Department of Environment and Agriculture

Efficacy of duckweed (*Lemna minor* Linnneus) integrated in barramundi recirculating aquaculture system (RAS)

Ardiansyah

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

January 2018

DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number: AEC_2013_16

Signature:

Date: 27 January 2018

ACKNOWLEDGEMENTS

I am immensely grateful to my supervisor Prof. Ravi Fotedar for his continuous support, guidance, and patience throughout my postgraduate program. His knowledge, foresight and hard work were essential to the completion of my project. I would also like to thank my co-supervisor Dr. Jane Fewtrell for her insightful comments and valued advice in the completion of my thesis.

There are many people that made this project possible. I am grateful to Mr. Simon Longbottom for his help, advice and endless encouragement. My exceptional CARL manager Rowan Kleindenst, thank you for all your hard work, friendship, and shared laughs during my study.

I would especially like to acknowledge my funding sources DIKTI, Indonesia. I am grateful for the support of many postgraduate students; Huy, Hung, Ha, Ilham, Sulaiman, Bin, Sulaiman, Irfan, and Ky during the bad times and for the endless talks over “many” cups of coffee.

Firmansyah Bin Abdul Jabbar, thank you for assisting me during my lab work, I will never forget your unconditional friendship and support. Thank you to everyone in the Curtin Aquatic Research Laboratory Unit, especially to Anthony Cole for all the fun and great moments during these years.

Finally, to my family, my wife, my son and my daughters for their everlasting love, Indonesian craziness, and unconditional support.

TABLE OF CONTENTS

DECLARATION Ii
ACKNOWLEDGEMENT Iii
TABLE OF CONTENTS Iv
PREAMBLE X
ABSTRACT Xiv
LIST OF FIGURES Xviii
LIST OF TABLES Xix
LIST OF ABBREVIATIONS Xxii
CHAPTER 1. INTRODUCTION 1
1.1. Background Information 1
1.2. Aim 5
1.3. Objectives 5
1.4. Significance 6
CHAPTER 2. LITERATURE REVIEW 8
2.1. Aquaculture 8
2.2. Recirculating Aquaculture Systems (RAS) 9
2.3. Integrated aquaculture systems (IAS) 11
2.3.1. Benefits of IAS 13
2.4. Integrated Recirculating Aquaculture Systems (IRAS) 15
2.5. Stocking density 16
2.6. Barramundi aquaculture 18
2.7. Duckweed (*Lemna minor* Linnaeus) 19
2.8. Phosphate solubilising bacteria (PSB) 26
2.8.1. Isolation and screening of PSB 27
2.9. Heterotrophic bacteria 29
2.10. Stress indicator 31
CHAPTER 3: Evaluating and Validating Optimum Harvest Frequency of Duckweed (*Lemna minor* Linnaeus) To Improve Growth Rates of Barramundi (*Lates calcarifer* Linnaeus) and Nitrogen Uptake Efficiency in An Integrated Recirculating Aquaculture System 34

3.1. Introduction …………………………………………………………… 34
3.2. Materials and Methods ………………………………………………... 36
3.2.1. Trial 1: TAN uptake efficiency of duckweed associated with different harvest frequency of duckweed ……………………………… 37
3.2.1.1. Fish and experimental condition ………………………….. 37
3.2.1.2. Experimental protocol ……………………………………. 38
3.2.2. Trial 2: TAN conversion rate and biomass production of duckweed when cultured with the juvenile barramundi in RAS ……... 39
3.2.2.1. Fish and experimental condition ………………………….. 39
3.2.2.2. Experimental protocol ……………………………………. 40
3.2.3. Statistical analysis ………………………………………………. 41
3.3. Results ………………………………………………………………… 41
3.3.1. TAN uptake rate and uptake efficiency associated with different harvest frequency of duckweed ……………………………………... 41
3.3.2. TAN conversion rate and biomass of duckweed and growth of barramundi ………………………………………………………………... 43
3.4. Discussion …………………………………………………………….. 46
3.4.1. TAN uptake rate and uptake efficiency associated with different harvest frequency of duckweed ……………………………………... 46
3.4.2. Growth and TAN conversion rate of duckweed ………………… 48
3.5. Conclusions …………………………………………………………… 49
CHAPTER 4: The Abundance and Diversity of Heterotrophic Bacteria as a Function of Harvesting Frequency of Duckweed (*L. minor* Linnaeus) in Recirculating Aquaculture Systems ……………………………………………………………………………… 50
4.1. Introduction …………………………………………………………… 50
4.2. Materials and Methods ……………………………………………….. 51
4.2.1. Experimental setup and design ………………………………….. 51
4.2.2. Identification and enumeration of bacteria from biofilter tanks … 52
4.2.3. Bacteria diversity index …………………………………………. 54
4.2.4. Statistical analysis ………………………………………………. 54
4.3. Results ………………………………………………………………… 54
4.3.1. Abundance and diversity of heterotrophic bacteria ……………... 54

4.3.2. Growth performance of duckweed ………………………………… 55
4.3.3. Morphological and biochemical charatcteristics of heterotrophic bacteria ………………………………………………………………... 56
4.4. Discussion ……………………………………………………………….. 60
4.5. Conclusions ……………………………………………………………… 62
CHAPTER 5: The Abundance and Diversity of Phosphate Solubilising Bacteria Associated with the Harvesting Regime of Duckweed (*Lemna minor* Linnaeus) In Integrated Recirculating Aquaculture Systems (IRAS) ………………………………………………………………………... 63
5.1. Introduction ……………………………………………………………… 63
5.2. Materials and Methods ………………………………………………….. 65
5.2.1. Experimental system ……………………………………………… 65
5.2.2. Isolation, enumeration, and evaluation of phosphate solubilising bacteria and phosphatase activity ………………………………... 66
5.2.3. Bacteria diversity index …………………………………………… 68
5.2.4. Statistical analysis ………………………………………………… 68
5.3. Results ……………………………………………………………………… 68
5.3.1. Abundance and diversity of PSB ……………………………….. 68
5.3.2. Phosphate solubilising efficiency, IAA, and phosphatase activity 70
5.3.3. Morphological and biochemical characteristic of PSB ………… 72
5.4. Discussion ……………………………………………………………….. 75
5.5. Conclusions ……………………………………………………………… 79
CHAPTER 6: Water quality, growth and stress responses of juvenile barramundi (L. calcarifer Bloch), reared at four different densities in integrated recirculating aquaculture systems ………………………… 80
6.1. Introduction ……………………………………………………………… 80
6.2. Materials and Methods ………………………………………………….. 82
6.2.1. Animals and stress factors ……………………………………… 82
6.2.2. Experimental design and rearing condition ……………………... 82
6.2.3. Water quality parameters ………………………………………... 83
6.2.4. Growth parameters ………………………………………………… 83
6.2.5. Chronic and acute stress response analysis ……………………… 83
6.2.5.1. Chronic stress responses ………………………………….. 84

6.2.5.2. Acute stress response ………………………………………... 85
6.2.6. Statistical analysis …………………………………………………….. 85
6.3. Results ………………………………………………………………… 85
6.3.1. Water quality parameters ……………………………………………... 85
6.3.2. Growth and feed utilisation of juvenile barramundi …………….. 86
6.3.3. Stress responses ………………………………………………………. 87
6.4. Discussion …………………………………………………………….. 91
6.5. Conclusions …………………………………………………………… 97
CHAPTER 7: The physiological responses of two–sized juvenile barramundi (*Lates calcarifer* Bloch) when duckweed (*Lemna minor* Linnaeus) as a biofilter medium is incorporated into the rearing system ……………………………………………………………………... 98
7.1. Introduction …………………………………………………………… 98
7.2. Materials and Methods ………………………………………………... 100
7.2.1. Fish and stress factors ………………………………………………… 100
7.2.2. Experimental design …………………………………………………….. 101
7.2.3. Growth performance …………………………………………………….. 101
7.2.4. Physiological response analyses ……………………………………… 102
7.3. Results ………………………………………………………………… 102
7.3.1. Physiological response ……………………………………………….. 102
7.3.2. Water quality and growth performance ……………………………. 105
7.4. Discussion …………………………………………………………….. 107
7.5. Conclusions …………………………………………………………… 111
CHAPTER 8: Can duckweed (*Lemna minor* Linnaeus) harvested from IRAS be recycled into barramundi (*Lates calcarifer* Bloch) diets as a partial replacement of protein from the fishmeal? 113
8.1. Introduction …………………………………………………………… 112
8.2. Materials and Methods ……………………………………………….. 114
8.2.1. Animals ……………………………………………………………. 114
8.2.2. Fermentation of duckweed ………………………………………… 114
8.2.3. Diet preparation ……………………………………………………. 115
8.2.4. Experimental design and rearing condition ……………………... 115
8.2.5. Chemical analyses and data collection ………………………….. 116

8.2.6. Physiological parameters ……………………………………….. 116
8.3. Results ……………………………………………………………… 116
8.4. Discussion ………………………………………………………….. 121
8.5. Conclusions ………………………………………………………… 125
CHAPTER 9: Summary and General Discussion ……………………… 126
9.1. Introduction ……………………………………………………….. 126
9.2. General discussion ………………………………………………… 126
9.2.1. Stocking desnity, water quality, stress, and growth interactions in barramundi IRAS …………………………………………………... 126
9.2.2. The use of duckweed (*Lemna minor*) harvested from IRAS as a partial replacement of fishmeal in the diets of barramundi (*Lates calcarifer*) ………………………………………………………………. 128
9.2.3. Duckweed (*L. minor*), heterotrophic, and phosphate solubilising bacteria interactions in IRAS ……………………………………………. 132
9.3. Conclusions ………………………………………………………... 135
9.4. Limitations of IRAS ……………………………………………….. 137
9.5. Recommendations ………………………………………………… 137
REFERENCES ……………………………………………………………… 138
APPENDIX A: THE CURRENT LIST PUBLICATIONS……….…… 178

PREAMBLE

The aim of this research is to evaluate the effectiveness of duckweed (*Lemna minor* Linnaeus) when cultivated in barramundi (*Lates calcarifer* Bloch) integrated recirculating aquaculture system (IRAS). The effectiveness of duckweed in nitrogen removal was accomplished by evaluating the water quality, nitrogen removal efficiency, the abundance, and diversity of heterotrophic bacteria and phosphate solubilising bacteria, growth, and physiological stress response of barramundi when they were integrated with duckweed in IRAS. The triple role of duckweed as (i) an effective biological filter media, (ii) a substrate to harbour beneficial heterotrophic bacteria that paly crucial roles in removing nitrogen and phosphorus and finally (iii) effectiveness of duckweed protein to replace dietary fishmeal protein in carnivorus fish, were evaluated in a series of six experiments.

This thesis consists of nine chapters. Chapter 1 in the form of an introduction briefly highlights the current issue of aquaculture, barramundi industry, recirculating aquaculture systems (RAS), and integrated recirculating aquaculture systems (IRAS). The negative impacts of intensive aquaculture systems and the currently published knowledge on the aquaculture effluents, and the nutrient uptake capacities of duckweed. This chapter also justifies and underlines the need to undertake the current research.

Chapter 2 reviews the research into aquaculture, barramundi aquaculture industry; recirculating aquaculture systems, integrated aquaculture systems, benefits of integrated aquaculture, the current knowledge of phosphate solubilising bacteria and heterotrophic bacteria, the current knowledge on the effect of stocking densities on physiological stress responses and published information on the nutrient uptake capacities of duckweed.

Chapter 3, 4, 5, 6, 7, and 8 detail the main research in this thesis and attempt to evaluate the relationship between harvesting frequency of duckweed and the nitrogen uptake efficiency, the abundance and diversity of heterotrophic bacteria and phosphate solubilising bacteria, physiological stress responses of barramundi and stocking densities, the growth performance, feed utilization, and physiological of barramundi when fed with the fermented duckweed incorporated in the formulated

feed. All these chapters form an essential component of this research and can be viewed as independent experiments bound by a common theme. The experimental design and certain section in the introduction in these chapters may appear as repetitive, as it was essential to maintain the flow of information independently within these chapters. Moreover, these chapters are either published or have been submitted in various journals (**Appendix A**) and hence the repetitiveness.

Chapter 3 includes an experiment that investigates the effects of different quantities of duckweed in real time (using different harvesting frequencies) as biofilter media on total ammonia nitrogen (TAN) uptake rates and growth of barramundi in IRAS. Chapter 4 presents an experiment with an aim to investigate the effects of different harvesting frequencies of duckweed in a RAS on the composition, abundance, and diversity of non-pathogenic heterotrophic bacteria, involved in the removal of nitrogenous waste from the IRAS. Chapter 5 describes an experiment that is aimed to evaluate the effects of different amounts of duckweed in real-time (using a different harvest regime) as the media on the composition, abundance, and diversity of phosphate solubilising non-pathogenic bacteria that are involved in the removal of phosphates in IRAS. Chapter 6 descibes an experiment to investigate the chronic responses of high stocking density of juvenile barramundi (*L. calcarifer*) on the growth performance and stress-related parameters in barramundi in an IRAS followed by challenging the fish with ammonia exposure in order to evaluate the acute stress responses. The most suitable harvest frequency of duckweed in chapter 4 and 5 are applied. Additionally, the most optimum stocking density of barramundi, water quality parameters, growth, and survival barramundi are investigated in this chapter.

Chapter 7 presents an experiment aiming to investigate the effect of high fish stocking density on the juvenile barramundi of two different fish size on growth performance and physiological parameters, which then is used to compare the physiological status under different rearing systems and to validate the efficacy of IRAS. This chapter highlights the difference between RAS and IRAS.

Chapter 8 details the experiment that is aimed to determine optimum inclusion level of the fermented duckweed in the formulated feed and to evaluate the growth performance, feed utilization, and physiological parameters of juvenile barramundi.

Water quality parameters and bio-physiological parameters are investigated in this chapter. Chapter 9 summarizes the nutrient retention in different integrated culture models. The chapter also synthesizes the main findings of this project. The data collected from this research are also compared to obtain a more complete picture in the application of integrated recirculating aquaculture system. Effects of different harvest frequencies on nitrogen uptake efficiency, and the abundance and diversity of heterotrophic bacteria and phosphate solubilising bacteria, effects of different stocking densities of barramundi on physiological stress responses of barramundi reared in RAS and IRAS are combined and presented. An assessment of the inclusion of fermented duckweed in the formulated feed is also discussed. The main conclusions are highlighted, which are then followed by the recommendations for future research.

ABSTRACT

Intensive aquaculture has a potential to generate some negative impacts on the surrounding aquatic environment by increasing water pollution, algal blooms, and reduction in biodiversity of the natural flora and fauna. The nutrient-rich effluents from aquaculture activities contain high nitrogen and phosphates that serve as a nutrient source for bivalves, algae, and some other invertebrates.

In this research the duckweed (*Lemna minor* Linnaeus) was integrated with barramundi (*Lates calcarifer* Bloch) in the recirculating aquaculture system (RAS) in order to remove the waste from the effluents, and this integrated system was termed as Integrated Recirculating Aquaculture System (IRAS). This research has explored the nitrogen uptake capacities of duckweed integrated with barramundi in this IRAS and has evaluated the nitrogen retention of duckweed in the system.

The research can be divided into three facets in order to understand the efficacy of IRAS integrating duckweed and barramundi (*Lates calcarifer*). A series of experiments were conducted under laboratory conditions to evaluate the nutrient uptake efficiency of duckweed associated with harvest frequency from 0-6 days from juvenile barramundi effluents, and to investigate effects of different stocking densities of juvenile barramundi on nitrogen conversion rates and biomass production of duckweed and barramundi. A total of six independent trials were conducted in an experimental unit of an IRAS that consisted of three tanks: a fish rearing tank, a biofilter tank harbouring duckweed, and a waste-collection tank. The results suggested that harvest frequency significantly affected the growth index (GI) and harvested biomass of duckweed, which in turn influenced the total ammonia nitrogen (TAN) uptake rate. A four-day harvest frequency contributed to the maximum uptake efficiency, equivalent to a 94.33% and 60.73% removal of the TAN and total nitrogen (TN), respectively. Stocking density and nitrogen load positively correlated with duckweed growth. The specific growth rate (SGR), feed conversion rate (FCR) and survival rates (SR) of juvenile barramundi were not adversely affected by the different stocking densities and nitrogen load.

In an experiment to investigate the effects of different stocking densities on water quality, growth and physiological stress responses of juvenile barramundi, showed that the TAN and nitrite-N levels were similar in all fish stocking densities, while TP, orthophosphate, total nitrogen and nitrate-N levels were significantly higher in the tank with a stocking density of 18.75 than 10.10 and 12.98 kg m^{-3}. The mean weight gain (WG) and SGR of the juvenile barramundi that were reared at 18.75 kg m^{-3} were significantly lower than at other fish densities, whereas the mean FCR was significantly increased at higher density. Stocking density had significant effects on basal plasma levels of total T_4 (thyroxine), total T_3 (triiodothyronine), cortisol, glucose, lactate and whole-blood Hb (haemoglobin). A stocking density of less than 18.75 kg m^{-3} of barramundi is recommended for culturing in IRAS. Furthermore, two other experiments investigated the interactions among duckweed's harvest frequencies up to 6 days and the abundance and diversity of heterotrophic bacteria and phosphate-solubilising bacteria (PSB). The results proved that the strongest correlations (R^2>0.65) between duckweed SGR and biomass harvest with the heterotrophic bacteria and PSB diversity were observed at 4-day harvest frequency, indicating that harvesting duckweed every four days provides desirable conditions for the attachment and growth of bacteria, thus increasing the abundance and diversity of both heterotrophic bacteria and PSB.

In another experiment that was performed to evaluate the physiological responses of juvenile barramundi of different size, under different rearing systems, showed high stocking density of 21.63 kg m^{-3} influenced the physiological stress responses in a differential manner according to fish size, but no differences were observed in the experimental fish reared in RAS or IRAS.

The optimum inclusion level of the fermented duckweed in the formulated feed for barramundi was determined by evaluating the growth and physiological parameters of barramundi when feed fermented duckweed replacing fishmeal protein. The results showed that the highest fish WG and SGR were in the group of fish reared on diet F2 (25% duckweed meals inclusion level). Fish fed with F3 (35% duckweed meals inclusion level) also showed good performance in terms of WG and SGR. Protein efficiency ratio (PER) was highest in fish fed F3 which was significantly different from those reared with other diets. PER value was lowest with the control

diet, while FCR value was lowest for fish fed F3 and highest for F4 (45% duckweed meals inclusion level) diet. Glucose, cholesterol, low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) levels were significantly influenced by the dietary treatments. Apparent net protein utilization (ANPU) was highest in juvenile barramundi reared on a diet F2 and lowest with diet F4. Decreased plasma alkaline phosphatase (ALP) observed in the fish fed on fermented duckweed meal diets may be an indication of the healthier state of the plasma membranes of these fish than those in the control groups. The results indicated that the use of IRAS is a step forward to achieve sustainability of barramundi aquaculture.

LIST OF FIGURES

Figure 3.1	Design of the RAS used in the experiment …………………………..	38
Figure 3.2	Total ammonia nitrogen (TAN) uptake rate of duckweed harvested every 0, 2, 4, 6 days in barramundi (L. calcarifer) RAS over the 36 day trial ………………………………………………………………..	42
Figure 3.3	TAN uptake efficiency of duckweed in trial 1 ………………………..	43
Figure 6.1	Total T_4 concentration of blood plasma before and after an acute stressor. Data are shown as the mean ± SE …………………………..	88
Figure 6.2	Total T_3 concentration of blood plasma before and after an acute stressor. Data are shown as the mean ± SE …………………………..	88
Figure 6.3	Cortisol contents of blood plasma before and after an acute stressor. Data are shown as the mean ± SE …………………………………….	89
Figure 6.4	Glucose contents of blood plasma before and after an acute stressor. Data are shown as the mean ± SE …………………………………….	90
Figure 6.5	Lactate contents of blood plasma before and after an acute stressor. Data are shown as the mean ± SE …………………………………….	90
Figure 6.6	The Hb contents of blood plasma before and after an acute stressor. Data are shown as the mean ± SE …………………………………….	91
Figure 7.1	Cortisol concentrations of plasma blood in small and large-size fish after 48-day experimental period. Data are shown as the mean ± SE...	103
Figure 7.2	Glucose concentrations of plasma blood in small and large-size fish after 48-day experimental period. Data are shown as the mean ± SE...	103
Figure 7.3	Lactate concentrations of plasma blood in small and large-size fish after 48-day experimental period. Data are shown as the mean ± SE...	104

LIST OF TABLES

Table 2.1	Selected data on nitrogen removal efficiencies of aquatic plants …………………………………………………………………	23
Table 2.2.	Summary of TN removal rates and efficiency of duckweed for waste water treatments ……………………………………..	25
Table 3.1	TAN uptake rate, nitrification rates and physicochemical parameters of water in the fish-rearing tank in trial 1………	41
Table 3.2	Growth characteristics of barramundi (*L. calcarifer*) and duckweed (*L. minor*) in trial 2. …………………………...	44
Table 3.3	Variations in TAN production, the volumetric TAN conversion rate (TCR) and mean TAN uptake rate (*UR*mean) of duckweed in trial 2. …………………………………………………..	45
Table 3.4	Physicochemical properties of the water as the function of duckweed usage as biofilter media in trial 2. …………….....	46
Table 4.1	Abundance of heterotrophic bacteria attached to the duckweed in the biofilter tank. …………………………	54
Table 4.2	Shannon diversity index, total harvested biomass of duckweed, Mean GI and SGR of duckweed with correlation coefficients (R^2) between growth performance represented by biomass harvest and SGR of duckweed with abundance and diversity of heterotrophic bacteria. ……………………...............................	55
Table 4.3	The morphological and physiological characteristics of bacteria isolated from duckweed in the biofilter tanks over the 36-day trial. ……………………………………………….	56
Table 4.4	Biochemical tests of the heterotrophic bacteria attached to the duckweed in the biofilter tanks. ……..	58
Table 5.1	Abundance of phosphate solubilising bacteria attached to duckweed in the biofilter tank. ……………………….	69
Table 5.2	Correlation between SGR and total biomass harvest of duckweed with abundance and diversity of phosphate solubilising bacteria over the 36-day trial. ………………………	70
Table 5.3	The phosphate solubilising efficiency and phosphatase activity recorded for the putative	

	PSB bacteria attached to duckweed in the biofilter tank. ……………………………………………………	71
Table 5.4	The morphology and physiological characteristics of the putative phosphate solubilising bacteria attached to duckweed.	72
Table 5.5	The morphological and biochemical characteristics of PSB isolated from the biofilter tanks. ……………………………...	74
Table 6.1	Effects of four stocking densities of juvenile barramundi (*L. calcarifer* Bloch) on physicochemical parameters of the water in IRAS. ………………………………………………	86
Table 6.2	Effects of four stocking densities on growth performance of the juvenile barramundi (*L. calcarifer* Bloch) reared in IRAS.	87
Table 6.3	Selected data on the effects of stocking density on growth of several important fish species in RAS. ………………………	95
Table 7.1	Alterations in thyroid hormones and plasma ions in smaller and larger juvenile barramundi (*L. calcarifer*) when reared at two different rearing systems. ………………………………..	105
Table 7.2	Summary of water quality assessment in *L. calcarifer* RAS and IRAS over the 48-day experimental period. ………….....	106
Table 7.3	Growth of juvenile barramundi (*L. calcarifer*) reared at two different rearing systems for 48 days. ……………………....	107
Table 8.1	Proximate composition of dried and fermented duckweed (% dry weight). …………………………………………….......	117
Table 8.2	Ingredient composition (% dry weight) and proximate analyses of dried and fermented duckweed incorporated feeds (on dry matter basis). ……………………………..........................	118
Table 8.3	Growth and feed utilization efficiencies in juvenile barramundi fed experimental diets for 63 days. …………………………….	109
Table 8.4	Proximate carcass compositions (% wet weight) of juvenile barramundi at the start and end of the 63-day feeding trial. …...	120
Table 8.5	Glucose, cholesterol, LDL-C, HDL-C, ALT, AST and ALP changes of juvenile barramundi in response to dietary fermented duckweed meal inclusion. ………………………….	120
Table 9.1	Summary of stocking density, TAN (total ammonia nitrogen),	

	cortisol and growth rate (SGR) of barramundi (L. calcarifer) in IRAS ………………………………………………………..	128
Table 9.2	Selected data on crude protein contents of aquatic plants.	129
Table 9.3.	Activities associated with the duckweed biofilter and participating microorganisms …………………………………	135

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
ANPU	Apparent Net Protein Utilization
AOAC	Association of Official Analytical Chemists
APD	Apparent Protein Digestibility
APHA	American Public Health Association
AST	aspartate aminotransferase
CARL	Curtin Aquatic Research Laboratory
CFU mL^{-1}	Colony forming unit per millilitre
D	Day
DO	Dissolved Oxygen
FAO	Food and Agriculture Organization
FCR	Feed conversion ratio
G	Gram
G	Gravity
GI	Growth Index
H	Hour
HDL-C	High Density Lipoprotein-Cholesterol
IRAS	Integrated Recirculating Aquaculture Systems
Kg	Kilogram
L	Litre
LDL-C	Low Density Lipoproptein-Cholesterol
m^2	Square meter
mg L^{-1}	Milligram per litre
mL	Millilitre
N	Nitrogen
P	Phosphorus
PER	Protein Efficiency ratio

PSB	Phosphate-Solubilising bacteria
RAS	Recirculating Aquaculture Systems
SGR	Specific Growth Rate
SPSS	Statistical Package for the Social Science
TAN	Total Ammonia Nitrogen
TN	Total Nitrogen
TP	Total Phosphorus
T3	Thyroxine
T4	Triiodothyronine
WG	Weight Gain

CHAPTER 1: Introduction

1.1 Background information

Similar to many other agricultural activities, aquaculture depends on the use of natural resources like water, land, seed, and feed (Barg, 1992; Phillips and Mcintosh, 1997). Future availability of the above resources will considerably influence the development of the industry. Aquaculture is also dealing with the challenge of sustainable development (Pauly et al., 2003). As the concerns over potential environmental problems have significantly increased, the challenge for aquaculture is to make it safer in terms of environmental sustainability (Waite et al., 2014). More intensive production systems are expected to reduce water-use but can cause pollution due to many material inputs and many waste outputs (Diana, 2009).

