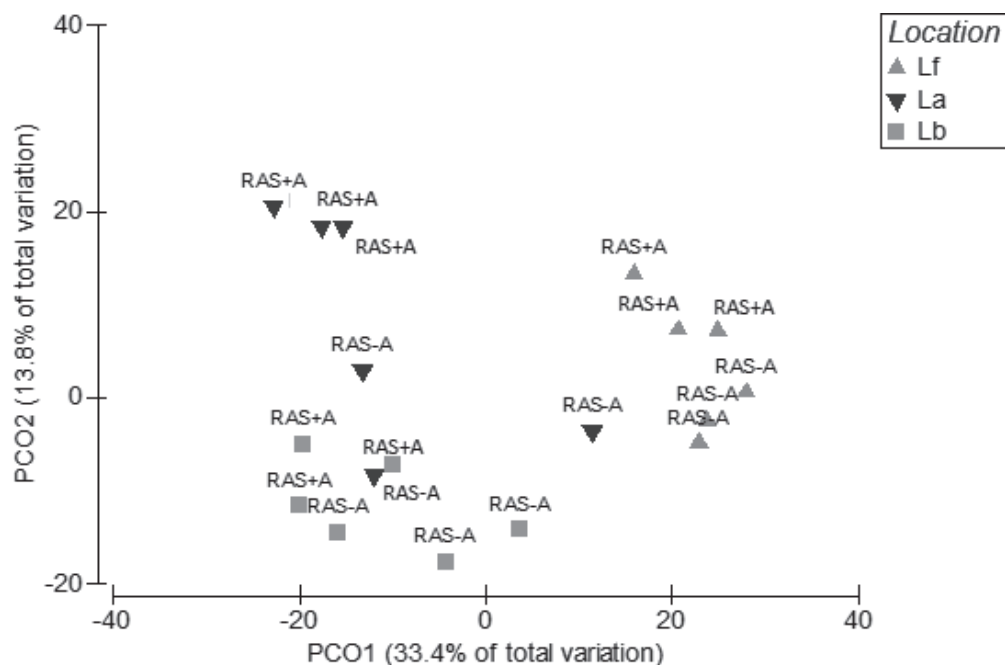


Fig 3 Principle coordinate analysis of bacterial communities in recirculating aquaculture systems (RAS). The bacterial communities were different among the fish (Lf), algae (La) and bio-filter (Lb) tanks. Samples were labelled with factors “algae”- with algae (+A), without algae (-A). Plots are based on the Bray-Curtis distance of a relative abundance of an operational taxonomic unit (OTU) data.

6.2.2 Effect of microalgae on bacterial composition

One of the hypotheses of the study was that microalgae influence bacterial diversity and species composition. The result showed that there was no significant difference in bacterial diversity, species richness and evenness between RAS+A and RAS-A (**Chapters 4 and Chapter 5**). However, the bacterial community composition was significantly different between the RAS+A and RAS-A. The function of the microbial community in the RAS+A was also significantly different than in the RAS-A (**Chapter 5**). Meanwhile, location and stress factors affected only the composition of the bacterial community, but not its functionality. These results suggest that algae can be used to steer the bacterial composition and function in a RAS. In the current study, it has been shown that with the addition of

microalgae in the RAS, the organic matter from live and dead microalgae cells could influence the nutrient dynamics in the system and consequently affected the heterotrophic bacterial community. Bacteria that were able to decompose microalgal metabolites were more abundant in the RAS+A than in the RAS-A. Meanwhile, RAS-A had a significantly higher abundance of bacteria that could degrade xenotic compounds than in RAS+A. Therefore, it is assumed that more xenobiotic compounds were available in RAS-A than in RAS+A. This deduction is in agreement with the study of Coutinho et al., (2015) who, based on 180 marine prokaryote metagenomic datasets, suggested that the abundance of the bacteria is positively or negatively correlated with the available nutrients. For example, bacteria that were more abundant in eutrophic waters were found less so in oligotrophic waters (Coutinho et al., 2015).

One of the major concerns in a RAS is whether the effect of algae on the bacterial community composition remains consistent. Hargreaves (2006) suggested that improving the bacterial communities in aquaculture systems, in terms of a desired composition, was an elusive effort. This opinion might be true considering that attempts to steer bacterial communities in aquaculture systems by the addition of probiotics have not been reliable (Qi et al., 2009). Hargreaves (2006) argued that the effects of a probiotic were ambiguous since bacterial growth depends on interacting factors of inocula and the environmental conditions of the culture system.

In contrast, the literature also suggests also that the effect of algae on the bacterial composition is consistent as algae-associated bacteria are species-specific and that environmental factors, such as light, temperature, and polyphenol concentration, could be overruled (Eigemann et al., 2012). Further, Krohn et al., (2013) observed a stable bacterial community including the classes of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and the phylum Bacteroidetes in association with *Chlorella vulgaris* and *Scenedesmus obliquus* cultured in a photo-bioreactor (Krohn-Molt et al., 2013), suggesting a consistent effect of algae on the bacterial community composition. These bacteria have the role of supplying B vitamins for the algae and depend on the compounds released by algae and bacteria for their growth.

In addition, the effects of algae were also consistent. A classic example was given by Pratt and his colleagues (1944) who found that *Chlorella* produces Chlorellin, which has an antibacterial property

with respect to many pathogenic bacteria for humans such as *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa* (Pratt et al., 1944). This effect of *Chlorella* was also found in aquaculture where the culture waters that contain *Chlorella* reduced or eliminated the presence of harmful pathogens, such as *Vibrio harveyi* and *V. anguillarum* (Sharifah and Eguchi, 2011; Sharifah and Eguchi, 2012; Tendencia and dela Peña, 2003). Also cultures of *Tetraselmis* spp. were found to exhibit antimicrobial activities against aquaculture pathogens (Austin et al., 1992). These findings may explain the consistent observation of a high survival rate described in the green water culture of fish larvae (Liao et al., 2001; Reitan et al., 1997).

6.2.3 Effect of microalgae on RAS stability

Bacterial communities play a key role in maintaining the water quality of a RAS. Thus, the stability of a RAS can be reflected by the stability of the microbial communities. Biological features that contribute to the stability of microbial communities are individual properties (plasticity, stress tolerance and dormancy), population properties (adaptation, growth rate, stochastic expression, survival and dispersal), and community properties (diversity, niche partitioning, community succession, interspecific interactions, turnover and emergent properties) (Shade et al., 2012). In this research, the focus is on the effect of algae in changing the diversity of bacteria. The insurance hypothesis states that diversity ensures stability (Yachi and Loreau, 1999). For example, Griffiths et al. (2000) while examining grassland soils found that the diversity of the microbial community was important to preserve functional stability in the grassland soils. They found that the decomposition rates of plant residues and responses to fertilization improved with decreasing biodiversity of the microbial community. However, most other processes, including nitrification, denitrification and methane oxidation, have been positively linked with increasing biodiversity. Griffiths et al. (2000) concluded that resilience, defined as the capacity of a community to recover from a sudden perturbation or stressor, tended to reduce with decreasing biodiversity.

However, the current study showed that microalgae did not affect the diversity, richness and evenness of a RAS bacterial community (**Chapter 4 and Chapter 5**), probably due to the low

microalgal density used. Therefore, in this study, the diversity could not be the premise to predict the RAS stability between the RAS+A and RAS-A. Nonetheless, from the 16S rRNA metagenomic result, the community structure was evaluated by employing Pareto-Lorenz evenness distribution curves (Fig. 4) with the cumulative proportion of operational taxonomic units (OTUs) on the x-axis and the cumulative proportion of OTU abundances on the y-axis (Cai et al., 2014). The curves allow for visualization of evenness of the bacterial communities. In Fig. 4, the 45° diagonal curve indicates a theoretical perfect evenness with all OTUs (bacteria species) equally abundant. However, in the current study, Fig. 4 shows that the bacterial communities in the RAS were dominated by a few species and the other species were present in low amounts. This could mean that the communities were highly functionally organized but also highly sensitive towards external disturbance (Marzorati et al., 2008). As reported by Wittebolle et al., (2009) a microbial community with a high evenness is more likely to preserve its functional stability in a changing environment.