Intensive aquaculture activity provides substantial quantities of high-protein feed to the cultured species, however, retention capacity for N and P is usually low and variable among the species, leading to significant releases of both dissolved and particulate wastes to the surrounding environment. Studies of intensive shrimp culture systems in Thailand found that only 21% of nitrogen used for the pond was assimilated as harvested biomass while 35% was discharged into the water (Briggs and Funge-Smith, 1994). The wastewater composition of intensive aquaculture systems mainly comprises of solid wastes, dissolved metabolic waste, dissolved nutrients from feeds and faeces, and biocide and pharmaceutical residues (Midlen and Redding, 1998). Troell et al. (1999a) reported that supplied N discharged into surrounding waters in the form of ammonium (NH_4^+) is about 50-60%. Only about 10% of dissolved nutrient is released from prawn pond into the environment (Briggs and Funge-Smith, 1994).

Recirculating aquaculture systems (RAS) are land-based intensive culture systems that apply less water exchange because of the internal recycling of water and use of mechanical and biological filters (ORR, 1999). RAS for intensive fish farming have been used for more than two decades (Molleda, 2007). RAS is developed to raise large numbers of high-value fish species in a small volume of water by circulating the water to different types of filters to remove toxic waste products and then reuse it. However, using the water manyfold can cause accumulation of organic substance

and non-toxic nutrients. Therefore, most water quality problems found in RAS were related to elevated concentrations of nitrogen metabolite in the water (Sanni and Forsberg, 1996), including total ammonia nitrogen (TAN), unionised ammonia (NH_3-N), nitrate (NO_3-N) (to a lesser extent), nitrite (NO_2-N), and non-biodegradable organic matter (Molleda, 2007). Of these nitrogen metabolites, fish approximately produce 1.0-1.4 mg L^{-1} TAN for every 10 mg L^{-1} of dissolved oxygen that they consume (Hagopian and Riley, 1998).

Although the dissolved nitrogens are an important nitrogen source for plant and other species in the aquatic environment, their increased availability may have a significant impact on the balance of natural ecosystems. The negative impacts of high rich-nutrient flux may result in increased algae growth and biomass, reduction of biodiversity, toxic and non-toxic algae blooms and stimulating dinoflagellate growth (Troell et al., 1997). Additionally, increased nitrogen metabolite concentrations can cause growth retardation, increase fish stress, increase the risk infectious disease, and other problems associated with water quality deterioration which in turn lower productivity (Timmons et al., 2002). Municipal wastewater treatment with protein production using duckweed represents a comprehensive solution (Oron et al., 1988; Hammouda et al., 1995). In the past several decades, researches have explored the role of duckweed in wastewater treatment and its potential for nutrient recovery (Tripathi et al., 1991; Al-Nozaily et al., 2000; Smith and Moelyowati, 2001; Cheng et al., 2002; Li et al., 2009). The use of duckweed (*Lemna minor* Linnaeus) for the wastewater treatment has been investigated in dairy waste lagoons (Landesman et al., 2000; Sammouth et al., 2008), raw and diluted domestic sewage (EI-Shafai et al., 2007), secondary effluent (Korner et al., 2003), sewage-loaded ponds (Alaerts et al., 1996) and fish culture systems (Kabir et al., 2009). Additionally, the economical use of harvested duckweed has been tested with variable results (Skillicorn, 1993).

Barramundi (*Lates calcarifer* Bloch) is a big predatory species found in freshwater, estuarine, and coastal environments in the Indo-Pacific region. Barramundi is a favorite species choice for aquaculture in RAS due to its fast growing, short production cycle, higher-value markets, and tolerance to a wide range of water quality conditions (DPI, 2008; Peterson et al., 2015). With the development of Asian markets, barramundi production has increased in recent years. The level of

intensification witnessed in the fish production system has raised several environmental issues that need to be addressed in the context of the sustainability of the barramundi aquaculture industry (Amirkolaie, 2011). Most of the environmental issue raised in RAS are related with nitrogen metabolites levels. However, the end products of fish metabolism (ammonia, nitrites, and nitrates) can also be used if they are allocated to secondary crops that have some economic values or in some way benefit the primary production. As reported by Martins et al. (2010), the latest developments in RAS focus on the recycling of nutrients through integrated recirculating aquaculture systems (IRAS) that grow additional crops by utilizing by-products from the production of the primary species (Rakocy and Masser, 2006). In IRAS, the main and additional crops are separated by different units and the effluents of main species circulate through the culture units of the additional crops (Chien and Tsai, 1985).

Aquatic macrophytes are an ideal secondary crop in IRAS because they rapidly grow in response to increased concentrations of dissolved nutrients that are generated from the microbial breakdown of fish wastes (Rakocy and Masser, 2006). Among floating macrophytes, duckweed is used due to its nitrogen and phosphorus assimilation potential and its performance in the elimination of carbon pollution (Vermaat and Hanif, 1998; Al-Nozaily et al., 2000; Kone, 2002). Duckweed (*L. minor*) can stimulate nitrogen (N) removal of bacteria by releasing root exudates that can serve as carbon sources and non-nutrient signals for denitrification (Lu et al., 2014). Abundant exudates are available around duckweed niches because all parts of root surfaces and ventral sides of fronds have the same opportunity to have contact with bacteria (Zhou et al., 2013). Gram-positive and gram-negative rhizobacteria colonized a small part of duckweed, mainly the junction between epidermal cells and areas where lateral roots appeared (Liu et al., 2006; Fan et al., 2011). The colonization of roots by bacteria is linked to the formation of robust biofilm, structured communities of the bacterial cells adherent to a surface of duckweed root epidermis cells (Dang et al., 2011), while the formation of biofilm at duckweed surface is found to be connected with synthesis of surfactant (Bais et al., 2004).

Earlier scientific researches have indicated that the removal of environmental pollutant including excessive N and P in aquatic environments is through the direct

impact of the interaction of aquatic plant and bacteria (Zhou et al., 2010; Sharma et al., 2013; Lu et al., 2014). Bacteria play foundational roles in the aquatic environment, through their role in decomposition, releasing nutrients stored in organic tissue. Bacteria present in any RAS can be divided into two main groups; autotrophic and heterotrophic bacteria. Autotrophic bacteria are categorised as primary producers in aquatic systems (as are true algae), while heterotrophic bacteria are involved in the decomposition of organic matter and the cycling of nutrient in aquatic systems (Sharrer et al., 2005). Heterotrophic non-pathogenic bacteria are growing five times faster, generating a biomass yield of two to three times higher than autotrophic bacteria (Aoi et al., 2000), and efficiently removing ammonia when the appropriate carbon source is available (Russel and Cook, 1995; Michaud, 2006).

Duckweed growth rate in optimal conditions can lead to a large biomass coverage percentage which may induce dysfunction of such ponds (Demirezen et al., 2007; Lasfar et al., 2007). Hence, to avoid an excess of biomass and any subsequent problem, such as the death and the settling of duckweed creating a secondary pollution (Korner and vermaat, 1998; Jupsin et al., 2004) or to valuate the biomass for animal feed, a regular harvesting should be conducted. Additionally, harvesting frequency is one of the factors that affects the nutrient removal efficiency of duckweed besides initial nutrient concentration, and duckweed density (Al-Nozaily et al., 2000; Nhapi, 2004; Chaiprapat et al., 2005). Harvesting of duckweed is required to maintain faster growth rates and high nutrient uptake capacity. Additionally, if duckweed is not harvested, the nutrients from the duckweed decomposing detritus are rapidly back into the water (Al-Nozaily et al., 2000; Chaiprapat et al., 2005).

Considering the organic materials, pathogens, and the removal of nutrients, duckweed has shown promise to be used in effluent treatment (Smith and Moelyowati, 2001). Additionally, duckweeds being floating weeds can reduce the suspended solids by blocking light penetration. The availability of light causes the algae to die off, which finally settle or disintegrate. Ran et al. (2004) showed the benefits of using duckweeds due to its high production rates, easy manual harvesting, high protein and low fiber contents. Given that, aquaculture effluent treatment should aim to reuse nutrients effectively, the use of the duckweed is cost-effective for

recycling as fertilizer and animal feed (Ran et al., 2004). Recycling of domestic waste in fish farming and agriculture is an effective approach to control pollution, which may contribute to cost recovery and also provide cheap protein food production (El-Shafai et al., 2007). Reuse of waste in fish farming has been applied in the experimental system as well as in a commercial scale (Shereif et al., 1995; El-Shafai et al., 2004). The main purpose of this practice is to fertilize the pond and increase the primary and secondary productivity (Mara and Cairncross, 1989). However, there is no available information on integration of duckweed with barramundi in the IRAS. In an integrated system, farming effluents from Nile tilapia (*Oreochromis niloticus*) ponds flow through duckweed ponds, which serve as biofilters for removing dissolved nitrogen and then part of effluents circulated back to the tilapia pond. This process reduces nitrogen concentration in the effluent but fails to remove nitrogen completely from the total nitrogen input in the system (El-Shafai et al., 2007; Mohapatra et al., 2012; Tavares et al., 2010). Additionally, published data on the optimum stocking density, the growth performance, and physiological stress responses of barramundi (*L. calcarifer*) in an IRAS are limited.

1.2 Aim

The aim of this study is to evaluate the efficiency and sustainability of using duckweed (*L. minor* Linnaeus) as a biofilter medium for nutrient removal and its use as a feed source in barramundi (*L. calcarifer*) RAS.

1.3 Objectives

The above aim is achieved by meeting the following specific objectives:

1. To evaluate the effects of different harvesting frequencies of duckweed as biofilter media on nitrogen uptake rates in IRAS culturing barramundi.
2. The effect of different stocking densities of juvenile barramundi on nitrogen conversion rates and biomass production of duckweed and barramundi cultured in IRAS.
3. To evaluate the effects of duckweed's harvesting frequencies on the abundance and diversity of heterotrophic bacteria in the biofiltration of IRAS culturing barramundi.

4. To evaluate the effects of duckweed's harvesting frequencies on the abundance and diversity of phosphate-solubilising bacteria (PSB) in the biofiltration system of IRAS culturing barramundi.
5. To investigate the effect of different stocking densities of juvenile barramundi on nitrogen conversion rates and biomass production of duckweed and barramundi in IRAS.
6. To determine carrying capacity of IRAS culturing barramundi.
7. To evaluate effects of different stocking density on stress-related parameters and growth performances of juvenile barramundi cultured in IRAS
8. To investigate the growth performance and physiological status of barramundi of different sizes cultured in IRAS
9. To compare growth performance and physiological status of juvenile barramundi reared at IRAS and RAS, and to validate the efficacy of IRAS.
10. To determine the optimum inclusion level of the fermented duckweed, harvested from IRAS in the formulated feed for barramundi.
11. To investigate the growth performance and physiological parameters of barramundi when fed fermented duckweed replacing fishmeal protein in the formulated diet for barramundi.

1.4 Significance

The research aims to make contributions in the management of barramundi aquaculture effluent for sustainable barramundi industry by contributing in the understanding of nutrient removal capabilities of duckweed. The significances of the current research are as follows:

1. The study will assist in understanding the mechanisms and principles of using duckweed as a biofilter medium in IRAS.
2. The research will assist in understanding the nutrient uptake capacities of duckweed in the experimental culture conditions.
3. The research will contribute in sustaining the production of IRAS culturing barramundi via an application of appropriate stocking density.
4. The research will assist in understanding the effect of stocking density on physiological stress responses of barramundi in IRAS.

5. The research will assist in providing the fundamental information of nutrient removal by either duckweed or bacterial communities in IRAS.
6. The research will assist in understanding the interaction of duckweed and bacterial communities in IRAS.
7. The research will add the value of IRAS by recycling duckweed by product as ingredients of aquafeed.

CHAPTER 2: Literature Review

2.1 Aquaculture

Aquaculture can be defined as the farming and husbandry of aquatic organisms in controlled or semi-controlled conditions (FAO, 2011). Aquaculture has been recognized as the fastest growing food production sector worldwide over the last decade (FAO, 2006). Aquaculture is becoming a primary source of cheaper protein and income, making it a significant contributor to the world's food supply and economy. Fish contribute a large amount of animal protein for human food worldwide. About 15 and 20 percent of all animal proteins derived from aquatic animals (FAO, 2012). Fish is food with high nutritional values, both as a source of protein and as a provider of essential fatty acids. Aquaculture production value accounts for more than half of the total world fish production (FAO, 2016).

Aquaculture can be used to produce both plants (including seaweeds and freshwater macrophytes) and animals (including crustaceans, finfish, and molluscs) (FAO, 2006). Aquaculture techniques vary and can be categoried according to the intensity of practice: extensive and intensive. The classification of intensity is based on the amount of biomass within the system and the amount of manipulation required to maintain the desired biomass density (Midlen and Redding, 1998). Extensive aquaculture uses minimal inputs or minimal human manipulation thereby operating with a lower total biomass. Intensive aquaculture practices require extensive human manipulation but can operate with high biomass densities. Between these two broad categories, a continuum of different production methods exists (Pullin, 1989).

Aquaculture systems can also be distinguished by way of the water system contact with the environment, defined as either open or closed (Flimlin et al., 2008). An open system directly discharges effluent water to another body. This can be either a pool or flowing system, but some water exchange with the environment occurs, resulting in an effluent discharge (Stickney, 1994). Closed systems utilize some treatment mechanisms, which allow these systems to reuse a majority of the water rather than discharging it directly to the environment (Cottee and Petersan, 2009). Intensive systems can be either open or closed. As the production methods become more intensive, greater quantities of waste are generated. Depending on the fish species,

large amounts of fishmeal might be required to support high biomass densities leading to the production of waste (Naylor et al., 2000). Intensive aquaculture practices can further exacerbate negative environmental impacts through habitat destruction, the capture of wild seed stocks, introduction of exotic species, transfer of pathogens, taxation of water and energy resources and pollution of waters with nutrients and other chemicals (Naylor et al., 2000). Closed intensive aquaculture systems provide the opportunity to reduce or eliminate many of these environmental concerns and recirculating aquaculture systems (RAS) hold this potential.

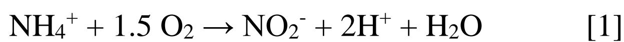
2.2 Recirculating Aquaculture Systems (RAS)

Recirculating aquaculture systems (RAS) can be used to reduce the use of water and the discharge of water in land-based aquaculture (Martins et al., 2009). Mantaining good water quality in RAS is essential for optimal fish growth and health. Treatment of recirculating water is, therefore, one of the limiting factors for a production capacity of a fish farm (Bjerknes, 2007). Solid waste treatment combined with control of dissolved minerals, dissolved gasses, dissolved organic matter, and various other water quality parameters contribute to ensuring optimal water quality criteria that support the survival and growth of aquatic organisms (Cripps and Bergheim, 2000). While the use of RAS has reduced the discharge of waste-water to the environment, the accumulation of potentially harmful substances within the system is receiving increasing attention. Efficient removal of minerals can prevent their accumulation to toxic concentrations (Bjerknes, 2007; Wood et al., 2012). Aluminum is of special concern because it accumulates on the gill surface at concentrations as low as 10 – 15 μg/L (Kroglund et al., 2007). Copper is also toxic to fish in low concentrations, and has been reported to accumulate in RAS (Martins et al., 2009).

RAS are intensive land-based systems where fish are raised in tanks, and the effluent can be treated by physical, chemical, and biological waste treatment systems. These systems can achieve up to 85 to 99 % of the treated water being continuously recycled back to the tanks nearly eliminating discharge (Wik et al., 2009; Badiola et al., 2012). A typical intensive aquaculture system will include aeration, oxygenation, and solids removal; however, the addition of biofiltration allows for recirculation in RAS (Piedrahita, 2003). The major purpose of the biofilter is to remove ammonium through nitrification. One of the most toxic compounds for fish is ammonia. When

ammonia levels are high, blood is less able to transport oxygen and ultimately results in fish kills (Chien, 1992). Unionized ammonia (NH_3) is the most toxic form, although both unionized and ionized ammonia (NH_4^+) forms can be deadly. Toxic values for ammonia depend on species, but on average acute toxicity values for freshwater fish are 2.79 mg L^{-1} NH_3 and 1.86 mg L^{-1} NH_3 for saltwater fish (Randall and Tsui, 2002).

Biofilters utilize a consortium of bacteria immobilized on the surface of a biofilm carrier media. While a diverse microbial community is present on the media, the growth of the organisms responsible for nitrification is critical for ammonium removal. Nitrification occurs in a two-step process. First, ammonium oxidizing bacteria convert ammonium (NH_4^+) to nitrite (NO_2^-) as shown in Equation 1. In the second step, nitrate oxidizing bacteria convert NO_2- to nitrate (NO_3-), as shown in Equation 2. Through this process, the harmful ammonium and nitrite are oxidized allowing for the recirculation of aquaculture water.



$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad [1]$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \qquad [2]$$

Several different biofilter configurations can be used for nitrification including moving bed bioreactors, trickling filters, submerged filters, rotating biological contractors (RBC), fluidized bed reactors, and bead filters (van Rijn, 1996; Midlen and Redding, 1998; Crab et al., 2007). Other forms of biofilters that utilize plants or algae have also been used successfully to remove ammonium and other nutrients with intensive aquaculture systems (van Rijn, 1996). While functional, bacterial biofilters are expensive to operate, and high-value fish species must be cultivated to offset the costs of the biofilter (Zucker, 1999). The high capital costs and the operation and maintenance costs associated with water treatment and recirculation have prevented rapid growth of this more sustainable aquaculture technique (Martins et al., 2010). Using plants and algae in combination with the biofilter instead, can reduce the costs of water treatment and provide additional opportunities for revenue (Neori et al., 2004; Graber and Junge, 2009). The use of plants or algae for biofiltration in this way is often referred to as an integrated aquaculture system (IAS).

2.3 Integrated aquaculture system (IAS)

Integrated aquaculture systems (IAS) are defined as the interaction between two or more agricultural activities that can result in similar levels of benefit to both components, of which at least one component is fish farming (FAO, 2011). In IAS, the output of one subsystem becomes inputs for other subsystems, producing a greater efficiency of the output of the desired product from land or water under controlled conditions (Edwards et al. 1988).

Integrated pond systems have been traditionally practiced in Asia (Schneider et al., 2005) and principally originated in China, Japan, and South Korea (Goldman et al., 1974a; Ryther et al., 1975). Wastes in the integrated system serve as nutrient for phototrophic and detritivorous/heterotrophic conversion into plants, bacteria, and invertebrates (Riise and Roose, 1997; Liu and Cai, 1998), higher nutrient retention is a result of nutrient re-use by primary and secondary producers (Liu and Cai, 1998). Consequently, integrated aquaculture system can limit the environmental impact of waste from fish/prawn cultivation and enhance the total productivity (Troell et al., 2003).

Integrated aquaculture models can be classified as fish-macroalgae (Cohen and Neori, 1991; Neori et al., 1991; Neori et al., 2000), fish-macroalgae-abalone (Neori et al. 2000), prawn-microalgae-oyster (Wang, 2003), fish-bivalve-macroalgae (Neori et al., 1993), prawn-fish (Tian et al., 2001; Muangkeow et al., 2007). An extensive review on analyzing nutrient conversions in integrated intensive aquaculture systems is provided by Schneider et al. (2005).

Integrated aquaculture can be land-based or open-water systems and may include several combinations of species (Neori et al., 2004). Both systems have mainly built on technical approaches involving mechanical separation, sedimentation, and filtration (Cripps, 1994). The land-based systems have been successfully implemented (Neori et al., 1991; Lin et al., 1993; Buschmann et al., 1994; Buschmann et al., 1996; Neori et al., 2000; Muangkeow et al., 2007) in small-scale (indoor/outdoor tank culture) systems (Buschmann et al., 1994; Buschmann et al.,

1996; Neori et al., 2003; Muangkeow et al., 2007) and large-scale (pond culture) systems (Maguire et al., 1981; Wang et al., 1998). Polyculture in earthen brackish water ponds has been practised for a long time, with extensive polyculture systems of prawn, fish, agriculture plants (including also mangroves and rice) found mostly in China, Indonesia, India, Ecuador, the Philippines, Taiwan Province of China, Thailand, Japan and Viet Nam (Brzeski and Newkirk, 1997; Neori et al., 2004).

Several studies investigated the use of macroalgae as a biofilter for marine RAS effluents before the development of integrated recirculating aquaculture systems (IRAS). Macroalgae, for example, can protect coastal areas from eutrophication while providing a valuable byproduct (Chow et al., 2001). As awareness of coastal degradation, due to shrimp and fish monocultures, became more prominent, so grew the interest in using algae for nutrient removal (Troell et al., 1999b). Some of the first experiments using macroalgae for the treatment of marine aquaculture wastewater were performed by Goldman et al. (1974a) and Goldman et al. (1974b).

The concept of using macroalgae for the treatment of aquaculture effluents was developed further in a series of experiments on multitrophic intensive aquaculture systems performed in Israel (Neori et al., 2004). Shpigel and Neori (1996) compared the potential revenue from three systems: integration of seaweed and abalone; fish, seaweed, and abalone; and fish, bivalves, seaweed, and abalone. They concluded that all three systems would cover the cost of operations. However, growers must keep in mind the variations in complexity. In a companion paper, Neori et al. (1998) examined the nitrogen budget for the simplest system: seaweed and abalone. When *Ulva lactua* was used as the biofilter, consistent performance was observed, with a removal efficiency of 49-56% of NH_4^+-N (Cohen and Neori, 1991). Much of the nitrogen was accounted for in growth of the algal biomass. Neori et al. (2000) studied a pilot system combining fish, seaweed, and abalone. As in Shpigel and Neori (1996), the combination of the three organisms effectively maintained water quality and produced three valuable products.

Integrated aquaculture has been used for centuries to culture organisms in relatively low densities (Li, 2003), but has only been considered for large-scale commercial aquaculture production (in China and elsewhere) in the past few decades (Troell et al., 2003). Increasingly, articles are appearing in the literature on the design and

effectiveness of integrated aquaculture systems of all types and sizes. Some studies are laboratory experiments to determine optimum species densities, feed, and water exchange rates, and farm design (Shpigel et al., 1993; Neori et al., 2000; Jones et al., 2001; Schuenhoff et al., 2003; Metaxa et al., 2006). Other studies examine the on-farm use of integrated aquaculture to assess ecological effects of commercial integrated culture systems (Gautier et al., 2001, Jones et al., 2002, Marinho-Soriano et al., 2002, Wu et al., 2003; Fei, 2004; Ryder et al., 2004; Yang et al., 2005). Another set of studies focuses on promoting the philosophy of integrated aquaculture, referring to it as potentially "sustainable" aquaculture, and emphasizing the need for fundamental changes in the aquaculture industry (Chopin et al., 2001, Paez-Osuna, 2001; Costa-Pierce, 2002; Frankic and Hershner, 2003; Fei, 2004).

2.3.1 Benefits of IAS

The economic value of integrated/polyculture systems has been investigated since 1985 (Neori et al. 2004). Integrated aquaculture systems can diversify the production- mix in changing market conditions and add another source of income (Buschmann et al., 1994; Neori et al., 1996; Troell et al., 1997; Neori et al., 2007). The production of species in integrated aquaculture systems is at least equal to, or greater than, those attained from monocultures systems (Neori et al., 2000; Newell, 2004). For example, by integrating seaweed, *Gracilaria* with salmon, approximately 48.9 kg m-2 year-1 of seaweed, *Gracilaria* was harvested annually in a pond with salmon, equivalent to US$ 34,000 (Troell et al., 1999a). Eventually, a farm model of land-based integrated seabream-shellfish-seaweed can harvest 25 tons of fish, 50 tons of bivalves and 30 tons of seaweed annually (Neori et al., 2004). Integrated mussel-salmon aquaculture system cultured in the cage can enhance productivity by 20%, compared to the monoculture of mussel or salmon. Investment in an integrated mussel-salmon aquaculture system is shown to produce a positive net present value (NPV) (£1.425 million) which exceeds the combined NPV of mussel monoculture (£0.353 million) and salmon monoculture (£0.922 million) (£0.353 million) (Whitmarsh et al., 2006). Hence, using bivalves/seaweeds for biofiltration in aquaculture systems has both ecological and economic benefits.

According to Muir (1986), the advantages of integrated aquaculture system are as follows: (1) multiple uses of resources, (2) reduced operating and maintenance cost,

particularly feeding costs, (3) multiple harvests of both plant and fish in the same place, (4) more effective removal or reduction of the effluent, (5) makes for effective monitoring since both fish and plant are within the same range.

Integrated aquaculture systems are practical and functional in most cases and, usually, lead to reduced effluent being discharged into open water bodies (Neori et al., 2004; Martínez-Porchas et al., 2010). The subordinate species in the integrated systems can retain the nutrients by assimilation most of the nutrient wastes generated from fish or prawn farmings. Thus, integrated aquaculture systems improve the water quality in the culture media and reduce the negative ecological impacts (Martínez-Porchas et al., 2010). Moreover, if the quality of the treated effluent is sufficiently improved, it can be re-used in recirculating aquaculture systems.

Several studies have shown that the presence of secondary species can improve the performance of the shrimp. Martínez-Córdova and Martinez-Porchas (2006) concluded that the integration of the Pacific oyster and black clam into white shrimp (*Litopenaeus vannamei*) aquaculture resulted in the increase of the productive performance of white shrimp and Pacific oyster, indicating that black clams are a good candidate to be kept in the white shrimp ponds. The integration of tilapia (*Oreochromis niloticus*) with shrimp showed that tilapia can utilize the excess organic matter, improve water quality and thereby increase production of shrimp (Akiyama and Anggawati, 1999). A similar conclusion drawn by Wang et al. (1998), which reared the Chinese shrimp-hybrid tilapia in a small scale polyculture.

The optimization of an integrated farming systems depends on the targets to achieve, that is, biofiltration versus biomass production (Neori et al., 2003; Schuenhoff et al., 2003). Maximising bivalves and seaweeds production and nutrient removal efficiency in an integrated aquaculture system could be difficult to achieve. Schuenhoff et al. (2006) pointed out that an integrated aquaculture system has a high biomass yield of seaweed, the drawback is that nutrient removal efficiency decreases. To achieve the elevated biomass yield of seaweed, water exchange rates should ensure the availability of both carbon and nitrogen for the growth of seaweed (Schuenhoff et al., 2006). However, Msuya et al. (2006) reported that the higher water exchange rates might result in the higher nutrient removal rates. The growth rates of the cultured species can reduce due to the fouling and mortality of the

seaweed in an integrated aquaculture system (Msuya et al., 2006). The biomass production per unit area of each species in an integrated aquaculture system may be lower than monoculture system. Besides, efficient treatment of waste water from aquaculture ponds usually involves a high level of technology which requires large investment regarding the setup and running costs (Neori et al., 2004; Matos et al., 2006). Adding a sub-ordinate species may increase the risk of transferring pathogens from one species to another. Naylor et al. (2001) emphasised that the transport and transmission of pathogenic organisms are often found in the aquaculture industry, resulting from the use of exotic species.

Considering the benefits of IAS, a farming system designed to rear high-value fish species in land-based tanks with continuous recycling of water is required to optimize water use, increase productivity, improve sustainability, and decrease environmental emissions.

2.4 Integrated recirculating aquaculture systems (IRAS)

Integrated recirculation aquaculture system implies a polyculture system where the main and subordinate species are separated by different units and the effluents of main species circulate through the culture units of the subordinate species (Chien and Tsai, 1985). The subordinate species use the uneaten feed, dissolved organic matters and other nutrients from the main species effluents.

IRAS is developed to improve the quality of the fish farming effluents by treating and recirculating the treated effluents back to the rearing systems, reducing the environmental impact of aquaculture activities (Martínez-Porchas et al., 2010). Researchers have intensively studied on semi-recirculating integrated aquaculture systems (Neori et al., 1998; Neori et al., 2003; Schuenhoff et al., 2003) where parts of the treated effluents recirculate back to the culture system. Shpigel and Neori (1996) have proposed three designs for the semi-recirculating integrated culture of mollusc with seaweed and fish in land-based facilities and showed that these designed systems significantly reduce the operational and environmental risks involved with mariculture. However, this process reduces the nutrient concentration in the effluent but fails to completely remove nutrients from the farming effluents (Buschmann et al., 1996; Schuenhoff et al., 2003).

Integrated closed recirculation systems have been developed because of growing concern about water pollution and avoidance of disease infection through water intake. In this system, wastewater with high organic particles and nutrients from intensive prawn pond flows to the treatment ponds and then recycles back to the prawn pond (Muangkeow et al., 2007). Lin (1995) proposed an integrated closed recirculation system composed of prawn, sedimentation, biological treatment (fish, mussel, etc.) and aeration ponds. The integrated and intensive culture of white prawn with herbivorous fish (mullet) and oysters in closed recirculation system have been proposed by Sandifer and Hopkins (1996). Recently, Muangkeow et al. (2007; 2011) have reported the integration of white prawn and Nile tilapia in an integrated closed aquaculture system.

2.5 Stocking density

The use of high stocking density as an approach to maximize the use of water and to increase stock production in fish culture has been shown to result in severe adverse effects on growth (Allen, 1974; Refstie and Kittelsen, 1976; Refstie, 1977; Trzebiatowski et al., 1981; Leatherland and Cho, 1985) and performance of teleosts (Schreck, 1982; Schreck et al. 1985). Stocking density is recognized as an important husbandry factor in intensive aquaculture systems because it is an important inducer of chronic stress, which may cause physiological and behavioural changes of the target species (Schreck et al., 1997; Wedemeyer et al., 1997; Ellis et al., 2002).

The magnitude of physiological and behavioural responses to stress are varied between fish species, but also on the strain and the individual level (Scholten et al., 2005). Various physiological changes in response to the stressor have been documented in many fish species as primary steps involved neuroendocrine response including catecholamine release and activation of corticotrophin interrenal axis, followed by secondary responses including hematology, metabolism, blood enzymes and changes osmoregulatory (Mazeaud et al., 1977; Sumpter, 1997).

Rearing fish at inappropriate stocking densities may impair the growth, reduce immune competence and induce abnormal behaviour (Ellis et al., 2002; Iguchi et al., 2003; Barcellos et al., 2004; Kristiansen et al., 2004; Schram et al., 2006). Due to variations in response to physiological stress on the fish (Barton, 2002), Effects of

stocking density seem to be species-specific, and become primarily dependent on the sensitivity of fish to the decrease in water quality at high stocking density, and the increase of social interaction at very low or very high stocking densities (Montero et al., 2001; Ellis et al., 2002; Kristiansen et al., 2004; Bjornsson and Olafsdottir, 2006; North et al., 2006; Papoutsoglou et al., 2006). Inappropriate stocking densities may result in a decrease welfare and compromise the health conditions of target species, affecting also the profitability of the aquaculture industry (Ellis et al., 2002; Conte, 2004; Turnbull et al., 2005; Huntingford et al., 2006; North et al., 2006).

Growth performance, survival and biomass production effects of stocking density on aquaculture have been well-documented in a diversity of species (Samad et al., 2005; Mazlum, 2007; Garr et al., 2011; Zhu et al., 2011; Khatune-Jannat et al., 2012) and seem to impact production differently. Both growth performance and survival rate tend to be higher in lower stocking densities in the African catfish (*Clarias gariepinus*) (Hecht et al., 1996), in freshwater prawn (*Macrobrachium rosenbergii*) (Cuvin-Aralar et al., 2007), in nila (*Oreochromis spp.*) (Sorphea et al., 2010), in Amur sturgeon (*Acipenser schrenckii*) (Zhu et al., 2011), in silver catfish (*Rhamdia quelen*) (Pouey et al., 2011), in the Thai climbing perch (*Anabas testudineus*) (Khatune-Jannat et al., 2012). However, only survival is higher observed in *Oreochromis* spp cultured under same conditions (Ridha, 2005). In some cases, the advantage of lower stocking densities is either non-existent, as reported in *Barbus luteus* (Gokcek and Akyurt, 2007), in channel catfish (Southworth et al., 2009), in *O. niloticus* (Osofero et al., 2009) or temporary and wanes after sometime so that generally no differences occur across different stocking densities, as in the apple snail (*Pomacea paludosa*) aquaculture (Garr et al., 2011).

Different efforts have been made to determine appropriate stocking densities to evade this limitation but until now recommended stocking densities still vary considerably (Russell et al., 2008). With limited land resources and high competition with agriculture for land, there is an urgent need for refining these recommendations, so those appropriate stocking densities are adopted in time to minimize production deficiencies per unit space available.

Furthermore, few studies reported the effects of stocking density on stress and welfare of sea bass. However, no significant differences have been found regarding

final body weight, specific growth rate, survival, some blood metabolites and chemical composition of sea bass reared at 20 and 40 kg/m3 for 18 months (Roncarati et al., 2006). On the other hand, some immunological effects including depression of peritoneal leukocyte cytotoxicity were described in adult sea bass crowded at 60 kg/m3 for 48 h (Vazzana et al., 2002). An increase of both plasma cortisol levels and stress genes expression was observed in juveniles held at 80 and 100 kg/m3 for 3 months (Gornati et al., 2004; Terova et al., 2005).

2.6 Barramundi aquaculture

The popularity and demand for barramundi (*Lates calcarifer* Bloch) make it an obvious candidate for aquaculture. The species is an incredibly robust, grows and thrives in a wide range of environments (Noble et al., 2014). Apart from these characteristics that endear it to the consumer; tender, mild tasting, boneless fillets, the fish is also fast-growing and euryhaline. The latter fact is seen as a valuable attribute for a species being raised in areas subject to varied conditions (Carter et al., 2010).

Techniques for the culture of barramundi were first established in Thailand in the beginning of 1970s at the Songkhla Marine Laboratories, and a considerable progress in barramundi aquaculture techniques has been achieved since that time (Schipp et al., 2007). Over the 80s and 90s, barramundi aquaculture expanded throughout the Asia-Pacific regions, including China, Taiwan, Vietnam, Malaysia, Indonesia, Malaysia, the Philippines, and Australia. More recently countries such as the USA, the Netherlands, the UK and Israel have also involved in barramundi farming. Most of those countries are supporting active research into culture techniques for barramundi (Glencross et al., 2013).

Australia's involvement in barramundi aquaculture began in 1983 with the establishment of a research program at Northern Fisheries Centre, Department of Primary Industries and Fisheries in Cairns. The main thrust of the initial research was to produce fingerlings for the enhancement of rivers and estuaries for recreational fishing. Early breeding programs used eggs and sperm hand stripped from wild spawners at Weipa in North Queensland. Later, spawnings were undertaken using captive as well as wild brood fish (Schipp et al., 2007). Furthermore, the first large

scale barramundi farm was developed near Innisfail in North Queensland in 1986 following the public float of Sea Hatcheries Limited. Since then many barramundi farms have been established around Australia, and in 2004-2005 the industry produced nearly 3000 tons of fish (Schipp et al., 2007).

Recently, barramundi has been successfully cultivated in several states in Australia. Production is characterized by a large number of relatively small producer, with about 8-900 tons of fish were produced in 1998-1999. Although the production of barramundi in WA is still in the early stages with less than 10 tons of fish were produced in 1997-1998, has grown rapidly in the last three to four years (Thorne, 2002).

In Asia, barramundi aquaculture is carried out in marine water, brackishwater, and freshwater, with most production based on hatchery-reared stock. Over the past 10 years, worldwide production of barramundi has been relatively constant (FAO, 2007). Even though barramundi production in Asia has decreased, barramundi production in Australia has increased during this period. Most barramundi is farmed in cages and ponds located in brackish estuaries and coastal areas (FAO, 2007). Furthermore, Australia has experienced a large-scale development of barramundi farming, where the cultivation of barramundi is located outside the tropics, and the production is commonly performed using recirculation aquaculture systems (RAS) (in southern Australia and north-eastern United States of America). Barramundi farming has also become important species cultivated in Iran, Guam, French Polynesia, United States (Hawaii, Massachusetts), and Israel (Mathew, 2009).

2.7 Duckweed (*Lemna minor* Linnaeus)

The term duckweed commonly refers to a group of aquatic vascular angiosperms of the family Lemnaceae. The plants are divided into four genera; *Spirodela, Lemna, Wolfiella,* and *Wolffia*. There are approximately 40 species worldwide, half of which are found in the United States. Lemnaceae plants are widely distributed in the world from the tropical to the temperate zones, from freshwater to brackish estuaries, and throughout a wide range of trophic conditions (Hillman and Culley, 1978).

Lemnaceae plants are common in the aquatic environment, especially in quiescent water bodies. Lemnaceae plants are composed of two parts, frond, and root. The

plants are colonial and form aggregates of two or more fronds in a colony. Duckweed (*Lemna minor* Linnaeus) has a single rootlet, while Spirodela has several rootless (Correll and Correll, 1972). Lemnaceae plants are small, *L. minor* is 2-4 mm across, while Spirodela thallus is 3-10 mm long (Correll and Correll, 1972). The plant size is small so that a large laboratory space is not required for culturing or testing. The size is sufficiently large to be visually observed, allowing nondestructive, repeated observations.

Lemnaceae plants are incredibly fast growing (Hillman and Culley, 1978). The plants form a thick mat, frequently dominated by a single species, in a lake or a pond. Duckweed and algal domination have been reported to cause fish mortality (Lewis and Bender, 1961). In an 18-month study, the doubling time for *L. minor* fronds ranged from 1.3 to 2.8 days (Wang, 1987). In comparison, Hughes et al. (1988) reported the doubling time for *L. gibba* to be 0.7 days. Lemnaceae can take up nitrogen as nitrite, nitrate, ammonium, urea and some amino acids. However, ammonium and nitrate are the main nitrogen sources for most species. Minimum, optimal, and toxic levels of nitrogen greatly vary between species and geographic isolates, and increased intensity of light is believed to elevate optimal nitrogen requirements for growth. Among the species studied, *L. minuscula* has the lowest tolerance (0.0016 $mM.L^{-1}$), and an unclassified species of *Lemna* was recorded to have the highest minimum requirements for nitrogen (0.08 $mM.L^{-1}$) (Landolt, 1986). Likewise, the maximum tolerated level of nitrogen was observed from 30 $mM.L^{-1}$ (*L. minuscula*) to 450 $mM.L^{-1}$ for *L. aequinoctialis* (Landolt, 1986). The optimal nitrogen requirements were recorded ranging from 0.01 $mM.L^{-1}$ for *Wolffia colombiana*, up to 30 $mM.L^{-1}$ for *Spirodela polyrhiza* (Landolt, 1986).

Phosphorus requirements for duckweed are variable (0.003 to 1.75 $mM\ L^{-1}$) among species, but apparently unassociated with the nitrogen requirement (Landolt, 1986). Duckweed can accumulate up to 1.5% by weight of phosphorus in waters rich in nutrients (Leng, 1999). Between-species, differences are also evident in potassium, with requirements also being influenced by light intensity. The pH of water exerts a profound effect on the growth of duckweed, affecting the uptake of nutrients and especially the nitrate: ammonium ratio and species availability (Caicedo et al., 2000). Both optimal and limiting values for growth vary widely, both between species and

between colonies or isolates. Both *Spirodela* and *Lemna* species require a neutral pH of 7 for their optimal growth, while *Wolffia* reaches its optimal growth at pH of 5 (McLay, 1976; Leng, 1999). Although the minimum and maximum pH values that can be tolerated by most duckweed species are still debatable, Landolt (1986) reported that pH 3 is a lower pH value, while Leng (1999) concluded that the lower and upper pH values are 5 and 10.5, respectively. Landolt (1986) has also recommended that the presence of chelating agents have a significant impact on the pH range at which duckweed grows.

Most of the duckweed species seem to have their optimum growth at the temperature between 20o and 30o C (Landolt, 1986). The rise in temperature from 12oC to 30oC has synergistic effects on the growth of most duckweed species. Additionally, most species are able to achieve maximum growth at 9,000 Lux and pH 24oC (Landolt, 1986). The data indicate that besides light intensity, the temperature affects photosynthesis and the nighttime respiration rates.

The water depth required to support the growth of duckweed is influenced by many factors, including the growing purposes and management approaches (Leng, 1999). The depth of the pool less than 0.5 m is strongly influenced by the diurnal temperature fluctuations. However, the greater the depth, the less likely duckweed have full access to the nutrients in the water column. Al-Nozaily et al. (2000) found that the surface area has a greater influence than the depth of water on the nitrogen removal capacity of duckweed.

Many studies have shown that duckweed can absorb 40 to 60% of the nitrogen in the solution over 12 to 24 days. Volatilization can result in a similar loss of nitrogen (Vermaat and Haniff, 1998), even though, a more recent study conducted in Israel, has suggested that duckweed directly absorbs less than 20% of nitrogen losses, and volatilization/denitrification can reach over 70%. (Van der Steen et al., 1998). Likewise, duckweed can absorb 30 to 50% of dissolved phosphorus, even though, a study of Alaerts et al. (1996) claimed more than 90% removal in the full-scale system. Phosphorus absorption (as measured by tissue phosphorous content), and crude protein, linearly elevated with the increase in nutrient concentrations up to 1.5 g P L^{-1}, and increased in absolute terms up to 2.1 g P L^{-1} (Sutton and Ornes, 1975). It was found in conjunction with a proportional rise in the concentration of nitrogen.

Thus the relationship between the concentration of nitrogen and phosphorus was still not clear.

Vandecasteele et al. (2005) claimed that aquatic plant-based wastewater treatment systems are low-cost technologies which may be adopted by developing countries. Several studies have reported the potential of various aquatic plants for reducing N in wastewater (Table 2.1).

Table 2.1. Selected data on nitrogen removal efficiencies of aquatic plants

Aquatic plants	Percentage of Nitrogenous removal (%)	Freshwater environment	References
Eichhornia crassipes	85% (TN)	Imboassica lagoon	Petrucio and Esteves (2000)
	81% (ammonia)	Minicipal wastewater	Kutty et al. (2009)
	60-80% (TN)	Urban stormwater retention ponds	Fox et al. (2008)
	80% (in organic N)	Agricultural drainage effluents	Reddy et al. (1982)
	32.6% (nitrite)	Fish farming effluents	Henry-Silva and Monteiro-Camargo (2006)
	43-98% (TN)	Fish farming effliuents	Schwartz and Boyd (1995)
	86.8% (nitrite)	Fish farming effluents	Rubim et al. (2015)
	77.37% (ammonia)	Fish farming effluents	Akinbile and Yusoff (2011)
	67.90% (ammonia)	Fish farming effluents	Alex-Diaz et al. (2010)
	30% (TN)	Fish farming effluents	Schulz et al. (2004)
	46.1% (TN)	Fish farming effluents	Henry-Silva and Monteiro-Camargo (2006)
Elodea dense	25% (TN)	Fish farming effluents	Ng et al. (1990)
Elodea nuttalli	8.2% (ammonia)	Fish farming effluents	Redding et al. (1997)
Pistia stratiotes	81.68% (ammonia)	Fish farming effluents	Akinbile and Yusoff (2011)
	Up to 95% (ammonia)	Polluted urban river	Olguín et al. (2015)
	Up to 95% (ammonia)	River water	Awuah et al. (2004)
	31-51% (nitrate)	River water	Ingersoll and Baker (1998)
	43.9% (TN)	Fish farming effluents	Henry-Silva and Monteiro-Camargo (2006)
Salvinia molesta	42.7% (TN)	Fish farming effluents	Henry-Silva and Monteiro-Camargo (2006)
Azolla filiculoides	4.3% (ammonia)	Fish farming effluents	Redding et al. (1997)
Roripa nasturtiumaquaticum	10.7% (ammonia)	Fish farming effluents	Redding et al. (1997)
Typha latifolia	66% (TN)	Agricultural runoff	Comin et al. (1997)
	27-47% (TN)	Wetland	Greenway and Woolley (2000)

Duckweed has also been applied for the treatment of a wide range of wastewater types in several countries, such as Bangladesh, Israel, and the U.S (Culley et al., 1981; Oron, 1994; Korner et al., 1998). Many studies have evaluated ammonia removal capacity of duckweed from domestic wastewater, and its assimilation into N compounds (Oron et al., 1987). Nutrient (N and P) removal efficiencies of duckweed have been investigated in dairy wastewaters (Culley et al., 1981), secondary effluents (Harvey and Fox, 1973; Sutton and Ornes, 1975), waste stabilization ponds (Wolverton, 1979), raw and diluted domestic sewage (Skillicorn et al., 1993; Oron, 1994; Hammoudo et al., 1995), and fish farming systems (Porath and Pollack, 1982). Ozengin and Elmaci (2007) reported the TN removal efficiencies of duckweed were 86.49% and 83.69% respectively in municipal and industrial wastewater. Previously, Korner and Vermaat (1998) and Erol Nalbur et al. (2003) reported nitrogen removal efficiencies of 34-99% and 56-67%, respectively. Values on N removal efficiency of duckweed are shown in Table 2.2.

Table 2.2. Summary of TN removal rates and efficiency of duckweed for waste water treatments.

Parameters	Removal efficiency (%)	Reference
TN/TKN	17.59	Shah et al. (2014)
	35	Gürtekin and Sekerdag (2008)
	56-67	Erol-Nalbur et al. 2003
	73	Cedergen and Madsen (2002)
	70.35	Willett et al. (2003)
	83-87	Ozengin and Elmaci (2007)
	34-99	Korner and Vermaat (1998)
	98	Mohedano et al. (2012)
	50-95	Korner et al. (1998)
	73-97	El-Kheir et al. (2007)
Parameters	**Removal rate (g N m^{-2} d^{-1})**	**Reference**
TN	0.54	Korner and Vermaat (1998)
	0.61	Lyerly (2004)
	0.77	Willett et al. (2003)
	0.95	Cheng et al. (2002b)
	1.2	Benjawan and Koottatep (2007)
	2.1	Cheng et al. (2002a)
	3.4	Cheng et al. (2002a)
	96	Selvarani et al. (2015)

Duckweed utilizes these nutrients and produces a significant amount of biomass which can be used for some beneficial purposes. Additionally, bioconversion of domestic sewage in fish farming can contribute to cost recovery and protein production (El-Shafai et al. 2007).

Over several decades, feeds from plant origin have been accepted for Indian major carps because the growth performance has been reported to be as good as that obtained with the feed contained fish meal (Mohapatra and Patra, 2013). In tropical countries, where macroalgae production rates are high, macroalgae have been receiving increasing attention as an alternative protein possessing relatively high protein content (50-60%), which may be regarded as balanced fish feeds (Devraj et al., 1986; Mohapatra and Patra, 2013).

Duckweed has been used as fresh food for ducks and other animals (Leng l., 1995), and in the formulated diets for poultry (Anderson et al., 2011), dogs (Brown et al., 2013), and fish (Bairagi et al., 2002; Yilmaz et al., 2004; He et al., 2013). Duckweed contains from 28 to 43 % crude protein, 5 % fiber in dry weight, and high concentrations of trace minerals, such as phosphorus and potassium, as well as xanthophylls and carotenes (Chaturvedi et al., 2003).

Duckweed protein has higher concentrations of essential amino acids; lysine and methionine than most other plant proteins, and its methionine and lysine level closely resemble that of animal protein (Journey et al., 1991). Guru et al. (2007) claimed that protein content of duckweed was estimated to be the highest in comparison to *Eichhornia crassipes* and *Pistia stratiotes*. Duckweed has been used for partial replacement of fish meal on the growth performance of Indian major carp (Ahamad et al., 2003; Said et al., 2005). Meanwhile, Porath and Agami (1977) reported that the weight of grass carp can be tripled (from 100 to 300 g) within 50 days when feeding a mixture of *L. gibba* and *L. minor*. Patra (2015) reported that 15% duckweed dietary inclusion is an optimum inclusion level to produce cost effective feed for the Indian carp (*Labeo rohita).*

However, limited information is available with regard to the interaction between duckweed root exudates and the bacterial communities (Huang et al., 2014). Duckweed roots release a variety of molecules, including fatty acid methyl esters and fatty acid amides that attract and select bacteria attached to duckweed (Lu et al., 2014).

2.8 Phosphate solubilising bacteria (PBS)

Phosphorus is an essential nutrient involved in several metabolic processes (Azcon and Barea, 1995). A correct P supply resulted in increased number and size of nodules, alfalfa (*Medicago sativa*) yield as well as N fixation. Rodriguez and Fraga (1999) stated after nitrogen (N), phosphorus (P) are the major essential macronutrients for plant growth and development. Most of the P exists in nature in a variety of organic and inorganic forms, but the concentration of available P in soil is usually very low, frequently at levels of 1 mg kg^{-1} or less (10 M H_2PO_4). Plants can only utilize fewer amounts of phosphate fertilizers that are often continuously applied, and the rest, which is about 70%, is rapidly converted into insoluble complexes such as calcium phosphate, aluminium phosphate and iron phosphate in the soil. The insoluble inorganic compounds of phosphorus may then be converted into plant usable forms (Reynolds and Davies, 2001).

To increase the availability of P, Phosphate solubilising bacteria (PSB) is currently used by the agro-industry (Bagyaraj et al., 2000; Trivedi & Sa, 2008). PSB are microorganisms that capable of solubilising mineral-bound insoluble phosphate, primarily by the production of various organic acids like gluconic acid, keto-gluconic acid, citric acid, and lactic acid (Nautiyal, 1999; Lin et al., 2006). Additionally, enzymes such as glucose dehydrogenase, citrate synthase, and lactate dehydrogenase involved in the oxidation of glucose and other intermediates of energy metabolism pathways are essential in the production of these organic acids (Goldstein, 1994; Chen et al., 2006). PSB is mostly effective in solubilisingsolubilising Ca-bound P (Ca–P), rather than the Fe-, Al- and Mn-bound forms (Kucey et al., 1989; Fankem et al., 2006). The main strains that can perform this conversion belong to the genera *Pseudomonas, Bacillus, Micrococcus, Mycobacterium, Achromobacter, Agrobacterium, Erwinia, Burkholderia, Flavobacterium, Rhizobium, Mesorhizobium,* and *Sinorhizobium* (Rodriguez and Fraga, 1999).

PSB poses the potential capacity to solubilize fixed P resulting higher crop yields (Gull et al., 2004). The rate of Phosphate solubilisation and Phosphorus uptake in plant tissues varied with the bacterial strain and P fertilizer used. They also found that *Bacillus* and *Xanthomonas* increased the height and biomass of canola but not P content of plants compared to uninoculated plants. Similarly, seed bacterisation with PSB strains raised root elongation and biomass of Chinese cabbage in seedling culture, although they had no effect on the P uptake of plants. Jeong et al. (2012) reported that *Bacillus megaterium* showed a potential for directly solubilising

phosphorus from soil more than 10 folds higher than the control without inoculation. Inoculation of *B. megaterium* as PSB increased the bioavailability of Cd and consequently boosted its uptake by plants. This was also good for improving phytoremediation using PSB in the Cd-contaminated soils.