To confirm the stability, the RASs were perturbed. (**Chapter 5**). In both systems, the RAS+A and the RAS-A had the same resistance. However, in this case, the author had no other study to benchmark the results and to compare the resistance of the RAS. When reflecting on the recovery process (resilience), it is possible to conclude that the higher resilience of the RAS+A than the RAS-A was contributed by the role of microalgae in removing ammonia (niche partitioning), and not by their role in influencing the diversity of the bacteria community.

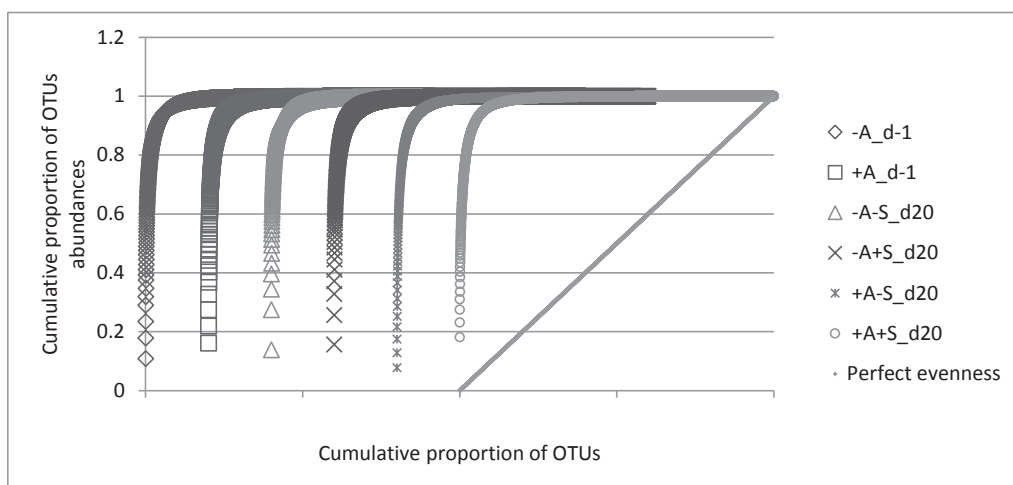


Fig 4 Pareto-Lorenz curves of the bacterial community in the recirculating aquaculture systems with (+A) and without (-A) microalgae, stressed (+S) and not stressed (-S) on day -1 (d-1) and on day 20 (d20). Each of the curves starts at 0 until 1 of the x-axis. Perfect evenness is the diagonal line.

In the opinion of the author, the process of nitrification can be considered as the most critical function to maintain RAS stability because the ammonia concentration must be kept low so as not harm the fish. This assumption has been supported by a survey which indicated that biofilters are regarded as the most difficult device to handle and to cause failure in a RAS (Badiola et al., 2012). In the current study, the author chose pH as the disturbance that would affect nitrification. However, there are other parameters that could disturb nitrification such as ammonia concentration, high carbon to nitrogen (C/N) ratio, and lack of dissolved oxygen (Eding et al., 2006). Badiola et al., (2012) found that the failure of managing a solid removal device had a large impact on the performance of the biofilter which indicated that carbon can cause significant disturbance to nitrification. Therefore, if the author had used C/N ratios or carbon levels in the water as the disturbant, it may be possible to find a stronger and more precise effect on the RAS stability.

6.2.4 Algae selection and growth

Species selection is important in algae culture (**Chapter 2**) and depends largely on the required function of the microalgae. Since the objective of using algae was to improve water quality in a RAS,

the author focused the selection on species robustness to find a species that would be able to thrive in highly contaminated water, and be easy to handle (**Chapter 3**). The author chose a periphytic microalga, *Stigeoclonium nanum* which was found to prefer ammonium rather than nitrate as the nitrogen source (**Chapter 3**). By knowing the preference of the microalgae, its role in an aquaculture system could be optimized.

There are many periphytic microalgae species that could replace *S. nanum*, such as *Spirogyra*, *Synedra*, and *Melosira* (Cardinale, 2011). Besides, there is a variety of periphytic microalgae species have been reported. For example, there were 155, 41, and 31 periphytic algae species reported from the Upper Parana River floodplain, Brazil (Dunck et al., 2016), the Ninféias Reservoir located in the Parque Estadual das Fontes do Ipiranga, Brazil (dos Santos and Ferragut, 2013), and the Dal Lake, Kashmir Valley, India (Pandit et al., 2014), respectively. *Bacillariophyceae*, *Zygnemaphyceae*, and *Chlorophyceae* were the common classes observed with *Scenedesmus* spp. and *Cosmarium* spp. amongst the common genera found. Depending on their preferences of flow rates and nutrients, these species can be selected for integration in a RAS. In addition, the use of a mixed microalgae species that use different forms of nitrogen could ensure that both ammonia and nitrate could be reduced.