Some bacterial species have mineralization and solubilisation potential for organic and inorganic phosphorus, respectively (Khan et al., 2007). Phosphorus solubilising activity is affected by the microbe's ability to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups remove the cation bound to phosphate, the latter being converted to soluble forms (Sharma et al., 2011).

2.8.1. Isolation and screening of PSB

PSB have been isolated from many soil samples taken from the rhizosphere of many plants as well as soil taken from other locations (Mehta et al., 2013). Illmer and Schinner (1992) reported that two of phosphate-solubilisingsolubilising bacteria (M7c and FM7d strains) isolated from the soil, showed a greater solubilisingsolubilising capacity than that of the control strain Pseudomonas putida SP22 under in vitro conditions. These isolates were identified as *Bacillus* spp. (M7c) and *Pseudomonas* spp. (FM7d) by PCR amplification and partial nucleotide sequencing of the ribosomal 16S DNA. Furthermore, Grayston et al. (1994) observed that the mycorrhizosphere activity exerts a significant stimulating effect on the population of fluorescent pseudomonads in the soil. This report clearly showed that most of the fluorescent pseudomonads strain isolated from the Pisolithus treatment had the ability to solubilize inorganic phosphate by contrast to the majority of those isolated from un-inoculated treatments.

Many factors including physical factors (pH, temp) and chemical factors (carbon source and nitrogen source) influence the phosphate solubilisation efficiency of microorganism. The correlation between the pH and soluble-P concentration indicates that organic acid production by PSB strains has a significant role in the acidification of the medium by facilitating the P solubilisation. Rashid et al. (2004) reported that a negative relationship was observed between the pH and soluble P concentration, the result suggested that acidification of the medium could facilitate Ca–P solubilisation. A similar negative correlation between pH and available P content was reported earlier by (Illmer et al., 1995).

The effect of phosphate solubilisingsolubilising bacteria various plants has been assessed by different researchers. In most of the cases, the growth has been enhanced by the PSBs as per De

Freitas et al. (1997), bacterial isolates which showed the minimum phosphate solubilization in vitro, resulted in higher shoot biomass production of plants grown in MRP amended the soil. The results support previous observations by Ekin (2010) that the rate of in vitro phosphate solubilization has no simple relationship with in vivo plant growth. The reason may be the production of plant growth promoting metabolites (indole acetic acid and siderophores) besides phosphate solubilization.

An increase in the yield of various crops (wheat, vigna, and chickpea) following seed or soil inoculation with phosphate solubilisingsolubilising organisms and other plant growth-promoting rhizobacteria (Afzal and Bano, 2008). Furthermore, Malboobi et al. (2009) planted potato seedlings into pots containing sterile or non-sterile soil that uniformly mixed with 30 ml suspension of either individual or combinations of two or three PSB containing 108 CFU/ml of each strain. Combinations of various PSB isolated from the potato rhizosphere led to the increase in the biomass and potato tuber.

Mamta et al. (2010) stated that industry needs huge amounts of quality biomass produced with the minimal usage of chemical fertilizers. They also reported in their previous work that the inoculations of PSB along with tricalcium phosphate (TCP) increased the plant growth, the liberation of P and steviol glycosides in *Stevia rebaudiana*. *B. gladioli* MTCC 10216, *B. gladioli* MTCC 10217, *Enterobacter aerogenes* MTCC 10208 and *Serratia marcescens* MTCC 10238 used as PSB in the present studies were isolated from the rhizosphere of *S. rebaudiana* plants grown commercially in the agricultural fields. Ngwene et al. (2010) reported the increase in P uptake by the cowpea plants along with the higher crop yield when they used arbuscular mycorrhizal fungi (AMF) and plant growth promoting bacteria (PGPB) as inoculants.

PSB has a crucial role in the cycling of minerals in each ecosystem. However, the abundance of bacteria normally varies and depends on a variety of environmental conditions (Wright and Coffin, 1984). Besides PSB, aquatic heterotrophic bacteria have a crucial link in the cycling of minerals. The bacteria promote organic degradation, decomposition, and mineralization processes in the overlying water, and releases dissolved organic and inorganic substances (Goldstein, 1986).

2.9 Heterotrophic bacteria

Heterotrophic bacteria refers to a group of bacteria that obtains carbon from organic sources. Although large inputs of feed to any intensive aquaculture systems, the growth of heterotrophic

bacteria is limited by dissolved organic carbon (Hargreaves, 2013). These types of bacteria are often considered nuisances in biofilters of RAS because heterotrophic bacteria outcompete autotrophic nitrifiers for oxygen when excessive organic material are available. Heterotrophic bacteria releases carbon dioxide as the final product, which provides a carbon source for autotrophs, which creates biomass that is eventually consumed by heterotrophs (McGraw, 2002).

Recently, the primary focus on heterotrophic bacteria in RAS has only been on pathogenic bacteria since these directly affect the fish health. The non-pathogenic bacteria has never been comprehensively studied. The heterotrophic bacteria can consist of more than 80% of the total bacterial population on biofilters (Hovanec and DeLong, 1996). However, Heterotrophic bacteria can have an indirect negative effect on the fish population. As mentioned earlier undigested food pellets, as well as the faecal matter, will be converted to ammonium. A constant problem in most RAS is heterotrophic growth on the nitrifying biofilm, which leads to a decrease in ammonia oxidation. UV radiation of water entering the biofiltration chambers decreases heterotrophic growth but despite these efforts, heterotrophic growth is still observed in biofiltration (Elasri and Miller, 1999). When heterotrophic bacteria invade the existing nitrifying biofilm, competition for dissolved oxygen will intensify. The slow growing nitrifies will most likely be outcompeted by faster-growing heterotrophs. Access to the bulk water will also be limited, decreasing the access to dissolved ammonia. A RAS with completely overgrown biofilters can be in serious threat of reaching toxic levels of TAN (Michaud et al., 2006).

In intensive aquaculture systems, nitrogen and phosphorus originating from dissolved and solid fish waste are converted into the valuable biomass by aerobic heterotrophic bacteria. This biomass can be re-utilized as aquatic feed. This re-use of otherwise wasted nutrients improves the sustainability of the system. This approach has been applied in intensive shrimp ponds, tilapia ponds, and recirculation aquaculture systems (RAS) (Avnimelech et al., 1989; Burford et al., 2003; Hari et al., 2004). For optimal biomass production, heterotrophic bacteria conversion requires carbon/nitrogen (C: N) ratios of 12–15 (w/w) (Lechevallier et al., 1991; Henze et al., 1996), through the addition of a supplemental source of carbohydrate or using low-protein feed. The C: N ratios of 12-15 favors the heterotrophic bacteria for ammonia control (Hargreaves, 2013).

In the case of RAS, where drum filters are used to separate solid and dissolved waste, the C: N ratios of the drum filter effluent are only three or lower (Schneider et al., 2006). It is because the slurry has high nitrate content. Under such conditions, organic carbon such as sodium acetate,

should be supplemented if the slurry is utilized for bacteria production. However, previous studies showed bacteria yields which were lower compared to yields reported in the literature (Henze et al., 1996; Schneider et al., 2006).

The increase in TAN conversion compared to nitrate when TAN was used as the main nitrogenous substrate for heterotrophic bacteria did not result in a detectable higher volatile suspended solids (VSS) production and higher yields under the experimental RAS conditions. This is in contrast to one of the major hypotheses of this study. Even though this assumption could not be validated, the preference of TAN over nitrate by the bacteria was confirmed. TAN flux was linearly related to TAN conversion and increasing TAN conversions resulted in negatively correlated nitrate-N conversions (Schneider et al., 2006).

At the high organic carbon to nitrogen (C/N) ratios of feeding, heterotrophic bacteria can directly take up ammonia-nitrogen from the air and produce cellular proteins. Heterotrophic bacterial biomass can also be used as an important source of protein feed, lower production costs and thereby increase the overall economy (McIntosh, 1999; Moss, 2000).

Increased C: N ratio of the fish feed result in a significant increase in the total heterotrophic bacteria and lower concentration of nitrate in water. Additionally, increased biomass of heterotrophic bacteria can efficiently immobilize total ammonia nitrogen (TAN) (Hari et al., 2004). Reduction of nitrogenous waste results in the minimization of stress, which in turn leads more profitability (Hochheimer and Wheaton, 1998).

2.10 Stress indicator

In general, stress is defined as a state of real or perceived threat to homeostasis. Maintenance of homeostasis due to the aversive stimuli (stressors) induces activation of a complex range of responses involving the endocrine, nervous, and immune systems, known as the stress response (Chrousos and Gold, 1992; Carrasco and Van de Kar, 2003). Fish response to stress is characterized by the activation of the neuro-endocrine systems which is followed by metabolic and physiological changes (Wedemeyer and Yasutake, 1997; Lowe and Davidson, 2005). These changes enhance the tolerance of an organism to face an environmental variation or an adverse situation while maintaining a homeostasis (Pickering, 1992).

Under stress condition, immediate responses of the fish body are known as primary and secondary stress responses. The primary stress response is the perception of an altered state of

the central nervous system (CNS) and the secretion of stress hormones (cortisol/adrenalin and catecholamines/noradrenalin) into the blood stream (Randall and Perry, 1992). The release of stress hormone triggers the secondary responses (Barton and Iwama, 1991), causing changes in the blood and tissue chemistry such as an increase in plasma glucose (Begg and Pankhurst, 2004), followed by increases in metabolic rate which then causes changes to blood lactate. This entire metabolic pathway produces a burst of energy to prepare the fish for an emergency situation (Rottman et al., 1992).

Some plasma chemicals can be valuable tools to evaluate stress condition of the fishes (Sadler et al., 2000; Campbell, 2004; Wagner and Congleton, 2004). Many studies highlighted that under stressful conditions, the fish always show increased levels of plasma cortisol and glucose (Balm et al., 1999; Barcellos et al., 1999; Hattingh, 1997). Hence, stress is considered to increase plasma cortisol (Wendelaar- Bonga, 1997; Pottinger et al., 2003; Haukenes et al., 2008) and glucose levels (David et al., 2005). Cortisol secretion is slower than catecholamines, but its effects are much longer (Gamperl et al., 1994; Waring et al., 1996) because the cortisol combines mineral and glucocorticoid actions to restore homeostasis (Maule et al., 1993; Colombe et al., 2000). Cortisol involved in activation of glycolysis and gluconeogenesis processes in fish and the chromaffin cells that release catecholamines which then increase glycogenolysis and modulate cardiovascular and respiratory functions (Reid et al., 1996). This whole process increases the glucose level (substrate) to produce enough energy according to the demand of the animal. Thyroid hormones affect various aspects of metabolism. The hormones play a substantial role in a stress related mobilization of glucose in fish. Thyroxine (T_4) treatment caused a hyperglycemic effect in some cases (Chan and Woo, 1978). However, other studies observed hypoglycemic effects in T_4 treatment (Murat and Serfaty, 1970). Closely related significant elevations of plasma T_4 and glucose induced by disturbance stress were reported (Himic and Eales, 1990). Administration of thyroid hormone is recognised to support the growth of fish (Higgs et al., 1979; Saunders et al., 1985; Woo et al., 1991) and support of this concept, elevated levels of plasma T_3 were detected during the periods of rapid growth (Gannam and Lovell, 1991; Soengas et al., 1992). The inconsistencies observed in these experiments may reflect the interaction of the thyroid hormones with the other hormones such as cortisol and growth hormones. T_4 administration resulted in increased levels of blood glucose (hyperglycaemic) in some cases (Chan and Woo, 1978).

However, another study observed a hypoglycemic response in thyroxine treatment (Murat and Serfaty, 1970). A significant increase of plasma T_4 and glucose levels induced by stress disorder reported (Himic and Eales, 1990). Administration of thyroid hormone is reported to accelerate the growth of fish (Higgs et al., 1979; Saunders et al., 1985; Woo et al., 1991) and the support of this concept, the increased concentration of T_3 was found in a period of rapid growth (Leatherland et al., 1987; Gannam and Lovell, 1991; Soengas et al., 1992). Inconsistency is evident in this trial may in part reflect thyroid hormone interactions with other hormones such as growth hormone and cortisol

Thyroidal administration has been reported to increase the anabolic effects of growth hormone (lower liver glycogen and high serum cortisol) in rainbow trout (*Oncorhynchus mykiss*) (Farbridge and Leatherland, 1988), whereas administration of growth hormone elevated plasma T_3 level of rainbow trout (Farbridge and Leatherland, 1988; Maclatchy and Eales, 1990), this indicates increased peripheral conversion of plasma T_4 to T_3. The sub- lethal concentration of arsenic decreased plasma T_4 level (Nichols et al., 1984). Many teleost fish rely mainly on lipid and protein sources for energy. Teleost fish possess enzyme system for the utilization of the carbohydrates (Cowey and Sargent, 1979; Walton and Cowey, 1992). In these species, amino acids such as arginine and lysine have been reported to be more effective than glucose in simulating the release of insulin (Higuera and Cardenas, 1986; Petersen et al., 1987). Nevertheless, increased carbohydrate metabolism is observed in high energy demand condition such as stress, in which, blood glucose increased due to both glycogenolysis and gluconeogenesis (Wendelaar- Bonga, 1997).

Stress has been identified as an energy drain of energy that might be utilized in growth diverted to catabolic utilization (Barton, 1988; Pickering, 1990; McDonald et al., 1993). Mobilization of readily available energy in the form of glucose aims to improve fish survival (Barton and Iwama, 1991; Pickering, 1993). This is because the increase in plasma glucose has been recognized as part of the common stress response in fish (Davis and Parker, 1990; Melotti et al., 1992; Barry et al., 1993) and fish exposed to pollutants (Goss and Wood, 1988; Whitehead and Brown, 1989; Al-Kindi, 1996).

CHAPTER 3: Evaluating and Validating Harvest Frequency of Duckweed (*Lemna minor* Linnaeus) To Improve Growth Rates of Barramundi (*Lates calcarifer* Linnaeus) and Nitrogen Uptake Efficiency in An Integrated Recirculating Aquaculture System[1]

3.1 Introduction

Recirculating aquaculture system (RAS) is an intensive fish farming system that continues to gain increasing attention due to certain benefits, such as reduced land and water requirements, a better control of the rearing environment (Molleda, 2007), higher biosecurity than the open system and its ease of placement nearer to potential markets (Martins et al., 2010). As RAS is expensive to construct and operate (Summerfelt et al., 2001), higher stocking densities and productivity are essential to recover the investment costs (Timmons et al., 2002).

The stocking density is an important factor affecting the growth, performance, and productivity of aquaculture activities (Tolussi et al., 2010). Many studies have investigated the effects of stocking density on the growth of different fish species (Schram et al., 2006; Merino et al., 2007; Tolussi et al., 2010). Rearing at high densities beyond the system capacity may cause chronic stress, increase the risk of infectious diseases and, consequently, loss of production due to the accumulation of particulate waste and ammonia resulted from the feed metabolism (Timmons et al., 2002). Ammonia is problematic, as it is a natural by-product of fish metabolism and a toxic compound that adversely affects fish health. Therefore, the high stocking densities and feeding rates essential for a typical RAS result in higher waste production.

To maintain good water quality and overcome the toxic effects of ammonia, frequent water exchange is the most common practice in any intensive fish farming systems. Nevertheless, frequent water exchange is expensive and laborious (Thompson et al., 2002) and the use of biological and chemical filters may convert the nitrogenous waste into less toxic forms without actually reducing the “output” of nitrogen to the environment. Besides, they are also expensive and involve a high degree of technology (Matos et al., 2006).

Although nitrogenous wastes are poisonous to the cultured species at certain concentrations, nitrogen compounds are physiologically required by autotrophs, including aquatic macrophytes, for the synthesis of amino acids (Noctor et al., 2002). The aquatic macrophytes require a supplementary energy expenditure to transform different nitrogen forms into ammonia;

[1] This chapter has been submitted to Aquaculture Research

therefore, the macrophytes prefer ammonia over other forms of nitrogen (Fang, 2007; Phillips and Hurd, 2004). In addition, light has no or little effect on ammonia uptake rates (Botella et al., 1994; Nelson et al., 1980).

Several researchers have reported the capability of many aquatic macrophytes from the family Lemnaceae in wastewater treatment, particularly for nutrient removal (Bergmann et al., 2000; Cedergen and Madsen, 2002; Cheng et al., 2002a). Xu and Shen (2011) showed that high-starch duckweed (*Spirodela polyrrhiza*) can remove approximately 84% and 89% of total nitrogen (TN) and total phosphorous (TP), respectively, from swine manure. Duckweed (*Lemna minor* Linnaeus), as one of the most widespread duckweed species, has been well documented to have a high nutrient removal capacity through biofiltration from an organic nutrient-rich water body (Sharma et al., 2000), with a removal rate of 2.1 g m^{-1} d^{-1} for TN and 0.6 g m^{-1} d^{-1} for TP (Cheng et al., 2002b). The biomass of duckweed produced during the wastewater treatment may contain a high nutritional value with high productivity (Mohedano et al., 2012), making it useful feed for some herbivorous fish species (Chowdhury et al., 2008; Nekoubin and Sudagar, 2013). Therefore, incorporating duckweed into RAS reduces the need for mechanical treatment and, at the same time, produces value-added biomass, thereby increasing the sustainability of RAS.

Studies have concluded that nitrogen uptake rates are mainly regulated by the availability and type of substrates (Kempd and Dodds, 2002; Kyambadde et al., 2005). The growth potential of macrophytes to develop sufficient root systems influences microbial transformation of various materials into microbial biomass and nutrient conversion into plant biomass (Heidenwang et al., 2001; Kemp and Dodds, 2002). Differences in biomass of macrophytes on the water surface show substansial effects on the degradation of nitrogenous waste materials and uptake of nutrients (Kyambadde et al., 2005). However, maximum macrophytes biomass does not positively correlate with maximum nitrogen uptake because macrophytes uptake is limited by biomass covers that are too dense (IWA, 2000).

Managing nutrients, particularly nitrogen in RAS, is not only a step forward towards sustainable development but also crucial for maintaining high stocking densities and productions (Martins et al., 2005; Crab et al., 2007). A proper management procedure is necessary to improve the efficiency of nitrogenous wastes processed by the biofilter media (Ardiansyah and Fotedar, 2016a), which can be achieved by maintaining optimum growth rates of duckweed on the surface of the water; this, in turn, can be influenced by the harvest frequency. However, research on the use of duckweed as a biofilter in RAS (Jo et al., 2002), as well as the effects of duckweed's

harvesting frequency on nitrogen uptake rate and efficiency, is limited. Therefore, the aim of this study was to evaluate the effects of different quantities of duckweed in real time (using different harvesting frequencies) as biofilter media on total ammonia nitrogen (TAN) uptake rates and growth of barramundi (*Lates calcaifer* Bloch) IRAS.

3.2 Materials and Methods

This study was conducted in Curtin Aquatic Research Laboratory, Perth, Western Australia. Duckweed was brought to the laboratory, rinsed and acclimatised in 200 L circular tanks containing freshwater for two weeks. During the acclimation period, epiphytes were removed every second day. At the end of the acclimation period, duckweed was pre-incubated in 100 L circular tanks for 40 h before being incorporated into the biofilter tanks to evaluate TAN uptake capacity of duckweed in IRAS.

The present study was divided in two separate trials. In trial 1, the interaction between harvest frequency of 6, 4, 2, 0 (unharvested control) of duckweed and TAN uptake efficiency was evaluated. The frequency of duckweed harvest that delivered the maximum TAN uptake efficiency was selected and applied in trial 2 to evaluate the conversion rate and biomass production of duckweed in barramundi IRAS. Both trials were undertaken using a 12-independent IRAS.

In both trials, the increased quantity of duckweed biomass on the water surface was indicated by GI, and calculated every second day using the following equation: $GI = W_t / W_0$ where: W_t = biomass at time t, W_0 = biomass at time 0 and t = the number of days, whereas the increase of duckweed biomass per unit of harvest time was indicated by SGR and calculated at each harvest time using the following equation Zhang et al. (2014): SGR (% d-1) = (Ln Wf – Ln Wi) / t * 100, where: Ln Wf = the natural logarithm of the final weight, Ln Wi = the natural logarithm of the initial weight t = time (days).

In this study, TAN referred to the amount of NH_3 and NH_4^+. TAN uptake efficiency was measured following TAN depletion in the water. TAN was colourimetrically measured using a salicylate method. Total nitrogen was colourimetrically determined using a Nessler method. Nitrite and nitrate assessments were colourimetrically performed using a ferrous-sulphate and cadmium reduction method, respectively. Additionally, pH (digital pH meter Cyber-scan 30 (IL, USA), temperature and DO (YSI-55 probe, Yellow Spring Instruments, Ohio, USA) were measured. All water quality analyses followed APHA (2005) recommendations.

3.2.1 Trial 1: TAN uptake efficiency of duckweed associated with different harvest frequency

3.2.1.1 Fish and experimental condition

The experimental condition used in the trial was similar to previously published work (Ardiansyah and Fotedar, 2016a). Twelve independent IRAS were constructed, each consisting of three tanks: a fish-rearing tank (400 L circular tank), a biofilter tank (100 L circular tank) and a waste collection tank (100 L circular plastic drum). Water was circulated between the three tanks through PVC pipes using a submersible pump. The fish-rearing tanks and waste-collection tanks were placed on the floor, while the biofilter tanks were placed above the waste-collection tanks (Fig. 3.1). Water from the bottom of the fish-rearing tank was circulated through a PVC pipe to the waste-collection tank, and water from 20 cm below the surface of the waste-collection tank was pumped through a PVC pipe to the filter tank with a submerged pump. Water from the biofilter tanks was then circulated back into the fish-rearing tanks by gravity.

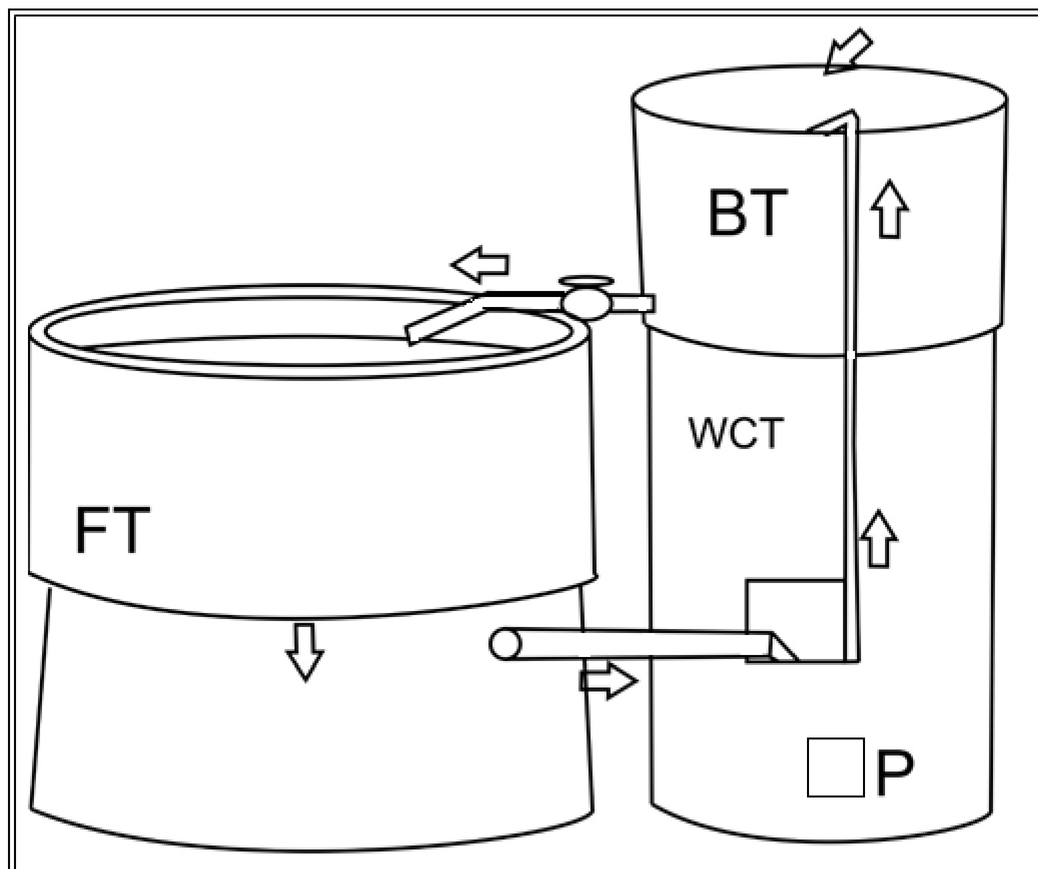


Figure 3.1. Design of the recirculating aquaculture system used in the experiment (Arrows show the direction of water flow), FT: fish-growing tank, BT: biofilter tank, WCT: waste collection tank and P: pump.

Before the start of the trial, 720 juvenile *L. calcarifer*, with an average size of 7.2 ± 0.17 g, were raised in the IRAS systems for 30 days (Ardiansyah and Fotedar, 2016a). At the end of the rearing period, all juveniles were removed from the IRAS systems, but the water in each tank was retained with no water exchanges. The water volume in each fish-rearing tank was maintained at 185 L, while that of the biofilter tanks and waste-collection tanks was kept at 40 and 30 L, respectively.

3.2.1.2 Experimental protocol

The experimental protocol was prepared following the method described in the previous study (Ardiansyah and Fotedar, 2016a). The trial was initiated by adding 18.51 g NH_4Cl as a source of ammonia-nitrogen into the fish-rearing tanks. Oxygen saturation in the fish-rearing tanks was maintained at above 80% by providing aeration. Water temperature was kept at 26 ± 2°C with photoperiods of 12 h. Water quality analysis of the systems was conducted every four days during the 36 day trial.