As described in **Chapter 2**, the microalgae species and cultivation method must match the RAS configuration to ensure high algal growth. In order to increase the efficiency of the microalgae, the biomass has to be increased. Light, CO₂, hydraulic retention time, tank mixing and addition of surface area for the periphytic microalgae can be considered to increase microalgae growth. However, photosynthesis only captures 5 to 7% of total light available (Peers, 2014). Since artificial light is expensive, it would be interesting to explore the feasibility of using heterotrophic algae. Some microalgae are capable of heterotrophic growth by using organic carbon instead of carbon dioxide (Perez-Garcia et al., 2011; Tuchman et al., 2006). In this case, microalgae may continuously remove inorganic nutrients regardless of light availability. However, there are very few species reported to have this capacity. Species which have been identified to be capable of heterotrophic growth are *Chlorella vulgaris* (Perez-Garcia et al., 2010), *Chodatella* sp. (Li et al., 2014), *Chlorella sorokiniana*, *Euglena gracilis* (Ogbonna and Tanaka, 1998), *Scenedesmus obliquus* (Girard et al., 2014) and diatoms (Prathima Devi et al., 2012; Tuchman et al., 2006). Heterotrophic growth nutrient requirements are

similar to those in phototrophic growth except that organic carbon is used as a carbon source (Lowrey et al., 2016). The types of carbon that can be used include glucose, acetate, ethanol (Ogbonna and Tanaka, 1998), and glycerols (Bumbak et al., 2011). Therefore, if an aquaculture system was able to encourage both phototrophic and heterotrophic growth, then organic carbon could be supplied to the system. Normally organic carbon is added continuously in small quantities to avoid bacteria from using the organic carbon that can lead to excessive bacterial growth (Perez-Garcia et al., 2011).

To facilitate management and control of the algae in a RAS, the author additionally opted for their immobilization in beads (**Chapters 3 and 4**). Though positive effects from the use of the immobilization technique were reported in many studies, including this current work, it was observed that the cost required for bead preparation was expensive. Therefore, the use of alginate beads as a microalgae substrate may not be suitable for a large-scale RAS. Additionally, the beads tend to dissolve in the RAS water when the salinity (which is indicated by the conductivity) increased as a consequence of the feed introduced. The dissolved beads added some carbon to the RAS which might encourage heterotrophic bacteria growth and reduce nitrification efficiency. Therefore, the use of other substrates which are more stable and do not produce any contamination in the RAS should be investigated. One of the solutions is by using a porous substrate bioreactor, also termed as a twin layer-system, for immobilizing the microalgae (Berner et al., 2015). The advantages of using this substrate are water saving and reducing energy consumption through limitation in gas exchange and light dilution, reducing sheer stress on the algae as well as facilitating harvesting of the microalgae (Kiperstok et al., 2017; Naumann et al., 2013; Schultze et al., 2015). Also, a thick concrete layer as used in the study of Ozkan et al., (2012) which also reduced water and energy use, can be tested (Ozkan et al., 2012). To sum up, there are many improvements that can be undertaken to stimulate the integration of microalgae in a RAS.

6.3 Research implications

Water quality is important to ensure fish health and sustainable aquaculture production. Current research focus on the microbial community dynamics in a RAS as microbes are regarded as one of the

most important biological components for controlling water quality. Topics investigated included; 1) the composition and functions of the microbial community in a RAS (Blancheton et al., 2013; Wold et al., 2014); 2) nitrifying consortia and nitrification (Bartelme et al., 2017; Keuter et al., 2011; Kruse et al., 2013); 3) 'r' and 'K' strategists for predicting the stability of the microbial community (Attramadal et al., 2012; Attramadal et al., 2014); 4) interactions between microbial communities in the gut and rearing water in fish larval culture (De Schryver and Vadstein, 2014; Giatsis et al., 2015; Giatsis et al., 2014) and 5) the role of microalgae in managing fish diseases (Defoirdt et al., 2011; Natrah et al., 2014).

The findings of this current study can complement and extend the researches mentioned earlier for two reasons: first, algae positively interact with bacteria to improve efficiency in nutrient recycling; second, algae influence the bacterial community composition through nutrient exchange (effects of compounds produced by bacteria and algae), signal transduction (activation or inhibition of gene expression and physiological activities), and gene transfer (the main process in evolutionary development) (Kouzuma and Watanabe, 2015). The role of microalgae to steer the water bacterial community can be extended to the study of bacteria colonization in fish larvae as the water bacterial community has a high correlation with gut microbiota (Giatsis, 2016). In another example, Natrah et al., (2011) found that *Chlorella saccharophila* was reported to produce quorum sensing-interfering compounds that inhibited acylhomoserine lactone-regulated bioluminescence in the aquaculture pathogen *Vibrio harveyi*. As *Vibrio* sp. can cause serious damage to fish, for example cod larvae (*Gadus morhua*) (Reid et al., 2009) the addition of *C. saccharophila* should be further tested in a RAS in order to control the population of *Vibrio* sp. in the culture water.

Even though challenges remain in managing the algae, the role of microalgae to improve water quality and to steer the bacterial community should be further studied and applied. There are many types of pollutants that can be removed by microalgae and interesting compounds discovered in microalgae that should motivate further research of microalgae in a RAS.

6.4 Limitations and suggestions

It has been reported that ammonia-oxidizing archaea (AOA), in contrast with ammonia-oxidizing bacteria (AOB) were the dominant ammonia oxidizers in most fresh water aquaria (Bagchi et al., 2014). A study of microbial communities in macroalgal biofilms found that bacterial *amoA* genes that code for ammonia monooxygenase which is responsible for the ammonia oxidation process, were 10 times more abundant than those from AOA (Trias et al., 2011). Therefore, in the future a study of RAS stability should include the analysis of archaea so that the link between archaea, bacteria, and algae in ammonia removal can be determined.

Interactions between bacteria and algae also occur via compounds produced by the algae. Therefore, the anti-microbial and anti-quorum sensing compounds which might be produced by *S. nanum* should be investigated.

6.5 Main conclusion

The main outcomes of this study can be summarized as follows:

- Algae performance in a RAS is determined by the RAS configurations that influence nitrogen loading and nitrogen species (ammonium versus nitrate), cultivation methods (suspended versus attached), and environmental conditions (light, temperature, pH, oxygen, and carbon dioxide).
- As a test species, *Stigeoclonium nanum*, a periphytic microalga prefers ammonium over nitrate. This information is helpful to understand the inorganic nitrogen removal processes in a RAS.
- The lower nitrite and nitrate in the RAS+A indicated that less nitrification occurred in the RAS+A than the RAS-A and that ammonia removal was partly contributed by the algae.
- When the bacterial processes were affected by perturbation, the algae played their role in removing the ammonia. This helped the RAS to recover earlier from the perturbation.
- The algae influenced the bacterial community composition in a RAS.

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Summary

Recirculating aquaculture systems (RAS) are becoming important for aquaculture due to land and water supply limitations, and due to their low environmental impact. Bacteria are important in a RAS as their role in nutrient recycling is the main mechanism for waste removal in these systems. The presence of microalgae, in addition to bacteria, in a RAS can further improve the water quality through the absorption of inorganic nitrogen (ammonium and nitrate) and phosphorus from the water. On top of that, microalgae can influence the bacterial community composition in the culture water. However, the effects of microalgae on the bacterial community and on the stability of the RAS are unknown. This study aimed at finding the effects of microalgae on the water quality and bacterial community in a RAS. Four main sub-objectives were included in order to achieve the general objective;

- 1- To review the state of the art of algae incorporation in a RAS
- 2- To measure the ammonia and nitrate removal by *Stigeoclonium nanum*, a periphytic microalga selected for this study
- 3- To observe the effects of microalgae inclusion on the bacterial community in a RAS
- 4- To study the effects of microalgae inclusion on the water quality and on the stability (resistance and resilience) of a RAS under both normal conditions and perturbed conditions