To measure TAN uptake efficiency and nitrification rates, uniform size of duckweed was selected, rinsed with deionised water and pre-incubated for 40 h in a 100 L fibre-glass tank with deionised water to elicit starvation, in order to induce maximal possible TAN uptake response during the subsequent measurements. After the starvation period, an initial amount of 35 g/tank of duckweed was randomly stocked into each of the 12 biofilter tanks. Four harvesting frequencies were employed by randomly harvesting every three tanks at a time (in triplicate) after 2, 4 and 6 days, whereas the remaining three tanks were not harvested (control) (Ardiansyah and Fotedar, 2016a). The harvested duckweed was then replaced by fresh prewashed duckweed. The reduction in water volume due to evaporation was compensated for by adding new freshwater (Ardiansyah and Fotedar, 2016a).

The biofilter performance was evaluated based on nitrification rates, TAN uptake rate and efficiency. Nitrification rates were calculated according to Kemp & Dodds (2002) by using the following equation: nitrification = (TANi – TANf/g) / t, where TANi = the initial TAN concentration, TANf = the final TAN concentration, g = grams dry weight (gdw) of substratum (duckweed) and t = time (days), whereas TAN uptake rate was determined according to Cohen &

Neori (1991): V = [TANi–TANf] x Qr; whereas uptake efficiency = [TANi-TANf/TANi]*100, where: V = TAN uptake rate (µmoles. $L^{-1}.h^{-1}$), Qr = the flow rate.

3.2.2 Trial 2: TAN conversion rate and biomass production of duckweed when cultured with the juvenile barramundi in RAS

3.2.2.1 Fish and experimental condition

Barramundi juveniles were obtained from Challenger Institute Technology, Western Australia, and transported to Curtin Aquatic Research Laboratory. Before the trial, barramundi juveniles were randomly stocked in twelve independent IRAS for the three-week acclimation period to reach an initial size of 26.67 ± 0.21 g $fish^{-1}$, in which the fish were divided into three stocking densities of 15.19, 18.87 and 22.55 kg m^{-3} per tank. 18.87 kg m^{-3} is the maximum stocking density in barramundi IRAS (Ardiansyah and Fotedar, 2016b). Over the acclimation period, the fish were fed with a high-protein commercial diet three times per day, at 09.00, 13.00 and 17.00 h at a feeding rate of 5% of the stock biomass, in order to increase TAN production. At the end of the acclimation period, TAN concentrations in the three-density treatments were 20.67, 23.67 and 26.67 mg L^{-1} TAN, respectively, in which the TAN ranged below and above the TAN concentrations recorded in trial 1 (23.67 mg L^{-1}). There was no water exchange throughout the acclimation period.

3.2.2.2 Experimental protocol

Three different stocking densities with three different TAN concentrations were designed to determine growth, nitrogen uptake rate and efficiency of duckweed. The trial was initiated by randomly stocking the initial amount of 35 g/tank of duckweed to each of the 12 biofilter tanks. Over the trial, the fish were hand-fed daily (ad libitum) at 09.00 and 16.00 h using a high-protein commercial diet (Skretting, Tasmania, Australia). The daily feeding rate was fixed at 3% of the stocked biomass. The approximate values of the commercial diet composition are provided by the feed manufacturer. Throughout the trial, oxygen saturation was kept at above 80% by providing aeration. Water temperature was maintained at 26 ± 2°C, with photoperiods of 12 h, and pH at 6.9-7.3. Water quality analysis of the systems was conducted every four days. At the end of the trial, barramundi in each experimental unit were weighed and their SGR was estimated by the following formula: $WG = W_2 - W_1$ where: W_2 = the final bodyweight (g), W_1 = the initial bodyweight (g). Specific growth rate (SGR) was calculated as follows: SGR = [(LnW_2

- LnW_1) /t] ×100 where: - Ln = the natural log, W_1= the initial bodyweight (g), W_2 = the final bodyweight (g) and t = period in days. Feed conversion ratio (FCR) was calculated as follows: FCR = FI (g) / WG (g) where: FI = dry weight (dw) of feed (g), WI = weight gain.

TAN production (TAN*p*) in the system was based on the barramundi feeding rate following the equation described by Pfeiffer and Malone (2006): TAN*p* = F x PC x 0.092, where TAN*p* = total ammonia nitrogen production ($g.d^{-1}$), F = daily feed ratio (g), PC = crude protein of the feed (%) and 0.092 = a constant in the equation derived from a series of estimates formulated from the percent nitrogen in protein and the protein assimilation (one day^{-1}).

The SGR of duckweed was measured every four days through manual harvesting and weighing of biomass. After estimating the four-day production, the same initial amount of duckweed was weighed and put back into the tank. This procedure was adopted and repeated every four days throughout the trial, in order to calculate and estimate duckweed SGR and ammonia uptake rate and efficiency.

The biofilter performance was determined based on the volumetric TAN conversion rate (TCR) and mean N uptake rate (*UR*mean). The TCR was calculated using following equation (Pfeiffer and Malone, 2006): TCR = [TANi–TANf] x Qr/Vb, where TCR $g\ m^{-3}$, TANi is the initial TAN concentration ($g\ m^{-3}$), TANf = the final TAN concentration ($g\ m^{-3}$), Qr = the flow rate through the filter ($m^3\ day^{-3}$) and Vb = the total volume of the biofilter medium (m^{-3}), whereas the mean N uptake rate (*UR*mean; $\mu mol\ N\ g^{-1}\ dw\ h^{-1}$) of duckweed was estimated based on N content of duckweed tissue according to the equation described by Zhou et al. (2006): *UR*mean = SGR x N tissue / 100, where SGR= specific growth rate (% day^{-1}) for the thalli; N tissue= N content of duckweed ($\mu mol\ N\ g^{-1}$ dw).

3.2.3 Statistical analysis

Data of TAN uptake efficiency and growth performance of duckweed were pooled for each treatment, computed and analysed using one-way variance (ANOVA), and the difference between means was examined using the Tukey HSD test. Critical values in all tests were $\alpha=0.05$.

3.3 Results

3.3.1 TAN uptake rate and uptake efficiency associated with different harvest frequency of duckweed

Table 3.1. Total ammonia nitrogen (TAN) uptake rate, nitrification rates and physicochemical parameters of water in the fish-rearing tank in trial 1.

Parameters	Harvest frequency			
	6 days	4 days	2 days	no harvesting
TAN uptake rates (µmoles. $L^{-1}.h^{-1}$)	33.00 ± 6.40	39.01 ± 7.21	38.05 ± 10.54	29.83 ± 4.71
Nitrification rates (mg TAN $gdm^{-1}.d^{-1}$)	37.10^{-3}±0.01	44.10^{-3}±0.01	42.10^{-3}±0.01	34.10^{-3}±0.01
The mean TN ($mg.L^{-1}$)	31.11 ± 0.72[b]	28.26 ± 0.88[a]	28.51± 1.00[a]	33.21 ± 0.75[c]
The mean nitrate (NO_3^{-1}) (mg L^{-1})	10.89 ± 0.56[b]	11.61 ± 0.63[c]	10.44 ± 0.55[a]	11.77 ± 0.67[d]
The mean nitrite (NO_2^{-1}) (mg L^{-1})	2.81 ± 0.39[b]	2.47 ± 0.37[a]	2.77 ± 0.41[b]	3.02 ± 0.42[c]
The mean temperature (°C)	26.46 ± 0.07[a]	26.38 ± 0.07[a]	26.39 ± 0.06[a]	26.55 ± 0.10[a]
The mean pH	6.18 ± 0.08[d]	6.11 ± 0.07[c]	5.91 ± 0.07[b]	5.78 ± 0.03[a]
The mean DO (mg L^{-1})	7.94 ± 0.10[d]	7.79 ± 0.13[c]	7.21 ± 0.10[b]	6.94 ± 0.08[a]

Variations in water quality and nitrification rates as a result of different harvesting frequencies are summarised in Table 3.2. Differences in the frequency of harvest influenced the TAN uptake rate, in which harvesting duckweed after every four days accounted for the highest TAN uptake rate of 39.01 µmoles L^{-1} h^{-1} compared to the other harvest frequencies. The TAN uptake rate was greatly influenced by duckweed biomass, in which the maximum TAN uptake rate was approximately 124.57 µmoles L^{-1} h^{-1} at biomass of 103.95 g fresh weight (fw) (Fig. 3.2); in these, at biomass below and or above 103.95 g fw, TAN uptake rates were not at the maximum.

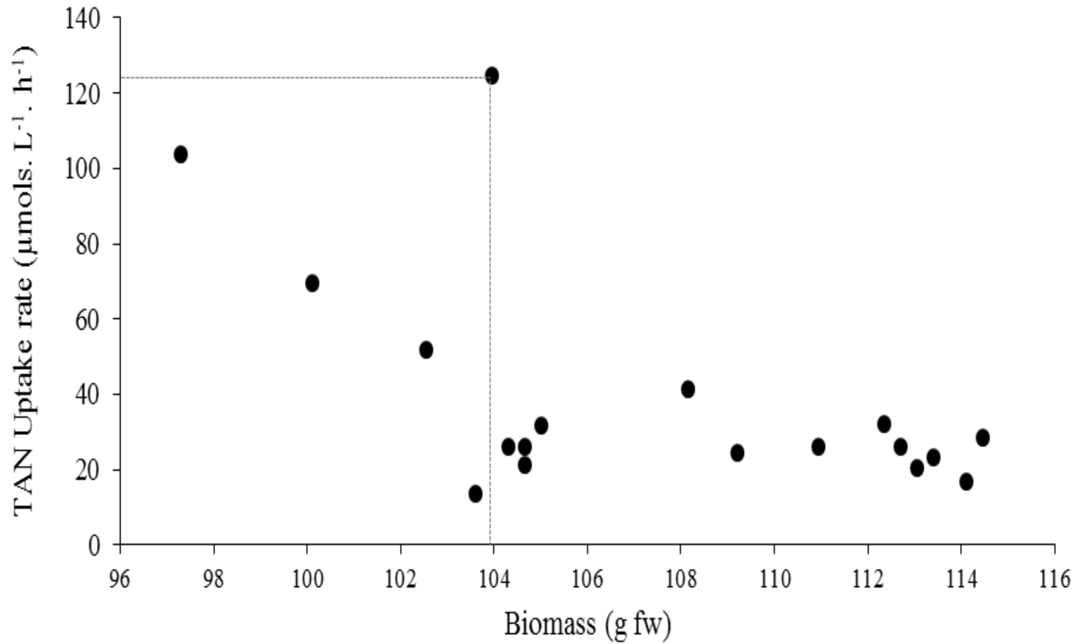


Figure 3.2. Total ammonia nitrogen (TAN) uptake rate of duckweed harvested every 0, 2, 4, 6 days in barramundi (*L. calcarifer*) RAS over the 36-day trial.

Nitrification rate was significantly affected by the harvest frequency of duckweed, in which the highest rate of 44 10^{-3} mg TAN gdw^{-1} d^{-1} was found in duckweed harvested every four days, while the lowest rate of 34.10^{-3} mg TAN gdw^{-1} d^{-1} was found in unharvested *L minor*. Furthermore, significant differences were also observed in total nitrogen (TN) and NO_3-N levels. The lowest TN was in duckweed harvested every four days, but no significant difference ($P >$ 0.05) was observed in 2-4-day harvest frequency. The lowest NO_3-N concentrations were in duckweed harvested every two days, while the highest were in unharvested-control.

A significant difference ($P \leq 0.05$) in dissolved oxygen (DO) concentration was observed during the 36-day trial. Harvesting duckweed every six days maintained the highest DO concentrations, whereas DO concentrations in unharvested duckweed tank was significantly lower ($P < 0.05$) than other harvesting frequencies. A similar trend was also observed in pH of the water. The results showed that different harvest frequencies resulted in significant ($P \leq 0.05$) differences in pH levels over the 36-day trial. The lowest pH level of 5.78 was observed in unharvested duckweed, whereas the highest pH of 6.18 was observed in duckweed harvested every six days.

Harvesting duckweed every four days generated the maximum TAN uptake efficiency, in which TAN reduced by around 94.33 % from 23.67 mg L^{-1} to 1.33 mg L^{-1}. Likewise, harvesting

duckweed every four days led to a reduction of 60.37% TN in the fish-rearing tanks, which was higher than those obtained from other harvest frequency (Fig. 3.3).

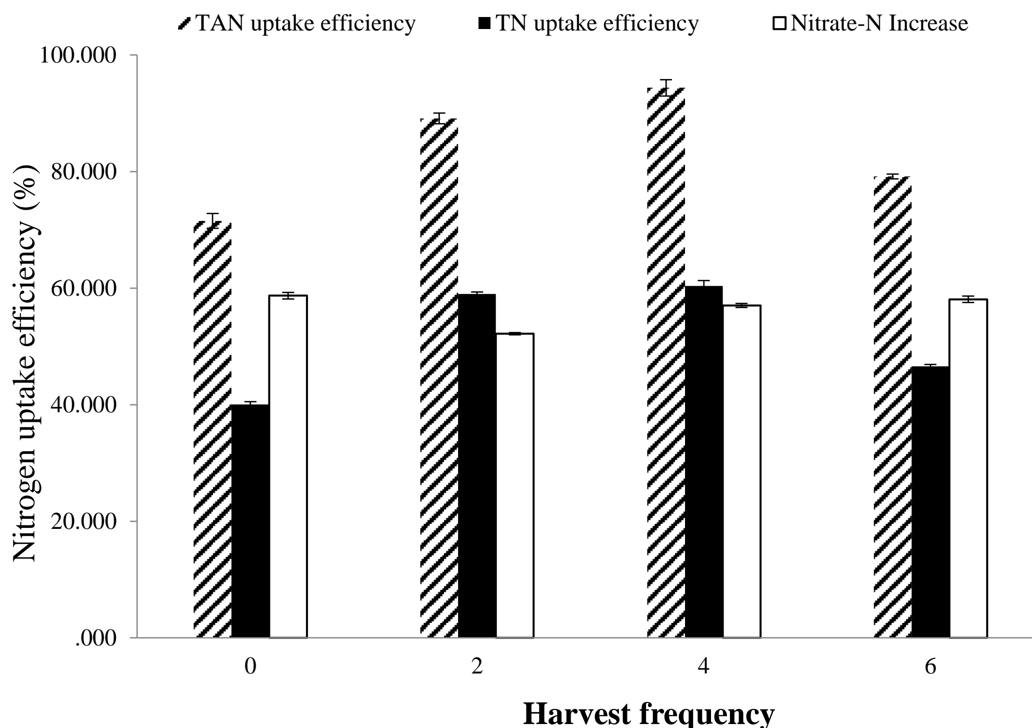


Figure 3.3. Total ammonia nitrogen (TAN) uptake efficiency of duckweed in trial 1.

3.3.2 TAN conversion rate and biomass of duckweed and growth of barramundi

Growth characteristics of barramundi and duckweed are summarised in Table 3.2. During the trial, no significant difference (P >0.05) was found in SGR, FCR and SR of the barramundi between three different densities. Total harvested biomass of duckweed in 15.19, 18.87 and 22.55 kg m^{-3} were 623.33, 796.33 and 814.33 g fw $tank^{-1}$, and the mean SGR was 23.92%, 27.80% and 28.62% day^{-1}, respectively. Even though, SGR and total harvested biomass of duckweed in the biofilter with high fish density were significantly higher ($P \leq 0.05$) than those at lower densities, the increase of SGR and biomass harvest from low to medium density were 16.22% and 27.75%, respectively; both were higher than the increase from medium to high density, 2.95% and 2.26%, respectively.

Table 3.2. Growth characteristics of barramundi (*L. calcarifer*) and duckweed (*L. minor*) in trial 2.

Stocking densities (kg m^{-3})	*Barramundi*			*Duckweed*	
	SGR (% day^{-1})	FCR	SR (%)	SGR (% day^{-1})	Total biomass yield (g $tank^{-1}$)
15.19	1.39±0.01[a]	1.98±0.01[a]	100±0.01	23.92±0.12[a]	623.33[a]
18.87	1.37±0.01[a]	1.99±0.01[a]	99±0.01	27.80±0.21[b]	796.33[b]
22.55	1.37±0.02[a]	1.99±0.00[a]	99±0.10	28.62±0.30[c]	814.33[c]

[a.] Values in the same column with the same letter are not significantly different (P>0.05)

The main nitrogen sources correlated positively with stocking density, TAN*p* and tissue N concentration (Table 3.3). Tissue N concentration was higher for duckweed grown at the high fish density than that for those grown at low fish density. The trend for N concentration in duckweed was similar to TAN*p*, in which significant differences were observed among different stocking densities. The increase of 32% in daily feeding rate of the fish at higher stocking densities resulted in an increase of approximately 32% in TAN production (TAN*p*), followed by an increase of approximately 31.03% and 31.58% TCR of duckweed biofilter at medium and high density, respectively; this indicates that TAN conversion rate of duckweed increased with higher stocking density and nitrogen load.

Table 3.3. Variations in total ammonia nitrogen (TAN) production, the volumetric TAN conversion rate (TCR) and mean TAN uptake rate (*UR*mean) of duckweed in trial 2.

Stocking densities (kg m^{-3})	Daily feeding rate (g)	TAN*p* (g TAN day^{-1})	TCR (g TAN removed day^{-1})	N tissue of duckweed (mmol N g^{-1} dw)	*UR*mean (μmol N g^{-1} h^{-1})
15.19	116.35±0.38	4.82±0.02	0.29±0.01	0.59±0.01	14.18 10^3±0.20
18.87	153.75±0.90	6.37±0.04	0.38±0.01	0.66±0.01	18.35 10^3±0.48
22.55	202.90±0.41	8.40±0.02	0.50±0.01	0.67±0.01	19.19 10^3±0.50

[a.] Values in the same column with the same letter are not significantly different (P>0.05)

Although, an increase in tissue N concentrations was only about 11.86% at medium stocking density, an increase of approximately 29.4% was observed in *UR*mean N uptake rate of duckweed. Similarly, when no significant increase was observed in tissue N concentrations of duckweed reared at the highest levels of fish stocking density, *UR*mean N uptake rate of duckweed increased by approximately 4.58%.

Variations in water quality as a result of different stocking density and feeding rates are presented in Table 3.4. The water quality data demonstrated that the acceptable levels of TAN and NO_2-N were attained at all stocking densities and feeding rates. Increased stocking density, coupled with an increase in feeding rates, did not significantly affect DO concentration. Similarly, the water pH was also maintained above 7.10 in all stocking densities.

Table 3.4. Physicochemical properties of the water as the function of duckweed usage as biofilter media in trial 2.

Parameters	Stocking density		
	15.19	18.87	22.25
DO (mg L^{-1})	7.87±0.01	7.84±0.01	7.82±0.01
pH	7.25±0.01	7.22±0.01	7.18±0.01
Temperature (°C)	26.72±0.10	26.79±0.08	26.82±0.09
TAN (mg L^{-1})	0.89±0.06	0.94±0.06	0.98±0.06
Nitrate nitrogen (mg L^{-1})	3.14±0.20	3.50±0.21	3.75±0.21
Nitrite nitrogen (mg L^{-1})	0.06±0.01	0.08±0.01	0.09±0.01
Total nitrogen (mg L^{-1})	5.05±0.25	5.48±0.28	5.93±0.27

[a.] Values in the same column with the same letter are not significantly different ($P>0.05$)

3.4 Discussion

3.4.1 TAN uptake rate and uptake efficiency associated with different harvest frequency of duckweed

Due to a free-floating habitat, duckweed receives nutrients from the water column and the atmosphere, as it cannot get them from the sediment below. Thus, duckweed density on the surface water, as indicated by growth index (GI) and surface area availability, may become limiting factors for their SGR. Different harvest frequencies influenced the biomass of duckweed on the surface layer of the water, at a given time, which in turn caused significant differences in TAN uptake rates and efficiency of duckweed. Harvesting duckweed every four days supported the maximum TAN uptake rates and efficiency compared to the other harvest frequencies. Harvesting duckweed every six days resulted in the highest GI of duckweed (Zhao et al., 2014), which in turn led to a slower SGR and reduced the total biomass harvested, whereas harvesting duckweed every two days resulted in the lowest duckweed GI, thereby reducing the uptake rate capacity of duckweed. Similarly, unharvested duckweed caused excessive growth and increased GI of duckweed at the water surface, thus slowing down the SGR of duckweed. Higher GI indicates higher biomass on the water surface beyond the system capacity (Reddy and DeBusk, 1985), as N uptake becomes limited by the light available (Chaiprapat et al., 2005). Matos et al. (2006) reported that as the stocking density of *Gracilaria bursa pastoris* increased up to 7 kg m^{-2} under N-sufficient conditions, the biomass harvested decreased significantly. In addition, higher

biomass cover of duckweed can limit the atmospheric dissolution of the oxygen coupled with oxygen reduction by algal respiration. Further, the extensively interlaced root of duckweed effectively retained suspended organic particles which provided sufficient substrates for the proliferation of heterotrophic bacteria (Azza et al. 2000).

Over long harvest intervals caused excessive proliferation of duckweed and increased biomass on the water surface of the tank beyond the limits of maximum uptake rate (Fig. 3.2). Too long a harvest interval increased biomass density above 103.95 g fw, which led to a decrease in TAN uptake rate of duckweed, whereas the difficulties of growing sufficient duckweed to achieve the maximum TAN rate may be due to too short a harvest interval. When the increase of duckweed was, as occurs in the current study, a reduction in SGR of duckweed due to the limited light penetration to the lower layers, increased competition for space grew. Although light penetration and competition for space were not examined in this study, they appeared to be related to the same source, the biomass per unit surface area of duckweed. Similarly, Reddy and DeBusk (1985) and Chaiprapat et al. (2005) stated that higher SGR of *Lemnaceae* were achieved at lower plant biomass. The biomass of duckweed was used as a last indicator to represent all the effects mentioned. The result suggests that sufficient duckweed biomass is required by the plant and access to light required by duckweed for N uptake and photosynthesis (Mohedano et al., 2012).

In the absence of regular harvest, signs of chlorosis, disconnection of duckweed fronds and a dense mat of unidentified species of the microalgal population was observed on the water surface. Increased microalgal population can further decrease duckweed productivity and result in an increase in oxygen consumption, as indicated by lower DO concentrations, and may increase inputs of remineralised nutrient from macroalgal decomposition (Frederic et al., 2006). Additionally, N uptake rate and uptake efficiency of duckweed associated with microalgal proliferation exhibit different behaviours. Frederic et al. (2006) reported that for duckweed biomass lower than 25 g dw, uptake rates were higher for increasing initial biomass of duckweed. At medium biomass (from 25 to 45 g dw), the light crossing the layer of duckweed on the surface water becomes the controlling factor of the microalgae growth. Beyond biomass of 45 g dw, duckweed biomass yield decreases, whereas the average age of individual duckweed increases. Subsequently, duckweed lose their protecting resin and become vulnerable to the competitive microalga (Cross, 2002).

Our research showed that a high TAN concentration can be removed efficiently with duckweed by manipulating its biomass quantities on the surface water. A lower pH level observed

throughout trial 1 did not affect TAN uptake efficiency of duckweed. Korner et al. (2003) reported that *L. gibba* can be used to treat wastewater containing very high TAN concentrations, as long as certain pH levels are not exceeded (7.8). Wastewater treatment using *Lemnacea*e becomes impossible at pH levels above approximately 9.8, as ammonia volatilisation is unlikely at pH above 9.4 (Reddy and Graetz, 1987).

3.4.2 Growth and TAN conversion rate of duckweed

Increased stocking density correlated with the high amount of feed given to the fish, which in turn increased nitrogen load in the water column. In our study, duckweed can be an efficient biofilter capable of removing most of the nitrogen originating from the cultivation of barramundi juveniles. Total harvested biomass of duckweed in low, medium and high fish stocking density indicate that different stocking density and nitrogen load positively affected the growth of duckweed, whereas SGR, FCR, and SR of barramundi juveniles were not negatively affected by the different stocking densities and nitrogen load. The result suggests that high nitrogen uptake efficiency of duckweed could maintain an optimal growth of barramundi juveniles reared at these three different stocking densities.

However, the percentage of the increase in duckweed SGR and biomass harvest from low stocking density to medium density was higher than that from medium stocking density to high density, indicating that at a high nitrogen load, the duckweed biomass on the water surface negatively affected SGR. This can be attributed to limited light penetration and high nutrient removal rates possibly negatively influencing the growth of duckweed growth. Under high biomass conditions, fronds can pile up in several layers subdivided into two parts, an upper part with nutrient limitation and a lower part with light limitation (Driever et al., 2005).

In the study, N concentrations in duckweed at low, medium and high stocking density were in line with the finding of Cadergen and Madsen (2002), who reported increased N concentrations in duckweed when cultivated at low and high N media from 0.40 to 0.71 mmol N g^{-1} dw. Mean N uptake rates (*UR*mean) of duckweed at low, medium and high stocking density were much higher that the finding of Copertino et al. (2009) on *Ulva clathrata*. The rates of TAN uptake obtained were much higher than the results of previous studies by Cedergen and Madsen (2002) on duckweed, Subandar et al. (1993) on *Laminaria saccharina* and Zhou et al. (2006) on *Gracillaria lemaneiformis*. Similar to ours, previous studies have indicated that the increased TAN concentrations in the biofilter tanks were proportional to the filter's conversion ability (Pfeiffer and Malone, 2006; Sandu et al., 2002).

An increase in N concentration in duckweed is associated with the increased TAN uptake (*UR*mean) and conversion (TCR) rates. Most studies conducted with N-limited macroalgae demonstrated strong correlations between N uptake rates and tissue N of the macroalgae (Liu and Dong, 2001). Furthermore, increased TAN production caused increased consumption of oxygen (Zhang et al., 1995; Intriago, 2012). However, this was not evident throughout this study. Minimal variation in DO and pH were measured during the study, and this suggests that the use of duckweed as biofilter media may maintain an optimal level of DO in the water column, thereby reducing the negative effects of stocking density and TAN production of ammonia.