In **Chapter 2**, nitrogen removal by algae and algae reactor operation in a RAS were reviewed. Although algae are widely used in waste water treatments, reports on the use of algae in a RAS are scarce. The size needed for the algae reactors and the cost of maintaining the algae are believed to limit the application of algae in a RAS. From the analysis, it is learned that with a surface area ratio of an algae reactor to a fish culture unit of 1:1 to 2:1 then the algae reactors removed between 6 to 25% of the nitrogenous waste produced in the RAS. In addition, factors influencing algae efficiency are (a) the RAS configuration that influences nitrogen loading and nitrogen species (ammonium versus nitrate),

(b) the cultivation methods (suspended versus attached), and (c) environmental conditions (light, temperature, pH, oxygen, and carbon dioxide).

A periphytic microalga, *Stigeoclonium nanum* isolated from the experimental facility was selected in this study (**Chapter 3**). This species is presumed to suit the RAS condition because the species can tolerate highly contaminated water, and is easy to manage. From the observations of the author, the growth and nitrogen uptake of *S. nanum* was found to be higher when cultured using an immobilization technique rather than cultured in a normal suspension. The results of this study also demonstrated that when both total ammonia nitrogen (TAN) and nitrate-N were added to the culture medium, the uptake of TAN by *S. nanum* was significantly ($p < 0.05$) more efficient than the nitrate-N uptake.

This research continued with the integration of *S. nanum* in a RAS (**Chapter 4**). The objective of this study was to determine the effect of microalgae on water quality (TAN, nitrite, nitrate, and phosphate) and bacterial composition in a fresh water small-scale RAS. The immobilization technique was applied to introduce the microalgae in the RAS. A rapid fingerprint analysis known as denaturing gradient gel electrophoresis (DGGE) was used to determine the bacterial community composition in the water. The results showed that the TAN concentration was not significantly different ($P > 0.05$) between the RAS with algae (RAS+A) and the RAS without microalgae (RAS-A). However, nitrite, nitrate and phosphate were significantly lower in the RAS+A than the RAS-A ($P < 0.05$). Pielou's evenness and the Shannon diversity index of the bacterial community between the treatments were not different ($P > 0.05$). However, the bacterial composition between the treatments was significantly different ($P < 0.05$).

The capability of *S. nanum* to improve the RAS was further tested by introducing an acute pH drop (from pH 7 to 4 over three hours) to the system (**Chapter 5**). The water quality and bacterial community were monitored during the experiment. A 16S rRNA metagenomic analysis was used to identify the bacterial community composition in the RAS. The RAS with microalgae (RAS+A) and the RAS without microalgae (RAS-A) were affected by the pH stressor which was indicated by the same level of resistance and resilience towards the stressor in the function of ammonia conversion to nitrite (a high ammonia level). The same resistance level was also observed in the function of nitrite conversion

to nitrate (a high nitrite level). However, the RAS+A had a higher resilience level than the RAS-A in the function of nitrite conversion to nitrate which was indicated by a faster recovery to a low nitrite level in the RAS+A than the RAS-A. In terms of overall bacterial communities, the composition and predictive function of the bacterial communities were significantly different between the RAS+A and the RAS-A. In the RAS+A, algae-associated bacteria were more dominant than in the RAS-A. Meanwhile, bacteria which involved in xenobiotic degradation were more dominant in the RAS-A than in the RAS+A.

In **Chapter 6**, the implications of the thesis findings were discussed. The microalgae improved the RAS water quality through the concept of partitioning where the removal of ammonia is partitioned into a nitrification process, heterotrophic bacterial assimilation and microalgae assimilation. When the bacterial processes were affected by the stressor, the microalgae played their role in removing the ammonia. This helped the RAS to recover earlier from the perturbation.

This study showed that the microalgae could influence the bacterial community composition of a RAS (**Chapter 4 and Chapter 5**). As elaborated briefly in **Chapter 1**, there are many compounds or metabolites originated from microalgae that could inhibit or stimulate bacterial growth. Therefore, in the future, microalgae which produce interesting compounds, which are beneficial for aquaculture, for example for controlling harmful bacteria and for steering larvae gut microbiota, should be further tested in a RAS.