3.5 Conclusions

In summary, this study demonstrated that duckweed can be integrated into juvenile barramundi RAS due to its high nitrogen bioremediation efficiency and assimilative capacity; and its integration with fish culture may be an effective measure to maintain optimum water quality for fish growth and survival, and to reduce nitrogen loading in the environment. The study suggested that excess TAN concentrations can be efficiently removed from barramundi juvenile RAS when duckweed is harvested every four days. These results indicated that TAN uptake is one of the potential factors reducing ammonia in the system.

CHAPTER 4: The Abundance and Diversity of Heterotrophic Bacteria as a Function of Harvesting Frequency of Duckweed (*Lemna minor* Linnaeus) in Recirculating Aquaculture Systems[2]

4.1 Introduction

Many fish farms operate using recirculating aquaculture systems (RAS), and are characterized by high stocking densities of the target species along with large quantities of feed, resulting in the generation of significant amounts of organic and inorganic substances mainly including ammonia and phosphates (Martins et al., 2010). The fish also excrete nitrogenous wastes, urine, and faeces, whose decomposition and assimilation are especially important in RAS because of the high toxicity of ammonia and nitrite, and the possibility of hypertrophication of the environment by high levels of nitrate.

Production systems that utilize aquatic plants to recover nutrients from wastewater have a promising future as an alternative technology for converting the recovered nutrients into potentially useful products and preventing nutrient overload in the environment (Cheng et al., 2002). Various aquatic macrophytes have been used as biofilters, important components of RAS (Zhang et al., 2011), to remove nitrogen, phosphorus, and other elements that cause eutrophication in natural aquatic ecosystems (Nahlik and Mitsch, 2006). The duckweed (*Lemna minor* Linnaeus) is considered a potential biofilter by aquaculturists because of its rapid specific growth rates (SGR), ability to adapt to a wide variety of environmental conditions, and use as stockfeed for many fish species (Mukherjee et al., 2010). Compared with other aquatic macrophytes, duckweed has a high nutrient recovery capacity (Landolt and Kandeler, 1987). The efficacy of using duckweed as nitrogen and phosphorus biofilters in fishponds has already been evaluated by Ferdoushi et al. (2008), who reported that the introduction of duckweed in a fish pond efficiently recovered nitrogen and phosphorus. Thus, incorporating duckweed into the RAS is expected to remove nutrients, thereby maintaining water quality while producing a useful by-product that can be used as a protein-enriched feed for fish species.

Biofilters not only remove excess nitrogen from RAS, but can also provide a suitable substrate for heterotrophic bacterial adhesion and growth (Schreier et al., 2010). Hence, the type of biofilter and the surface area of the biofilter media per unit volume influences the abundance and diversity of bacterial communities in RAS, as these biofilters contribute to the growth and

[2] This chapter has been published in Letters in Applied Microbiology 63 (2016), 53-59.

structure of bacterial communities (Leonard et al., 2000). As such, biofilters can increase the overall effectiveness of the media in RAS through the support of heterotrophic bacterial communities.

The bacterial communities in RAS consist of both autotrophic and heterotrophic bacteria. The role of autotrophic bacteria in processing waste has been widely documented (Ikeda-Ohtsubo et al., 2013; Yao et al., 2013), but their abundance represents only 20% of the total eubacterial rRNA in biological filters. On the other hand, reports on the abundance and diversity of non-pathogenic heterotrophic bacteria in fish production systems are still limited, and practically no attention has been paid in regard to their role as RAS biofilters. High dissolved organic carbon in RAS and particulate organic carbon trapped in biofilters are the major sources of carbon available for heterotrophic bacterial growth (Michaud et al., 2006).

In any RAS, a proper management strategy is required to improve the efficiency of nitrogenous wastes processing by heterotrophic bacteria, which can be achieved by manipulating their abundance and diversity (Blancheton et al., 2013). This in turn is controlled by the quantity of biofilter media such as duckweed. Therefore, this study aimed to evaluate the effects of different harvesting frequencies of duckweed in a RAS on the composition, abundance, and diversity of non-pathogenic heterotrophic bacteria, involved in the removal of nitrogenous waste from the RAS.

4.2 Materials and Methods

4.2.1 Experimental setup and design

Twelve independent recirculating systems were constructed, each consisting of three tanks: a fish-rearing tank (400 L circular tank), a biofilter tank (100 L circular tank), and a waste-collection tank (100 L circular plastic drum). Water was circulated between the three tanks through PVC pipes using a submersible pump (Fig. 3.1).

The biofilter tanks were filled with free-floating duckweed that was taken from a stock tank. Initially, 35 g of freshly weighed duckweed per tank was washed with filtered fresh water and randomly stocked into each of the 12 biofilter tanks. Prior to the start of the trial, 720 juvenile barramundi (*Lates calcarifer* Bloch), with an average size of 7.2 ± 0.17 g, were raised in the RAS for 30 days. At the end of the rearing period, all juveniles were removed from the RAS, but the water in each tank was retained with no changes to water circulation. The water volume in each fish-rearing tank was maintained at 185 L, while the water volume in the biofilter tanks and

waste-collection tanks was kept at 40 and 30 L, respectively. Oxygen saturation in the fish-rearing tanks was maintained at above 80% by providing aeration. The water temperature was kept at 26 ± 1°C with photoperiods of 12 h dark and 12 h light.

The trial was initiated by adding 18.51 g of NH_4Cl as a source of ammonia-nitrogen into the fish-rearing tanks. Four harvesting frequencies were employed by randomly harvesting 3 biofilter tanks at one time (in triplicate) after 2, 4, and 6 days, while the remaining 3 tanks were not harvested (control). The harvested duckweed was then replaced by fresh, prewashed duckweed. The reduction in water volume due to evaporation was compensated for by adding new freshwater.

The health of the duckweed was visually observed by looking for signs of chlorosis and disconnection of the fronds (Khellaf and Zerdaoui, 2010). The increase in biomass of the duckweed on the water surface was indicated by GI, as calculated every second day using the equation: $GI = W_t / W_0$ where: W_t = biomass at time t, W_0 = biomass at time 0 and t = number of days. The increase in duckweed biomass per unit of harvest time was indicated by SGR, calculated at each harvest time using the equation: $SGR = (Ln(W_f) - Ln(W_i) * 100) / t$, where: $Ln(W_f)$ = the natural logarithm of the final weight, Ln(Wi) = the natural logarithm of the initial weight, and t = time (days).

4.2.2 Identification and enumeration of bacteria from biofilter tanks

Isolation and identification of heterotrophic bacteria were performed based on the methods of Forbes et al. (1998). As it takes 4–6 weeks for any bacterial community to get established in a new biofilter tank (Costa-Pierce, 2002), a 10 g wet weight of duckweed was randomly collected using 40 mL sample jars (each containing 10 mL of distilled water) on day 12 from all biofilter tanks after the duckweed was placed in the biofilter tanks for over 4 weeks to allow the establishment of a sufficient number of bacteria. The samples were homogenized in a homogeniser at 17,000 *g* for 5 min. The homogenate water (10 mL) was diluted in 90 mL of phosphate buffered saline (PBS), and homogenized for 2 minutes at 1900 *g*. The resulting homogenates were diluted 10 times with PBS. Using an aseptic technique, 0.1 mL of diluted samples were transferred separately onto solid trypticase soy agar (TSA) media using the conventional pour-plate technique.

After incubation at 20°C for 48 hours, colonies with different morphology were selected for species identification, of which only plates having between 30 and 300 colonies were considered.

The colonies were counted and expressed as CFU per mL for the attached bacteria as previously described (Leonard et al., 2000). A representative of each colony was removed from the plates and purified by streaking on fresh media with three serial streakings of each culture being done to ensure purity of the strains. The purified colonies were then characterized using a variety of biochemical tests, including oxidase, catalase, hydrogen sulfide production, ornithine decarboxylase, tryptophan hydrolysis, gelatin liquefaction, methyl-red and voges-proskauer, starch hydrolysis, citrate utilization, urease, nitrate reduction, carbohydrate (glucose, fructose, sucrose, mannose, maltose, lactose, and xylose). The identification results obtained from the conventional biochemical tests were then compared with those obtained by the analytic profile index (API) 20E identification system (bioMerieux). Data obtained were analysed using the API Web program.

4.2.3 Bacteria diversity index

The bacteria diversity index assumes that individual bacteria are sampled randomly from a large population so that all species are represented in the sample. The species diversity was calculated using the Shannon diversity index with the following formula: $H = - [\Sigma P_i * Ln(P_i)]$, where H = Diversity index, P_i = the number each species in the sample/total number of samples, and $Ln(P_i)$ = the natural logarithm of this proportion.

4.2.4 Statistical analysis

All data were expressed as mean ± standard error (SE), and group mean differences were compared using a one-way analysis of variance (ANOVA) and the Tukey HSD test. A regression analysis was performed to analyse the relationship of duckweed growth with gains on bacterial abundance and diversity. Values of $R^2 < 0.65$ were considered to have poor correlations, whereas $R^2 > 0.65$ indicated good correlations. A $P < 0.05$ was considered significant for all analyses.

4.3 Results

4.3.1 Abundance and diversity of heterotrophic bacteria

Table 4.1. Abundance of heterotrophic bacteria attached to the duckweed in the biofilter tank.

No	Heterotrophic bacteria (cfu x 10^3/mL)	Harvest frequency			
		6 days	**4 days**	**2 days**	**No harvesting**
1	*Bacillus licheniformis*	4.06 ± 0.11^b	4.10 ± 0.02^b	2.10 ± 0.04^a	3.69 ± 0.27^b
2	*Pseudomonas stutzeri*	3.54 ± 0.03^b	3.54 ± 0.06^b	3.10 ± 0.03^a	3.47 ± 0.01^b
3	*Aeromonas salmonicida*	2.32 ± 0.08^a	2.35 ± 0.09^a	2.05 ± 0.05^a	2.35 ± 0.05^a
4	*Chryseobacterium indologenes*	2.41 ± 0.12^a	2.36 ± 0.19^a	2.09 ± 0.13^a	2.45 ± 0.15^a
5	*Bacillus subtilis*	3.22 ± 0.11^b	3.40 ± 0.26^b	2.21 ± 0.26^a	3.20 ± 0.08^b
6	*Pseudomonas mendocina*	3.65 ± 0.14^a	3.55 ± 0.17^a	2.89 ± 0.48^a	3.65 ± 0.03^a
7	*Azotobacter vinelandii*	2.56 ± 0.13^b	2.57 ± 0.16^b	1.50 ± 0.10^a	2.07 ± 0.10^b
8	*Acinetobacter calcoaceticus*	1.21 ± 0.02^a	1.44 ± 0.04^a	1.06 ± 0.02^a	1.11 ± 0.08^a
9	*Achromobacter xylosoxidans*	1.41 ± 0.08^a	1.20 ± 0.17^a	1.10 ± 0.07^a	1.34 ± 0.05^a
	Total	24.38 ± 0.42^b	24.53 ± 0.61^b	18.11 ± 0.72^a	23.30 ± 0.09^b

[a.] Values in the same column with the same letter are not significantly different ($P>0.05$)

The abundance of bacteria attached to duckweed is summarized in Table 4.1, in which 4 out of 9 bacterial communities, *Bacillus licheniformis, B. subtilis, Pseudomonas stutzeri,* and *Azotobacter vinelandii*, were significantly affected ($P < 0.05$) by different harvest frequencies. Duckweed harvested with the shortest interval of 2 days had lower abundance than those with longer harvest intervals of 4 and 6 days and unharvested duckweed (control).

The highest Shannon diversity index value of bacteria was observed on duckweed that was harvested every 6 days while the lowest value was from the control (Table 4.2). Duckweed biomass harvest and the heterotrophic bacteria abundance were poorly correlated ($R^2 < 0.65$) to harvest frequencies. Significantly stronger correlations between duckweed biomass harvest and bacterial diversity were evident at the 4-day harvest frequency and unharvested control than in the other groups. Likewise, the diversity of heterotrophic bacteria was significantly correlated

(R^2 > 0.65) with SGR of duckweed at the 2 and 4-day harvest frequency, and the unharvested (control). Meanwhile, harvesting duckweed every 6 days resulted in a poor relationship between SGR and biomass harvest with the abundance and diversity of heterotrophic bacteria.

Table 4.2. Shannon diversity index, total harvested biomass of duckweed, Mean GI and SGR of duckweed with correlation coefficients (R^2) between growth performance represented by biomass harvest and SGR of duckweed with abundance and diversity of heterotrophic bacteria over the 36-day trial.

Harvest frequency	6	4	2	0
R^2 (biomass harvest vs abundance)	0.57±0.02[a]	0.57±0.06[a]	0.58±0.04[a]	0.60±0.00[a]
R^2 (biomass harvest vs diversity)	0.50±0.01[a]	0.65±0.01[c]	0.62±0.05[b]	0.68±0.03[c]
R^2(SGR vs abundance)	0.58±0.06[a]	0.67±0.06[a]	0.69±0.03[a]	0.69±0.03[a]
R^2(SGR vs diversity)	0.50±0.01[a]	0.65±0.01[b]	0.62±0.05[b]	0.68±0.03[b]
Shannon diversity index (H)	2.27±0.01[c]	2.17±0.01[bc]	2.14±0.01[b]	2.13±0.00[a]
Mean GI	3.14± 0.04[b]	3.07± 0.04[b]	2.97± 0.02[a]	3.09± 0.03[b]
Mean SGR	8.14 ± 0.00[b]	9.31 ± 0.00[c]	11.05 ± 0.00[d]	3.04 ± 0.00[a]
Total biomass harvest	656.37 ± 0.51[b]	999.37 ± 0.51[c]	1871.57 ± 2.43[d]	104.53 ± 0.11[a]

[a.] Values in the same column with the same letter are not significantly different (P>0.05)

4.3.2 Growth performance of duckweed

Different harvest frequencies also had a significant influence on duckweed's growth performances such as growth index (GI), SGR, and biomass harvest (Table 4.2). Harvesting duckweed after 2 days resulted in the lowest duckweed GI, while 6-day harvests resulted in higher duckweed GI than 2-day harvests, which in turn led to a slower SGR, and a lower harvested biomass than the 2 and 4-day harvest frequency. Significantly higher SGR and total biomass harvest of duckweed were achieved in the biofilter tanks when duckweed was harvested every 2 days compared with those of unharvested duckweed, where the unharvested control resulted in the lowest SGR and biomass harvest. Furthermore, in the absence of regular harvest, signs of chlorosis, disconnection of duckweed fronds, and a dense mat of unidentified species of the microalgal population on the water surface were observed.

4.3.3 Morphological and biochemical characteristics of heterotrophic bacteria

The morphological and physiological characteristics of the isolates identified to the genus level showed that Gram-negative bacteria dominates the bacterial communities (Table 4.3).

Table 4.3. The morphological and physiological characteristics of bacteria isolated from duckweed in the biofilter tanks.

Sample	Morphology	Gram stain	Spore	Motility	Pigmentation	No. of clones	Genus
1	Bacilli	+	+	+	White	7.8	*Bacillus*
2	Bacilli	-	-	+	Yellow	13.16	*Pseudomonas* sp.
3	Bacilli	-	-	-	Yellow	10.53	*Chryseobacteriu*
4	Bacilli	-	-	-	Brownish-	10.53	*Aeromonas* sp.
5	Ovoid	-	-	+	Yellow	18.	*Azotobacter*
6	Coccobacilli	-	-	+	White	10.53	*Achromobacter*
7	Cocci	-	-	-	White	7.89	*Acinetobacter*
8	Bacilli	-	-	+	Brownish-	7.89	*Pseudomonas*
9	Bacilli	+	-	+	Translucent	13.16	*Bacillus* sp.

Specifically, 42.11% of Gram-negative bacilli isolates were identified as *Pseudomonas*, *Chryseobacterium*, and *Aeromonas*; 36.84% of isolates of Gram-negative ovoid, cocci, or coccobacilli were identified as *Azotobacter*, *Acinetobacter*, or *Achromobacter*; and 21.05% of isolates of Gram-positive bacilli belonged to *Bacillus*. Non-pigmented isolates belonged to *Bacillus, Aeromonas, Achromobacter*, and *Acinetobacter*, whereas non-motile isolates were characterised as *Chrysobacterium*, *Aeromonas*, and *Acinetobacter*. In addition, the most common genera in the biofilter tanks were *Bacillus* and *Pseudomonas* (42.10%), whereas the least number of genera belonged to the genus *Acinetobacter* (7.89%).

Out of 38 different isolates, 16 species of heterotrophic bacteria were biochemically characterized from duckweed (Table 4.4). Most of the isolates were characterized as the same species by both the conventional biochemical tests and API 20E, where 15 of 16 isolates had the same identification result. Out of 16 isolates as shown in Table 4.4, *A. vinelandii* had the maximum number of isolates as three. Another five species, such as *B. licheniformis*, *P. mendocina, A. salmonicida, Chrysobacterium indologenes* and *Achromobacter xylosoxidans* had

two isolates each, whereas remaining three species, *B. subtilis, P. stutzerii,* and *Acinetobacter calcoaceticus* consisted of only one isolate each.

Out of 16 isolates as shown in Table 4.4, *A. vinelandii* had the maximum number of isolates as three. Another five species, such as *B. licheniformis*, *P. mendocina, A. salmonicida, Chrysobacterium indologenes* and *Achromobacter xylosoxidans* had two isolates each, whereas remaining three species, *B. subtilis, P. stutzerii,* and *Acinetobacter calcoaceticus* consisted of only one isolate each.

Table 4.4. Biochemical tests of the heterotrophic bacteria attached to the duckweed in the biofilter tanks.

Biochemical characteristics	No. of bacterial isolates															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase test	-	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+
Ornithine decarboxylase	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-
Tryptophan hydrolysis	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
H_2S	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-
Gelatin liquefaction	+	-	+	+	-	-	+	-	-	-	-	+	-	-	+	-
Starch hydrolysis	+	+	-	+	+	-	-	+	+	+	-	+	-	+	+	-
Gas	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	+
MR	-	-	+	-	-	-	+	+	-	+	-	-	-	+	-	-
VP	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
Citrate utilization	+	-	-	-	+	+	-	+	+	+	+	+	+	+	-	+
Nitrate reduction	+	+	+	-	+	+	+	+	+	+	+	+	_	+	-	+
Carbohydrates utilization																
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
Sucrose	+	-	+	-	+	-	+	+	-	+	-	+	-	+	-	-
Mannose	+	+	+	+	+	-	+	+	-	+	-	+	-	+	+	-
Maltose	+	+	+	+	+	-	+	+	-	+	-	+	-	+	+	-
Lactose	+	-	-	-	+	-	-	+	-	+	-	+	-	+	-	-
Xylose	-	-	-	-	+	+	-	+	+	+	+	-	-	+	-	+

4.4 Discussion

The present study showed that different harvest frequency significantly influenced the abundance of heterotrophic bacteria. Harvesting duckweed after every 2 days resulted in the lowest GI of duckweed, lowering the availability of organic substrates for bacterial attachment and growth (Itoi et al., 2006). A short harvest interval negatively affected the abundance of heterotrophic bacteria. The abundance of *B. subtilis, B. licheniformes, A. beijerinckii,* and *P. stutzeri* were lower at 2 days of harvest frequency. The results indicated that shorter harvest interval did not provide sufficient opportunity for the bacterial attachment and growth, affecting the growth and colonization of bacteria on duckweed. Davey and O'Toole (2000) and O'Toole et al. (2000) reported that bacterial communities are closely associated with the surface or submerged substrates.

Meanwhile, the longer harvest interval of 6 days resulted in the highest duckweed GI, which in turn caused a slower SGR and reduced the harvested biomass. The 6-day harvest and unharvested duckweed caused chlorosis and disconnection of duckweed fronds. The results indicated that too long harvest interval negatively affected growth and biomass harvest of duckweed. Frequency of harvest is required to encourage growth (Reddy and Smith, 1987) and maintain a healthy duckweed (Landolt and Kandeler, 1987 and Zhao et al., 2014).

Furthermore, a 4-day harvest frequency resulted in stronger correlation between harvest and bacteria diversity and a significant correlation between SGR and diversity of heterotrophic bacteria. Significantly stronger correlations between duckweed biomass harvest and bacterial diversity were evident at the 4-day harvest frequency and unharvested control than in the other groups. Likewise, the diversity of heterotrophic bacteria was significantly correlated ($R^2 > 0.65$) with SGR of duckweed at the 2 and 4-day harvest frequency, and the unharvested (control). Zhao et al. (2014) suggested that harvest frequency of 4 days is desirable to maintain the optimum biomass harvest of duckweed. Therefore, the results indicated that the 4-day harvest frequency might provide the desirable duckweed biomass and optimal availability of the organic substrates as carbon and energy source for the heterotrophic bacteria.

Shannon-Wiener diversity index of heterotrophic bacteria decreased with increasing frequency of harvest. The heterotrophic bacteria abundance and duckweed biomass harvest were poorly correlated ($R^2 < 0.65$) to harvest frequencies. The result indicated that harvest frequency was closely linked with changes in the availability of organic substrates, in which the bacteria

communities in the biofilter tank may have responded to various changes in the availability of substrates through physiological adaptations and changes in community composition. Pinhassi et al. (1999) and Eiler et al. (2003) reported that even small additions or reductions of organic substrates can cause a shift in the composition of the bacterial community.

The most dominant bacterial species in this study was *A. vinelandii*. This species is a common nitrogen-fixing bacterium, essential for maintaining the nitrogen cycle homeostasis in the biosphere. Most members of the genus *Azotobacter* can be used as biofertilizers, fish food organisms, detritus processors, bioremediators, bioameliorators, and, importantly, as biofilters (Tripathy and Ayyappan, 2005).

The *Bacillus* consisted of obligate aerobes (*B. subtilis*), or facultative anaerobes (*B. licheniformis)*, which are known to have a major role in heterotrophic nitrification (Kundu et al., 2014). Meanwhile, the *Pseudomonas,* the most abundant heterotrophic bacteria in marine RAS nitrification biofilters (Borges et al., 2008), was represented by two species: *P. mendocina* and *P. stutzeri*. Certain species of *Pseudomonas* have the ability to metabolize an extensive number of organic compounds, whereas others have metabolic properties such as denitrification, degradation of aromatic compounds, and nitrogen fixation (Lalucat et al., 2006).

The *Aeromonas* is commonly found in aquatic environments and is dominant in waters with high levels of faecal pollution. *A. salmonicida* is an etiological agent for furunculosis that severely impacts salmonids populations worldwide (Faisal et al., 2007). Heterotrophic bacteria, including *A. salmonicida*, obtain energy from the oxidation of carbon- and nitrogen-containing compounds. Thus, the presence of *A. salmonicida* may indicate excessive nitrogenous waste in the system. However, many members of the genus *Aeromonas* are capable of heterotrophic nitrification and aerobic denitrification, and are able to simultaneously remove organic carbon and nitrogen with high efficiency (Razzolini et al., 2008).

Chryseobacterium has been isolated from a variety of habitats such as freshwater, seawater, soil, and sediment. Members of the genera are abundant in freshwater and marine ecosystems and become dominant in response to the input of organic substrates (Kundu et al., 2014). *Chrysobacterium* spp. have been shown to reduce levels of ammonia-nitrogen and carbon oxygen demand (COD). These bacteria also have the ability to take advantage of aerobic denitrification in the presence of ammonia nitrate. *Achromobacter* spp. have been isolated from brackish water and have been known to degrade or convert various carbon sources (Ying-Ning

et al., 2012). Similar to *Chrysobacterium*, *A. xylosoxidans* is also capable of stabilizing levels of ammonium-nitrogen and reducing COD. *A. xylosoxidans* is a more potent nitrifier than the other bacteria with similar capabilities (Kundu et al., 2012).

A. vinelandii was one of the least dominant species found in the duckweed-based biofilter tanks. The *Acinetobacter* has been frequently isolated from soils and water. Members of the genus such as *A. vinelandii* has been reported to involve in a single step nitrogen removal by simultaneous heterotrophic nitrification and aerobic denitrification in wastewaters (Zhao et al., 2010).

4.5 Conclusions

All the heterotrophic bacteria from the present study have been known to play a fundamental role in heterotrophic nitrification and aerobic denitrification processes in a diverse range of ecosystems (Chen et al., 2012a). Our research suggests that duckweed biofilters in RAS are suitable substrates for the attachment, survival, and growth of heterotrophic bacteria involved in nitrification. A short 2-day harvest frequency reduced heterotrophic bacteria abundance in biofilter tanks, whereas a 6-day harvest frequency or no harvest decreased duckweed biomass due to chlorosis and disconnection of their fronds. The results showed that 4-day harvest frequencies maintained an optimum biomass of duckweed.

CHAPTER 5: The Abundance and Diversity of Phosphate Solubilising Bacteria Associated with the Harvesting Regime of Duckweed (*Lemna minor* Linnaeus) In Integrated Recirculating Aquaculture Systems[3]

5.1 Introduction

Aquaculture effluents with a high concentration of phosphorus (P) and nitrogen (N) create major problems for the aquaculture industry, as they contribute to eutrophication (Smith, 2003) and environmental degradation (Paez-Osuna et al., 2003). Although, recirculating aquaculture systems (RAS), which re-uses the water after removing solids and ammonical nitrogen, considerably reduces the effluent load (Losordo et al., 1998), however, significant amounts of N and P are still discharged through protein skimming mechanism (Martins et al., 2010). Phosphorus as an effluent is usually present at high concentrations in the RAS effluents as a significant portion of the phosphorus from the feed is unutilised by the cultured species. The phosphorus accumulation is further compounded due to the use of less appropriate methods for its removal from the production systems, including RAS (Barak and Rijn, 2000).