Overall, this thesis gives an insight into the possible mechanisms of improving the water quality and stability of a RAS by use of microalgae. Although microalgae cannot totally replace nitrification and denitrification in a RAS, microalgae can partly contribute to other water quality benefits. Microalgae functions in a RAS are not only restricted to eliminate inorganic nitrogen, phosphorus and other contaminants, but also to influence the RAS bacterial community that could further improve fish health.

About the Author

Norulhuda Mohamed Ramli was born on 4th September 1980 in Tanjong Karang, Selangor, Malaysia. She obtained her bachelor degree in Biological and Agricultural engineering at Universiti Putra Malaysia in 2004. After graduation, she worked as an officer of economic affairs with the Malaysian Farmers' Organization Authority for a year. During this period, she engaged directly with the activities of the farmers. In 2005, Norulhuda joined Universiti Putra Malaysia, UPM as a tutor. In 2006, she received a scholarship from the Ministry of Higher Education, Malaysia to pursue her MSc degree in aquaculture at the Aquaculture and Fisheries Group, Wageningen University, The Netherlands. After completion of her MSc degree in 2008, Norulhuda served UPM for another two years before embarking on her Ph.D. in the same research group. Norulhuda intends to will continue her research in UPM after the completion of her Ph.D.



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List of Publications

Nieuwegiessen, P.G. van de; **Ramli, N.M.**; Knegtel, R.M.A.; Verreth, J.A.J.; Schrama, J.W. (2010)
Coping strategies in farmed African catfish *Clarias gariepinus*. Does it affect their welfare?
Journal of Fish Biology 76 (10). - p. 2486 - 2501.

Mohamed Ramli, N., Verdegem, M.C.J., Yusoff, F.M., Zulkifely, M.K., Verreth, J.A.J., (2017).
Removal of ammonium and nitrate in recirculating aquaculture systems by the epiphyte *Stigeoclonium nanum* immobilized in alginate beads. Aquaculture Environment Interactions, 9, 213-222.

Mohamed Ramli, N., Giatsis, C., Md Yusoff, F., Verreth, J., Verdegem, M., (2018). Resistance and resilience of small-scale recirculating aquaculture systems (RAS) with or without algae to pH perturbation. PLoS ONE, 13, e0195862.

Mohamed Ramli, N., Md Yusoff, F., Giatsis, C., G.Y.A. Tan, Verreth, J., Verdegem, M., (2018). Effects of *Stigeoclonium nanum*, a fresh water periphytic microalga on water quality in a small-scale recirculating aquaculture system. Aquaculture Research. Accepted for publication.

Training and supervision plan¹⁰

The basic package (3 ECTS)¹¹

WIAS Introduction Course	2011
Ethics and Philosophy in Life Sciences	2015

Scientific Exposure

International Conferences (5.4 ECTS)

Malaysia-Thailand Graduate Forum in Life Science, Food Science and Agriculture, Bangkok, Thailand	2012
World Aquaculture, 2015, Jeju, South Korea	2015
International Conference on Marine Science and Aquaculture, Sabah, Malaysia	2016
Third International Conference on Agricultural and Food Engineering, Kuala Lumpur, Malaysia	2016
Asian-Pacific Aquaculture 2017, Kuala Lumpur, Malaysia	2017

Seminars and workshop (2.1 ECTS)

Third series of vaccine and therapeutic seminar and workshop: Fish disease, Universiti Putra Malaysia	2012
Data management, analyses, and interpretation using statistical software workshop, Universiti Putra Malaysia	2013
Seminars and workshops on utilization of microbial resources and phylogenetic analysis, Universiti Putra Malaysia	2016

Presentations (5 ECTS)

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Oral presentation at the WIAS Science Day	2015
Oral presentation at the World Aquaculture, Jeju	2015
Poster presentation at the International Conference on Marine Science and Aquaculture, Sabah, Malaysia	2016
Oral presentation at the Asian-Pacific Aquaculture, Kuala Lumpur	2017

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¹⁰ Completed in the fulfilment of requirements for the education certificate of the Graduate School, Wageningen Institute of Animal Science (WIAS)

¹¹ One ECTS equals a study load of 28 hour

Survival guide to peer review	2015
Thesis writing and reference management	2016
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Research Skills Training (6 ECTS)	
Preparing own PhD research proposal	2011
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Didactic Skills Training (1 ECTS)	
Supervising BSc thesis	2013
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Total ECTS	37.5
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Colophon

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