The removal of phosphorus to an acceptable level by various chemical methods is expensive (Liberti et al., 2001) and requires for high chemical dosages (Hultman et al., 2001). Even though, the chemical removal of phosphorus can be rapid and improve the effluent quality (Ebeling, 2004) than the multi-step biological process, the chemical removal of the phosphorous produces more sludge than its biological removal (Gillberg et al., 2003). Another biological way to enhance the phosphorus-removal process is to involve polyphosphate accumulating bacteria in the activated sludge in response to a change from aerobic to anaerobic conditions (Henze et al., 2002). Under anaerobic conditions, the bacteria are capable of storing organic compounds especially short chain fatty acids including acetic and propionic acids as internal storage compounds (Liu et al., 2007). Under subsequent aerobic conditions, the previously stored carbon is used for the cell growth and poly-P formation (Li et al., 2007), indicating that a higher amount of phosphorus is absorbed and utilised under aerobic conditions (Carvalho et al., 2007). However, in all cases, phosphorus can be converted into a solid fraction (microbial mass in activated sludge) (De-Bashan and Bashan, 2004). The phosphorus is removed by converting the dissolved inorganic phosphorus to a low-soluble metal-phosphate complex (Hansen et al. 2000). However, it fails to recycle the phosphorus to a sustainable product but is rather gained together

[3] This chapter has been submitted to Aquatic Living Resources

with a mixture of different other waste products of which some are toxic (De-Bashan and Bashan, 2004).

The integration of the macrophytes into the RAS is possible as RAS can house sufficient nutrients to sustain the growth of macrophytes. In any integrated RAS (IRAS), substantial amount of organic and inorganic matter from uneaten feed and faecal matter is accumulated and may be subsequently used by macrophytes. Turcois and Papenbrock (2014) stated that phosphates can be reduced in IRAS if the target species is cultivated with plants that can convert nutrients into valuable biomass. In addition, the combination of fish culture with phototrophic and herbivorous organisms can increase nutrient retention in the system (Schneider et al., 2005).

The roots of various plant species incorporating phosphate-solubilising bacteria (PSB) are recognised as having an important ecophysiological role (Chen et al., 2012b). The PSB can mobilize insoluble inorganic phosphates so that they can easily be absorbed by plant roots. The plants, in turn, may supply root-borne C compounds (mainly sugars) that can be metabolized for the bacterial growth (Deubel et al., 2000). Furthermore, PSB can convert the insoluble phosphate into soluble forms (Pradhan and Sukla, 2005) and increase its availability to the plants. The microorganisms with phosphate solubilising potential increase the soluble phosphate availability and enhance the plant growth by increasing the mineralization capacities of the organic P and solubilising precipitated phosphates (Chen et al., 2006) or by increasing the supply of other trace elements, such as iron, zinc, etc., and by the production of plant growth-promoting regulators (Ponmurugan and Gopi, 2006).

Duckweed (*Lemna minor* Linnaeus) is a small, free-floating aquatic plant belonging to the *Lemnaceae* family (Cheng et al., 2002) and is well known for its high productivity and high protein content in temperate climates. A variety of *Lemnaceae* species have been used for wastewater treatments and nutrient recovery due to their rapid multiplication and high protein content (Caicedo et al., 2000). Total phosphorus removal rates of between 70-85%, and orthophosphate removal rates of between 83-95%, have been reported in different types of wastewaters (Ozengin and Elmaci, 2007) by using duckweed. Additionally, the use of duckweed species as a biofilter media in RAS decreases the significant amount of orthophosphate and total phosphorus in water and increases the growth of the cultivated fish species (Sirakov and Velichkova, 2013).

As PSB can be found in the water column, the sediment or at the substrate (Sushanta et al., 2012), the use of biofilter media not only removes excess nutrients, including phosphorus, from the RAS, but can also provide a suitable substrate for the bacterial adhesion and growth (Schreier et al., 2010). Thus the abundance and diversity of the bacterial communities that may act as PSB in RAS are affected by the type of biofilter and biofilter media's surface area per unit volume, as biofilters contribute to the growth and structure of bacterial communities (Leonard et al., 2000).

Although any intensive fish culture systems are major source of phosphorus pollution, relatively a few studies have been conducted on the phosphorus removal in these systems (Valeta and Verdegem, 2009). Previous research demonstrated that harvest frequency of duckweed affects the abundance and diversity of heterotrophic bacteria communities, which are involved in nitrogen removal from IRAS (Ardiansyah and Fotedar, 2016a). Hence, the use of appropriate management strategies, including the manipulation of the abundance and diversity of the PSB communities in any IRAS, are required to enhance P absorption by rhizoplants (Maitra et al., 2014). This can be controlled by the quantity of biofilter media including duckweed present in real time. Therefore, this study aimed to evaluate the effects of different amounts of duckweed in real-time (using a different harvest regime) as the media on the composition, abundance, and diversity of phosphate solubilising non-pathogenic bacteria that are involved in the removal of phosphates in IRAS.

5.2 Materials and methods

5.2.1 Experimental system

The experimental system used in this experiment was similar to our previously published article (Ardiansyah and Fotedar, 2016a). The experiment was carried out in twelve small independent recirculating systems at the Curtin Aquatic Research Laboratory, Curtin University, Australia. Each recirculating system consisted of three tanks: a fish-rearing tank (400 L circular tank), a waste collection tank (100 L circular plastic drum), and a biofilter tank (100 L circular tank). A submersible pump was used to recirculate the water among the three tanks through PVC pipes.

The biofilter tanks were filled with free-floating duckweed that was taken from the stock tank. 35 g wet weight duckweed per tank was weighed, washed with water, filtered and randomly stocked into each of the 12 tanks biofilters. Before the start of the experiment, a total of 720 juvenile barramundi (*Lates calcarifer* Bloch) with an average size of 7.25 ± 0.27 g were cultured in the RAS for 30 days, and were then removed from the RAS system, while the water in every

tank was retained without any water exchanges and circulation. The volume of water in each fish-rearing tank was kept at 185 L, whereas the water volumes in the biofilter tanks and the waste collection tanks were kept at 30 and 40 L, respectively. During the study, the fish rearing tanks were provided with aeration to ensure oxygen concentration remained above 80% saturation, and a heater was used to keep the water temperature at 26 ± 2°C. Photoperiod was kept at 12 h dark and 12 h light.

185 mg of di-ammonium phosphate $(NH_4)_2 HPO_4$, as a source of phosphate was added to the fish-rearing tanks. Four *L.minor* harvesting regimes were employed during the 36-day experimental period. Three preselected random biofilter tanks were harvested every 2 days. Three other biofilter tanks were harvested after 4 days, and 3 others after 6 days. All harvesting was carried out in triplicate simultaneously at one pint of time. The remaining 3 tanks were not harvested and served as controls. The harvested duckweed was then replaced by freshly prewashed duckweed. The reduction in water volume due to evaporation was compensated by adding new freshwater (Ardiansyah and Fotedar, 2016a).

The health of the duckweed was visually observed, based on any signs of chlorosis and disconnection of the fronds (Khellaf and Zerdaoui, 2010). The increase in biomass of duckweed on the water surface was calculated using growth index (GI) on alternative days using the equation: GI = Wt / W0, where: Wt = biomass at time t, W0 = biomass at time 0, and t = number of days. The increase in duckweed biomass per unit of harvest time was indicated by specific growth rates (SGR), calculated at each harvest time using the equation: SGR = (Ln(Wf) - Ln(Wi) * 100) / t, where: Ln(Wf) = the natural logarithm of the final weight, Ln(Wi) = the natural logarithm of the initial weight, and t = time (days).

5.2.2 Isolation, enumeration, and evaluation of phosphate solubilising efficiency and phosphatase activity

Isolation, enumeration, and evaluation of the bacteria were performed following the procedure described in Ardiansyah and Fotedar (2016a). A 10 g fresh-weight of duckweed was randomly collected using 40 mL sample jars, containing 10 mL distilled water, and then kept at 4ºC before analyses. The samples were homogenized in a homogeniser at 17,000 *g* for 5 min. A 10 mL sample of the homogenate water was diluted with 90 mL of phosphate-buffered saline (PBS) and again homogenized for 2 minutes at 1900 *g*. The resulting homogenate was diluted ten times with PBS. Using an aseptic technique, 0.1 mL of the diluted samples were plated in triplicate by

the spread plate method onto a Pikovskaya agar (PVK) medium (Mehta and Nautiyal, 2001) using a conventional pour plate technique.

The bacterial colonies were selected according to their morphological appearance and maximum halo zone around them as an indicator of phosphate solubilisation after 120 h of incubation at 30°C (Nautiyal, 1999). The phosphate solubilisation efficiency of the selected isolate was estimated by plating onto a fresh PVK medium containing 0.5% insoluble tricalcium phosphate and incubating at 30ºC for 10 days, and an analysis of the PSB trait was made by measuring the zone of solubilisation around the colony growth. The solubilisation efficiency (%) of the selected isolates was determined following the method of Nguyen et al. (1992). The selected colonies were removed from the plates and purified in triplicates on LB agar. Enumeration of the PSB communities was determined using the total plate count method. The colonies were counted and expressed as CFU per mL for the attached bacteria, as previously described (Leonard et al., 2000).

Phosphatase activity was determined using 0.1 M disodium p-nitrophenyl as a substrate. For the assay, 3 mL of PSB culture on the PVK medium, 1 mL universal buffer (5x) adjusted to pH 6.5 (Skujins et al., 1962), and 0.5 mL of the substrate, were pipetted into a 20 mL reagent vial and incubated at 30°C for 90 min. The reaction was stopped by cooling at 2°C for 15 min. Then 20 mL of 0.5 N NaOH was added, and the mixture was transferred to a 50 mL volumetric flask, where the volume was increased to 50 mL with distilled water. The p-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremmer, 1969). Controls were made in the same way using 20 µg/mL disodium p-nitrophenyl.

Quantitative estimation of indole acetic acid (IAA) was performed according to Maria Guineth et al. (2000) and Tsavkelova et al. (2007). The PSB isolates were inoculated in Czapek's dox broth and incubated at 28° ± 2°C for 72 h. The culture was centrifuged at 3000 rpm for 15 min, and 2 ml of the supernatant was mixed with 1 ml of Salkowsky's reagent. The optical density was determined using a spectrophotometer at 535 nm.

Putative PSB were identified by the biochemical method described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). A variety of biochemical tests, including oxidase, catalase, hydrogen sulfide production, tyrosine hydrolysis, gelatinase test, ornithine decarboxylase, arginine dihydrolase, methyl-red and Voges-Proskauer, casein, starch hydrolysis, citrate utilization, nitrate reduction, TSI agar test, and motility test, were conducted.

5.2.3 Bacteria diversity index

The bacteria diversity index assumes that individual bacterium is sampled randomly from a large population so that all species are represented in the sample. The species diversity was determined using the Shannon diversity index with the following formula: $H = - [\Sigma Pi * Ln(Pi)]$, where H = Diversity index, Pi = the number each species in the sample/total number of samples, and Ln(Pi) = the natural logarithm of this proportion.

5.2.4 Statistical analysis

The abundance and diversity of PSB were examined by a one-way analysis of variance (ANOVA) and the Tukey HSD test. A regression analysis was performed to analyse the relationship between the growth of duckweed and bacterial abundance and diversity. Values of $R^2 < 0.65$ were considered to have poor correlations, whereas $R^2 > 0.65$ indicated good correlations. A $P < 0.05$ was considered significant for all analyses.

5.3 Results

5.3.1 Abundance and diversity of PSB

Forty-seven strains of the putative PSB were isolated and screened from duckweed in the biofilter tank. Among the isolates, 10 morphologically different bacterial strains were observed (Table 5.1). Different harvest regimes caused significant differences ($P < 0.05$) in the bacterial abundance. The abundance of six types of PSB (PSB1, PSB3, PSB15, PSB17, PSB26, and PSB35) were significantly impacted by the harvest regime. Duckweed harvested every 2 days had the lowest abundance of the putative PSB.

Table 5.1. Abundance of phosphate solubilising bacteria attached to duckweed in the biofilter tank.

Putative PSB bacteria (cfu x 10^3/mL)	**Harvest regime**			
	6 days	**4 days**	**2 days**	**No harvesting**
PSB1	5.23 ± 0.21[b]	5.34 ± 0.26[b]	4.76 ± 0.34[a]	4.70 ± 0.32[a]
PSB3	3.74 ± 0.03[b]	3.85 ± 0.06[b]	3.29 ± 0.03[a]	3.37 ± 0.01[a]
PSB7	1.79 ± 0.08[a]	1.75 ± 0.09[a]	1.64 ± 0.05[a]	1.59 ± 0.05[a]
PSB15	4.73 ± 0.12[b]	4.85 ± 0.19[b]	4.12 ± 0.13[a]	4.24 ± 0.15[a]
PSB17	5.17 ± 0.11[b]	5.09 ± 0.26[b]	4.53 ± 0.26[a]	4.98 ± 0.08[b]
PSB26	4.60 ± 0.14[b]	4.52 ± 0.17[b]	3.85 ± 0.48[a]	3.97 ± 0.03[a]
PSB29	1.84 ± 0.21[a]	1.79 ± 0.27[a]	1.65 ± 0.35[a]	1.71 ± 0.46[a]
PSB30	2.16 ± 0.13[a]	2.01 ± 0.16[a]	1.87 ± 0.10[a]	1.95 ± 0.10[a]
PSB32	2.89 ± 0.02[a]	2.94 ± 0.04[a]	2.75 ± 0.02[a]	2.80 ± 0.08[a]
PSB35	3.47 ± 0.08[b]	3.52 ± 0.17[b]	3.05 ± 0.07[a]	3.13 ± 0.05[a]
Total	35.62 ± 0.43[b]	35.66± 0.44[b]	31.51± 0.37[a]	32.44± 0.39[a]

[a] Values in the same row with the same superscript letter are not significantly different ($P > 0.05$)

Harvesting duckweed every 4 days resulted in the highest Shannon diversity index value (H=2.2910), whereas the unharvested (control) had the lowest index value (H=2.2257) (Table 5.2). PSB abundance and duckweed biomass harvest were poorly correlated ($R^2 < 0.65$) with harvest regimes, but at the 4-day harvest regime, significantly stronger correlation between the bacterial diversity and the harvested duckweed biomass was observed. Similarly, SGR harvest of duckweed was significantly correlated with the abundance and diversity of PSB at the 4-day harvest regime ($R^2 > 0.65$: $P < 0.05$), whereas the SGR and biomass harvest of the -duckweed harvested every 6 days had a poor relationship with the abundance and diversity of PSB ($R^2 < 0.65$).

Table 5.2. Correlation between SGR and total biomass harvest of duckweed with abundance and diversity of phosphate solubilising bacteria over the 36-day trial.

Harvest regime	6	4	2	0
R^2 (biomass vs abundance)	0.5971±0.02[a]	0.6192±0.06[b]	0.5941±0.04[a]	0.6185±0.00[b]
R^2 (biomass vs diversity)	0.6141±0.01[a]	0.6502±0.01[b]	0.6193±0.05[a]	0.6835±0.03[b]
R^2(SGR vs abundance)	0.5852±0.06[a]	0.6984±0.06[b]	0.6248±0.03[a]	0.6894±0.03[b]
R^2(SGR vs diversity)	0.6242±0.01[a]	0.6623±0.01[b]	0.6193±0.05[a]	0.6835±0.03[b]
Shannon diversity index (H)	2.2745±0.01[bc]	2.2910±0.01[c]	2.2365±0.01[b]	2.2257±0.00[a]
GI	3.15±0.02[c]	3.09±0.03[bc]	3.15±0.04[a]	3.04±0.03[ab]
Total biomass harvest	674.45 ± 0.54[b]	1003.45 ± 1.29[c]	1880.2 ± 0.67[d]	99.75 ± 0.11[a]
SGR	8.14 ± 0. 00[b]	9.31 ± 0.00[c]	11.05 ± 0.00[d]	3.04 ± 0.00[a]

[a] Values in the same row with the same superscript letter are not significantly different ($P > 0.05$)

Different harvest regimes also had a significant influence on the GI of duckweed. Duckweed harvested every 6 days had the highest GI of 3.15, whereas those harvested every 2 days had the lowest GI of 3.04 (Table 5.2). However, significantly (P <0.05) higher SGR and total harvested biomass of duckweed were obtained when duckweed was harvested every 2 days than the unharvested duckweed. The unharvested control resulted in the lowest SGR and harvested biomass (Table 5.2).

5.3.2. Phosphate solubilisation efficiency, IAA, and phosphatase activity

The results of the solubilisation efficiency of the selected PSB are shown in Table 5.3. Among the ten bacterial isolates, the highest efficiency was observed for PSB1 (E = 83.33%), followed by PSB17 (E = 75.00%) and then PSB15 (E = 62.50%), whereas PSB29 (E = 33.33%) and PSB32 (E = 34.47) had comparatively lower efficiencies. All putative PSB isolates produced IAA. The highest IAA (7.60 µg L^{-1}) was produced by PSB15, followed by PSB17 (7.45 µg L^{-1}), while the lowest IAA (6.07 µg L^{-1}) was produced by PSB29. Furthermore, the maximum phosphatase activity was observed in PSB1 (33.68 µmol g^{-1} h^{-1}), followed by PSB17 (31.25 µmol g^{-1} h^{-1}), while the minimum phosphatase activity was observed in PSB32 (15.84 µmol g^{-1} h^{-1}). The remaining bacterial isolates secreted phosphatase enzyme in the range of 17.65-30.85 µmol g^{-1} h^{-1}. Correlation coefficient values (R^2=0.921) showed a significant (P≤0.05) correlation between phosphate solubilising efficiency and phosphatase enzyme production.

Table 5.3. The phosphate solubilising efficiency and phosphatase activity recorded for the putative PSB bacteria attached to duckweed in the biofilter tank.

Putative PSB Bacteria	**Phosphate solubilisation efficiency (E, %)**	**Indole acetic acid production ($\mu g\ L^{-1}$)**	**Phosphatase activity ($\mu mol\ g^{-1} h^{-1}$)**
PSB1	83.33	7.35	33.68
PSB3	53.59	7.45	28.92
PSB7	43.93	7.00	23.92
PSB15	62.50	7.60	30.85
PSB17	75.00	7.23	31.25
PSB26	60.83	7.00	29.84
PSB29	33.33	7.08	17.65
PSB30	44.82	6.55	18.85
PSB32	34.47	7.08	15.84
PSB35	50.00	6.07	25.32

5.3.3 Morphological and biochemical characteristics of PSB

Of forty-seven visually different bacterial isolates, 10 bacterial strains were determined by colony morphology, gram staining, and pigmentation (Table 5.4). The morphological and physiological characteristics of the isolates identified to the genus level showed that gram-negative bacteria dominated the bacterial communities. Specifically, 40.41% of gram-negative bacilli isolates were identified as *Pseudomonas* and *Chryseobacterium*; 29.79% of isolates of gram-positive bacilli belonged to *Bacillus*; 23.41% of isolates of gram-negative ovoid, cocci, or coccobacilli were identified as *Azotobacter*, *Acinetobacter*, and *Achromobacter,* and 6.39% of isolates of gram-positive cocci were identified as *Micrococcus* (Table 4). Non-pigmented isolates belonged to *Bacillus*, *Achromobacter*, and *Acinetobacter*, whereas non-motile isolates were characterized as *Chrysobacterium*, *Acinetobacter*, *Azotobater*, and *Micrococcus*.

Table 5.4. The morphology and physiological characteristics of the putative phosphate solubilising bacteria attached to duckweed.

Strains	Morphology	Gram stain	Spore	Motility	Pigmentation	No. of clones (%)	Genus
PSB1	Bacilli	+	+	-	Yellowish-green	19.15	*Bacillus* sp.
PSB3	Bacilli	-	-	+	Translucent	6.39	*Bacillus* sp.
PSB7	Bacilli	+	+	+	White	4.25	*Bacillus* sp.
PSB15	Bacilli	-	-	-	Yellow	12.76	*Chrysobacterium* sp.
PSB17	Bacilli	-	-	+	Yellowish-green	14.89	*Pseudomonas* sp.
PSB26	Bacilli	-	-	+	Brownish-yellow	12.76	*Pseudomonas* sp.
PSB29	Cocci	+	-	-	Yellowish-orange	6.39	*Micrococcus* sp.
PSB30	Ovoid	-	-	+	Yellow	4.45	*Azotobacter* sp.
PSB32	Cocci	-	-	-	White	6.39	*Acinetobacter* sp.
PSB35	Coccobacilli	-	-	+	White	10.54	*Achromobacter sp*

Of the 47 different isolates, 10 species of putative PSB were characterized by both biochemical tests and confirmed with the API 20E (Table 5.5). All isolates were characterized as the same species by both conventional biochemical tests and API 20E. Out of 10 species, as shown in Table 5, *Bacillus* was represented by 3 species. *Pseudomonas* consisted of 2 species, whereas *Chrysobacterium, Micrococcus, Azotobacter, Acinetobacter,* and *Achromobacter* and the remaining 5 genera, had only one species each. Therefore, the putative PSB found in the biofilter tanks were *Bacillus cereus* (PSB1), *B. licheniformis* (PSB2), *B. subtilis* (PSB7), *Chrysobacterium indologenes* (PSB15)*, Pseudomonas fluorescence* (PSB17)*, P. mendocina* (PSB26)*, Micrococcus luteus* (PSB29)*, Azotobacter vinelandii* (PSB30)*, Acinetobacter calcoaceticus* (PSB32), and *Achromobacter xylosoxidans* (PSB35).

Table 5.5. The morphological and biochemical characteristics of PSB isolated from the biofilter tanks.

Test	The PSB Strain									
	PSB1	PSB3	PSB7	PSB15	PSB17	PSB26	PSB29	PSB30	PSB32	PSB35
Colony morphology	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Cocci	Ovoid	Cocci	Coccobacilli
Pigmentation	Yellowish-green	Translucent	White	Yellow	Yellowish-green	Brownish-yellow	Yellowish-orange	Yellow	White	White
Spore	+	-	+	-	-	-	-	-	-	-
Motility	-	+	+	-	+	+	-	+	-	-
Gram stain	+	+	+	-	-	-	+	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	-	-	+	-	+	+	+	-	+
H_2S production	-	-	-	-	+	-	-	-	-	-
Gelatinase	+	-	+	+	+	-	+	+	-	-
Nitrate reduction	-	+	+	+	-	+	+	+	-	+
Starch hydrolysis	+	+	+	+	+	-	-	+	-	-
Citrate utilization	-	-	+	+	+	+	+	+	+	+
Ornithine	-	-	-	-	-	-	-	+	-	-
Indole	-	-	-	+	+	-	-	+	-	-
Tyrosine hydrolysis	+	-	-	-	+	-	-	-	+	+
Arginine dihydrolase	-	+	-	-	+	+	-	+	-	+
Methyl Red	-	-	+	-	+	+	-	-	-	+
Voges-Proskauer	-	-	+	+	+	-	-	-	+	-
Urease	+	-	-	-	+	+	-	+	+	-
Glucose	+	+	+	+	+	+	+	+	-	-
Fructose	+	+	+	+	+	+	-	+	-	+
Lactose	-	-	-	-	+	-	-	-	-	-
Maltose	+	+	+	+	+	-	-	+	-	-
Mannose	+	+	+	+	+	+	-	-	-	-
Sucrose	+	+	+	-	+	-	-	+	-	-
Xylose	-	-	+	-	-	+	+	+	-	+

5.4 Discussion

Different harvest regimes significantly affected the abundance of bacteria of six putative PSB (PSB1, PSB3, PSB 15, PSB 17, PSB 26, and PSB35). The results showed that harvesting duckweed after every 2 days resulted in a lowest GI of duckweed, reducing the availability of organic substrates for bacterial attachment and growth (Itoi et al., 2006). Meanwhile, the longer interval of 6 days resulted in the highest duckweed GI, which in turn caused a slower SGR and reduced the harvested biomass. The 6-day harvest and no harvest regimes caused chlorosis and disconnection of duckweed fronds. Similar trends had been previously observed in the abundance of the heterotrophic bacteria associated with the different harvesting regime in barramundi RAS (Ardiansyah and Fotedar, 2016a). Therefore, the 4-day harvest regime may provide the desirable duckweed biomass and optimal availability of the organic substrates as carbon and energy source for the PSB.

In this study, only 10 isolates from 47 isolates showed the formation of halo zones around the growing colonies on PVK media plates. Halo zones are formed because of the transformation of glucose into organic acids, in which glucose is the carbon source used in the test of PSB isolated from the biofilter to dissolve the bonded phosphor marked by halo zones (De Souza et al., 2000). However, production of organic acids was not determined in the present study. Thus the authors cannot confirm whether all PSB isolates are capable of producing organic acids.

There is increasing evidence that PSB improves the plant growth due to the biosynthesis of plant growth substances rather than to their action in releasing available phosphorus. All PSB isolated from duckweed were capable of producing IAA. Davies (1995), Luthen et al. (1999), and Glick (2005) stated that IAA is the biologically active form of auxins that stimulates radical system growth (Dobbelaere et al., 2003; Vessey, 2003) and increases the uptake of the nutrients by the plants. Thus, it has prominent effects on plant growth and development. The results indicated that all PSB isolates are able to secrete physiologically active auxins. Shahab et al. (2009) reported that PSB isolated from mung bean (*Vigna radiata*) excreted phytohormones, including auxins. Other researchers found similar results, that isolated strains from rice fields have potential to produce IAA (Naher et al., 2009). These results were supported by the findings of Woo et al. (2010) that the PSB strains isolated from the rhizophore of Chinese cabbage (*Brassica rapa*) were found to solubilise P in the media, and besides this, they were able to produce IAA. However, the capacity to synthesize IAA is widespread among the PSB. Production of IAA

greatly varies among different species and is influenced by the culture conditions, growth stage, and substrate availability (Vijila, 2000).

Phosphatase enzyme activity showed that all the selected PSB isolates solubilised inorganic phosphates into various degrees in the PVK culture medium by producing phosphatase enzymes. The PSB have been shown to increase the solubility of the P insoluble compound through the secretion of extracellular enzymes such as organic acids, phytase, and phosphatase enzymes (Khan et al., 2014). These enzymes are present in all organisms, but only bacteria, fungi, and some algae are able to secrete them outside their cells. However, the PSB are more effective in phosphorus solubilisation than fungi (Alam et al., 2002). Among the whole microbial population in the aquatic environment, PSB constituted 1 to 50%, while phosphorus solubilising fungi (PSF) constituted only 0.1 to 0.5% in P solubilisation potential (Chen et al., 2006).

Furthermore, during the conversion process, phosphorous is partially assimilated by PSB, but the amount made soluble and released is in excess of the requirement of the PSB. The excess amount released is made available to plants. During this conversion process, the extracellular enzymes convert calcium phosphate to *di-* or *mono*basic phosphates and are then easily made available to plants phosphates (Jones, 2002; Kang et al., 2002; Pradhan and Sukla, 2005).

In the study, the stronger correlation between phosphate solubilising efficiency and phosphatase enzyme activity may indicate an increase in the availability of phosphorous in the medium, which is assumed to be facilitated through increased phosphatase enzyme activity (Barik and Purushothaman, 1998; Singh and Prakash, 2012). The results suggested that all selected PSB can produce phosphatase enzymes to convert the insoluble phosphate into the soluble form, which leads to increased content of available phosphate for the plants (Gyaneshwar et al., 2002). Microorganisms increase the P availability to the macrophytes by mineralizing organic P and by solubilising precipitated phosphates (Pradhan and Sukla, 2005; Chen et al., 2006). Additionally, Ponmurugan and Gopi (2006) found a positive correlation between phosphate solubilising activity and phosphatase enzyme activity. These previous findings were in agreement with the present results, which showed that the increase in inorganic phosphate levels was due to the phosphate solubilisation did not repress the phosphatase production by the PSB isolates found in the study.

In the present study, 40.41% of gram-negative bacilli isolates were identified as *Pseudomonas* and *Chryseobacterium*; 29.79% of isolates of gram-positive bacilli belonged to Bacillus; 23.41%

of isolates of gram-negative ovoid, cocci, or coccobacilli were identified as *Azotobacter, Acinetobacter*, and *Achromobacter,* and 6.39% of isolates of gram-positive cocci were identified as *Micrococcus*. Non-pigmented isolates belonged to *Bacillus, Achromobacter*, and *Acinetobacter*, whereas non-motile isolates were characterized as *Chrysobacterium, Acinetobacter, Azotobater* and *Micrococcus*. Leonard et al. (2000) and Michauld et al. (2006), who reported that *Pseudomonas, Chryseobacterium* (formerly known as *Flavobacterium*), *Aeromonas*, and *Acinetobacter,* are common inhabitants of various aquatic environments. Naik et al. (1982), Rodriguez and Fraga (1999), and Maitra et al. (2014) reported that among the bacterial genera with the capacity to solubilise insoluble inorganic phosphate compounds in aquatic environments are *Bacillus, Enterobacter, Agrobacterium, Pseudomonas, Acinetobacter, Achromobacter, Micrococcus, Chrysobacterium,* and *Aereobacter*.

The most common bacterial genera in this study was *Bacillus*. The majority of the *Bacillus* species showed high phosphatase activity, but the proportion varied with species. The *Bacillus* consisted of obligate aerobes (*B. subtilis*) or facultative anaerobes (*B. cereus* and *B. licheniformis*). The genus is known to have more characters promoting plant growth: synthesize phytohormones such as IAA (auxins); nitrogen fixation; antifungal activity by siderophores production; antibiotics, enzymes and phosphorus solubilisation (Nabti et al., 2013). Among the bacterial genera, *Bacillus* was the most efficient P-solubilising bacteria from P-Ca source culture solution (Oliveira et al., 2009).

Rodriguez and Fraga (1999) reported that the genus *Pseudomonas* was the suitable strain in dissolving the bonded P from any source of P and was dominant in the mineralization of organic phosphorus. This type of bacteria can also be found in extreme saline and alkaline water habitats (De Souza et al., 2000). In this study, Pseudomonas consisted of *P. licheniformis* and *P. mendocina*. Bacteria from the genera *Bacillus* and *Pseudomonas* are among the most powerful phosphate solubilisers (Subbarao, 1998).

A considerable number of bacteria from different genera are capable of solubilising phosphate, including *Chrysobacterium*, which has been isolated in a wide variety of habitats such as soil, plant roots, sludge, fish, sewage, fresh water, freshwater sediment, marine water, and marine sediment. The genus *Chryseobacterium* was first created for five species formerly classified as members of the genus *Flavobacterium* (Weon et al., 2008). Various *Chrysobacterium* species have been shown to solubilise phosphate by releasing organics acids that mobilize phosphorus

and by releasing phosphatase to release phosphate groups bound to organic matter (Chen et al., 2006).

Phosphorus availability depends on the degree of solubilisation by various organic and inorganic acids produced by microorganisms, and the genus *Micrococcus* is one of the most important microorganism that produces a substantial amounts of phosphates. *Micrococcus* inhabits a wide range of environments, including soil, water, and dust, and can sustain well in environments with high salt concentration and little water. This genus has been shown to possess multiple plant growth traits, like P-solubilisation, IAA and siderophore production (Dastager et al., 2010).

Different bacterial genera are involved in various biotic activities, important to make the nutrient turnover dynamics (Ahemad et al., 2009). Thus, there is ongoing research to discover various rhizobacteria that possess novel traits such as phosphate solubilisation (Ahemad and Khan, 2012) capacities. Among the bacterial genera with this capability is *A. xylosoxidans* (Jha and Kumar, 2009). This species shows a considerable level of nitrogenase activity, IAA production, and P solubilisation ability. This species may also increase Cu uptake by plants and increase the shoot length, the root length, and the fresh weight and dry weight of plants (Ma et al., 2009).

Azotobacter is a gamma-proteobacterium belonging to the family *Pseudomonadaceae*. It is an obligate aerobic, free-living Gram-negative bacterium that is broadly dispersed in various environments, including water, soil, and sediments (Staley et al., 2005). In fact, *Azotobacter* has favourable effects on plant yields due to its capability of fixing nitrogen and solubilising phosphates (Aquilanti et al., 2004; Tejera et al., 2005). *A. vinelandii* produces metabolically dormant cysts, which are formed under unfavourable environmental conditions. Thus it is suitable for use in diverse environments (Sabra et al., 2000). Several *Azotobacter* species isolated from wheat rhizophore have shown the ability to solubilise tricalcium phosphate, Mussoorie rock phosphate, and also to produce IAA. Additionally, the use of *Azotobacter* may increase seed yield, plant height, and microbial population in soil (Ma et al., 2009; Peng et al., 2013).

A. calcoaceticus has demonstrated the potential capability of P solubilisation by the production of organic acids, and can provide plant growth-promoting factors such as the production of IAA and siderophores. This species also exhibits Pb and antibiotic resistances. The use of *A. calcoaceticus* effectively increases the available Pb in the rhizosphere soil and promotes the growth of the host plant, which leads to an increase in Pb uptake (Ren et al., 2013).

Acinetobacter has been reported to be a PSB with high efficiency and acid tolerance. The bacteria also produce IAA, which is a phytohormone for plant growth.

All the putative PSB from the present study have been known to play a fundamental role in the phosphate solubilisation process in a diverse range of ecosystems (Anand et al., 2016). Our research suggests that duckweed biofilters in IRAS are suitable substrates for the attachment, survival, and growth of PSB.

5.5 Conclusions

A short 2-day harvest regime reduced the PSB abundance in biofilter tanks, whereas a 6-day harvest regime, or no harvest, decreased duckweed biomass due to chlorosis and disconnection of their fronds. The results showed that a 4-day harvest regime maintains an optimum biomass of duckweed. Further, the different harvesting regimes influence the abundance and diversity of PSB in IRAS. The selected PSB strains should be further evaluated in larger scale in vivo trials regarding the mechanism and efficiency of P solubilisation, and their potential application in managing a sustainable aquaculture systems.

CHAPTER 6: Water quality, growth and stress responses of juvenile barramundi (*Lates calcarifer* Bloch), reared at four different densities in integrated recirculating aquaculture systems[4]

6.1. Introduction

An increasing number of consumers have started making their seafood consumption decisions based on the safety and ethical values practiced by producers (Ashley, 2007). The use of unethical animal production practices may affect animal health and downgrade the quality of the products (Ormandy et al., 2011). Similarly, certain types of aquaculture production systems may cause stress and affect the welfare and health of the cultured species. Various factors that can compromise the welfare of the cultured fish are stocking density, malnutrition, genetic manipulation, nutrient deprivation, transport and handling (Conte, 2004). Therefore, minimising the stress in cultured species is crucial for welfare, health and productivity, as stress has been associated with reduced growth performance, decreased feed intake, decreased feed conversion efficiency (Ellis et al., 2002), and reduced survival (Lefrancois et al, 2001; Rowland et al., 2006).

The recirculating aquaculture system (RAS) is typically used for commercially important species with high stocking densities and low water exchange rates in order to cover the high investment costs (Timmons et al., 2002). However, the increased stocking density may lead to the reduced efficiency of the biological filter (Gullian-Klanian and Arámburu-Adame, 2013), increased stress levels due to nutrient overloading (Gonçalves et al., 2010), and the loss of productivity (Iguchi et al., 2003; Ferreira et al., 2007). In order to tackle the problems associated with nutrient overloading, in combination with addressing the animal welfare issues in a RAS, integrated RAS (IRAS) are gaining popularity (Troell, 2009). IRAS incorporate an additional plant or animal species into the RAS that has the ability to absorb nutrients (Endut et al., 2011). This additional species can have varying impacts on the water quality (Jo et al., 2002; Sipauba-Tavares et al., 2002) and biological oxygen demands (Lu et al., 2008; Akinbile and Yusoff, 2011) and hence on the final carrying capacities or the stocking densities in IRAS. However, little-published information is available on the requirements of the optimum stocking densities of the cultured species in IRAS (Muangkeow et al., 2010).

---

[4] This chapter has been published in Aquaculture 458 (2016) 113–120

Cortisol is generally accepted as a good indicator of stress in fish (Barton, 2002) due to the rapid elevation in its levels that occurs in response to various stressors, including poor water quality (Wandelaar-Bonga, 1997). The impact of poor water quality on endocrine function has attracted growing interest in recent years due to the crucial role of the endocrine system in the coordination of physiological processes and the maintenance of homeostasis. Hypothalamic–pituitary–thyroid and hypothalamic–pituitary–interrenal (HPI) axes play central roles in a broad range of important homeostatic mechanisms in fish. Thyroid hormones regulate growth and hydromineral balances (Van Anholt et al., 2003), while cortisol is involved in the regulation of energy metabolism, anti-inflammatory responses, immune competence and metamorphosis in fish (Hontela et al., 1995; Blanton and Specker, 2007). Stress has been shown to inhibit the thyroidal activity in teleost fish, in which the thyroid gland handles the secretion of thyroid hormones such as thyroxine (T_4) and triiodothyronine (T_3). Chronic stress induces activation of the HPI axis, which results in inhibition of T_3 levels (Brown et al., 1991; Pickering, 1993).

Both thyroid hormones and cortisol interact and influence carbohydrate metabolism (Hontela et al., 1995). Changes in carbohydrate metabolism as a result of stress can be indicated by changes in plasma glucose levels. Glucose is an energy substrate whose production is believed to help the animal metabolism to cope with the elevated energy demands caused by stress. Furthermore, lactate, which is used for glycogen synthesis, and the breaking down of lactate may be used as indicators of general stress in fish (Teles et al., 2007).

Most of the studies on the impact of stocking density on teleost fish performance and stress responses have been conducted in RAS (Sharma and Chakrabarti, 2003; Sammouth et al., 2009; Lupatsch et al., 2010). However, little has been published on the species reared in IRAS. The present study investigated the chronic responses of high stocking density on growth performance and stress-related parameters in juvenile barramundi (*Lates calcarifer* Bloch) in an IRAS followed by challenging the fish with ammonia exposure in order to evaluate the acute stress responses.

6.2 Materials and methods

6.2.1 Animals and stress factors

A total of 1400 juvenile barramundi with an average weight of 13.34 ± 0.01 g were randomly stocked in 12 independent IRAS with four different stocking densities. The study was conducted over 84 days in Curtin Aquatic Research Laboratory (CARL). Experimental fish were maintained to ethical and welfare standards (approval number of AEC-2013-16).

6.2.2 Experimental design and rearing conditions

The level of stocking density was evaluated in a factorial model distributed in a completely random design with three replicates. The stocking densities that were tested at the start of the trial were 10.10, 12.98, 15.86 and 18.75 kg m^{-3}, respectively.

The experimental unit comprised 12 independent IRAS. Each IRAS consisted of three tanks: a fish-rearing tank, a waste-collection tank and a biofilter tank that contained duckweed. All three tanks were connected and operated under an IRAS. The fish and waste-collection tanks were placed on the floor, while the filter tanks were placed above the waste-collection tanks. Water from the bottom of the fish-rearing tank was circulated through a PVC pipe to the waste-collection tank, and water from 20 cm below the water surface in the waste-collection tank was pumped through a PVC pipe to the biofilter tank using a submerged pump. Water from the biofilter tank was then circulated back to the fish-rearing tank by gravity. The water volumes in the fish-rearing and waste-collection tanks were maintained at approximately 185 and 40 L, respectively, while water volume in the biofilter tanks was maintained at 30 L. Two air stones suspended mid-depth in the water column of the fish-rearing tanks were used to diffuse atmospheric air into them and to keep dissolved oxygen (DO) levels at > 6 mg L^{-1}, pH between 7 and 8, total ammonia nitrogen (TAN) < 5 mg L^{-1}, and nitrite < 1 mg L^{-}1. In each fish-rearing tank, an automatic heater was used to maintain the water temperature at 26–28°C. The fish-rearing tanks were covered with polyethylene mesh (size: 1.5 cm diameter) in order to prevent any fish from jumping out and escaping. Depending on the water quality conditions, approximately 20–30% of the water system was exchanged weekly until the end of the trial.

The 12 biofilter tanks were stocked with 35 g/tank of free-floating duckweed (*Lemna minor* Linnaeus), harvested from the stock tanks. Duckweed was washed with filtered water and acclimatised to laboratory conditions for 2 weeks before starting the experiment. All fish were manually fed twice daily at 9.00 a.m. and 4.00 p.m. at the same fixed rate of 2% of total body weight per day with a commercial diet. The uneaten feed was collected after 30 min of

feeding and weighed. Uneaten feed and faeces were then removed in order to avoid the accumulation and decomposition of the particle waste. The feeding was stopped 24 h prior to blood sampling.

6.2.3 Water quality parameters

The temperature, DO and pH of the water in each tank were measured daily using a DO meter YSI-55 (OH, USA) and the pH meter Cyber-scan 30 (IL, USA), respectively. Measurements of total ammonia nitrogen (TAN), nitrite (NO_2), nitrate (NO_3), orthophosphate and total phosphate (TP) were performed weekly. All water samples were collected and analysed under laboratory conditions. TAN, NO_2 and NO_3 were determined using salicylate, ferrous-sulphate and. cadmium reduction methods, respectively. The orthophosphate level was determined using the molybdovanadate method, while TP was determined using a persulphate acid digestion method. All measurements of water quality were in accordance with standard methods for the examination of water and wastewater (APHA, 2005).

6.2.4 Growth parameters

At the end of the 84-day trial, the fish were anaesthetised (60 mg L^{-1} Aqui-S, containing 50% isoeugenol, New Zealand Ltd., Lower Hutt, New Zealand), harvested and individually weighed. The body weight gain (WG) was calculated as the increase in weight of barramundi at the end of the trial and calculated as follows: $WG = W_2 - W_1$, where W_2 = the final body weight (g) and W_1 = the initial body weight (g). Specific growth rate (SGR) is the most common method used to estimate fish growth rate for production. The SGR was determined as follows: $SGR = [(LnW_2 - LnW_1)/t] \times 100$, where Ln = the natural log, W_1 = the initial body weight (g), W_2 = the final body weight (g) and t = period in days. Feed conversion ratio (FCR) was calculated as follows: FCR = FI (g)/WG (g), where FI = dry weight of feed (g) and WG = weight gain (g).

6.2.5 Chronic and acute stress response analysis

Before blood sampling, the fish were fasted for 24 h. Ten fish from each experimental unit were randomly selected and prepared for the blood sampling. Blood was drawn from the caudal peduncle by 2-ml heparinised capillary tubes in order to prevent haemocoagulation and placed on ice. Blood sampling from the ten fish within each unit was completed within 10 min. A 20-µL aliquot of blood sample of each fish was stored frozen at –20°C and used to

determine the total haemoglobin (Hb) content. The remaining blood was transferred to Eppendorf tubes containing ammonium heparin. Plasma separation was obtained by centrifugation (10,000 g, 4°C, 5 min). The plasma was examined for changes in stress-related parameters, including total T_4, total T_3, cortisol, glucose, and lactate. The values obtained represent the plasma basal levels.

6.2.5.1 Chronic stress responses

Plasma cortisol was determined by indirect enzyme immunoassay (ELISA), validated for rainbow trout and gilthead sea bream (Tintos et al., 2006) and expressed as nmol.L^{-1}. Briefly, 25 µL of plasma blood and 200 µl of 20× enzyme conjugate containing 0.5% proclin were mixed into the wells and incubated for 60 min at 20°C. The mixture was removed and washed with 20× wash buffer concentrate. 100 µl of tetramethylbenzidine hydrochloride (TMB) was added to the wells and incubated for 15 min at 25°C. 50 µl of 0.5 M H_2SO_4 was added in order to stop the reaction. The absorbance was measured at 450 nm using a microtiter plate reader (Titertek plus MS212, ICN Biomedicals, Germany).

Total plasma T_4 and T_3 were measured by ELISA following the manufacturer's instructions (Pointe Scientific, No. T1007-96 and T1005-96, respectively, Lincoln Park, IL, USA). T_4 was assayed by mixing 25 µL and 100 µL of conjugate reagent in the wells. The mixture was incubated for 60 min at room temperature and then rinsed with deionised water. 100 µL of TMB was added, mixed and incubated for another 20 min at room temperature. The reaction was ended by adding 100 µL of stop solution. The absorbance was read at 450 nm using a microtiter plate reader. Meanwhile, the reaction mixture for T_3 consisted of 25 µL of samples, 25 µL of antibody reagent and 50 µL of conjugate reagents. The mixture was incubated for 60 min and the wells were rinsed with deionised water. 50 µL of TMB was added to each well, mixed and incubated at room temperature for 20 min, and the reaction was stopped by adding 50 µL of 1 N HCl. The optical density was determined at 450 nm using a microtiter plate reader (Titertek plus MS212, ICN Biomedicals, Germany).

Plasma glucose and lactate levels were measured by the enzymatic colorimetric method using a commercial kit (Pointe Scientific, No. G7521 and L7596, respectively, Lincoln Park, IL, USA) and expressed as mmol L^{-1}. Briefly, 20 µL of plasma blood was transferred into a cuvette containing 2 mL of glucose reagents (1.5 mmol L^{-1} NAD, 1 mmol L^{-1} ATP, 1000 U L^{-1} hexokinase, 0.05% sodium azide, 2.1 mmol L^{-1} magnesium ions and phenol buffer pH 7.5). The mixture was mixed and incubated for 5 min at 30°C. The absorbance was

spectrophotometrically recorded at 340 nm (UV-Visible Spectrophotometer, 1201, Shimadzu Co. Ltd., Japan). The reaction mixture for plasma lactate consisted of 10 μL of plasma blood, 500 μL of reagent I (100 mM TRIS buffer, 1.7 mM 4-aminoantipyrene, 10,000 U L^{-1} peroxidase and 0.09% sodium azide) and 334 μL of reagent II (100 mM TRIS buffer, 1000 U L^{-1} lactate oxidase, 1.5 mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS) and 0.09% sodium azide). 10 min after incubation at 37°C, the absorbance of the mixture was spectrophotometrically recorded at 546 nm.

The Hb concentration was determined spectrophotometrically at 540 nm (UV-Visible Spectrophotometer, 1201, Shimadzu Co. Ltd., Japan) using the standard cyanmethemoglobin method recommended by Baker and Silverton (1976) and expressed as mg dL^{-1}.

6.2.5.2 Acute stress responses

In addition, other samples of ten fish from each tank were subjected to acute stressful testing by dipping the fish for 1 h in a 1 L plastic container containing ammonium chloride (NH_4Cl) in order to achieve TAN concentrations of 55 mg.L^{-1}, which corresponds with an unionised ammonia concentration of 1.76 mg.L^{-1} (96 h LC_{50}). Water pH and temperature were maintained at 7.8 and 26°C, respectively. Finally, the fish was anesthetised (60 mg L^{-1} Aqui-S, containing 50% isoeugenol, New Zealand Ltd., Lower Hutt, New Zealand) and blood samples were collected by syringe from the caudal vein. The blood was analysed for altered stress responses, including total T_4, total T_3, cortisol, glucose, lactate, and Hb.

6.2.6 Statistical analyses

All of the data obtained were expressed as means ± standard error (SE), and group mean differences were compared using one-way analysis of variance. The group means were further compared with Tukey's honestly significant difference (HSD) tests. All computations were performed with IBM SPSS Statistics 22.0. Statistical significance was measured at $P \leq 0.05$ in all cases.

6.3 Results

6.3.1 Water quality parameters

There were no significant differences ($P > 0.05$) in water temperature, DO and pH among treatments of different densities (Table 1). Water temperature was between 26°C and 27°C during the 84-day grow-out period, while DO never dropped below 7.2. Similarly, the pH values in all treatments were stable at above 7.4, with the mean level ranging from 7.54 to

7.56 in all treatment densities. The mean TAN and nitrite-N levels were similar for all treatment densities, while TP, orthophosphate, total nitrogen and nitrate-N levels were significantly higher ($P < 0.05$) in the tank with a stocking density of 18.75 than 10.10 and 12.98 kg m^{-3} (Table 6.1).

Table 6.1. Effects of four stocking densities of juvenile barramundi (*L. calcarifer* Bloch) on physicochemical parameters of the water in IRAS.

	Stocking density (kg m^{-3})			
	10.10	12.98	15.86	18.75
Dissolved oxygen (mg L^{-1})	7.81±0.03	7.79±0.04	7.79±0.04	7.78±0.04
pH	7.56±0.01	7.54±0.01	7.54±0.01	7.54±0.01
Temperature (°C)	26.99±0.22	26.99±0.22	27.00±0.22	27.03±0.23
TAN (mg L^{-1})	0.83±0.03	0.83±0.06	0.90±0.06	0.91±0.06
Nitrate nitrogen (mg L^{-1})	7.17±0.73^{a}	7.34±0.68^{a}	8.21±0.69^{ab}	9.21±0.70^{b}
Nitrite nitrogen (mg L^{-1})	0.23±0.02	0.25±0.02	0.26±0.02	0.26±0.02
Total nitrogen (mg L^{-1})	10.25±0.80^{a}	10.33±0.80^{a}	12.28±0.92^{ab}	12.89±0.92^{b}
Orthophosphate (mg L^{-1})	0.90±0.11^{a}	0.95±0.12^{a}	1.18±0.14^{ab}	1.33±0.14^{b}
Total phosphorus (mg L^{-1})	1.37±0.13^{a}	1.39±0.13^{a}	1.49±0.11^{ab}	1.78±0.12^{b}

[a] Values in the same column with the same letter are not significantly different ($P>0.05$)

6.3.2 Growth and feed utilisation of juvenile barramundi

The mean WG and SGR of the juvenile barramundi that were reared at 18.75 kg m^{-3} were significantly lower than at other fish densities, whereas the mean FCR was significantly increased at SD_4. The mean final body weights were 25.51, 25.46, 25.07 and 23.99 g in 10.10, 12.98, 15.86 and 18.75 kg m^{-3}, respectively. However, no significant differences ($P > 0.05$) in the mean final body weights were observed in the fish stocked at 10.10, 12.98 and 15.86 kg m^{-3}. The fish survival rate, which was above 97%, was also not significantly different ($P > 0.05$) among different treatment densities (Table 6.2).

Table 6.2. Effects of four stocking densities of the juvenile barramundi (*L. calcarifer* Bloch) reared in IRAS.

	Stocking density (kg m^{-3})			
	10.10	12.98	15.86	18.75
Mean final weight (g.$fish^{-1}$)	25.51±0.45^a	25.46±0.02^a	25.36±0.01^a	23.99±0.01^b
Mean weight gain (g. $fish^{-1}$)	12.17±0.01^a	12.13±0.01^a	12.02±0.02^a	10.65±0.01^b
Feed consumption (g.$fish^{-1}$.day^{-1})	0.25±0.01	0.25±0.01	0.25±0.01	0.23±0.01
SGR (%.day^{-1})	0.78±0.01^a	0.78±0.01^a	0.78±0.01^a	0.70±0.01^b
Food conversion ratio (FCR)	1.81±0.04^a	1.81±0.01^a	1.82±0.01^a	1.90±0.01^b
Survival rate (%)	98.33±0.63	98.33±0.49	98.03±0.15	97.95±0.13

6.3.3 Stress responses

Stocking density had significant effects on basal plasma levels of total T_4, total T_3, cortisol, glucose, lactate and whole-blood Hb. Basal plasma total T_4 levels were significantly higher in the fish stocked at 18.75 kg m^{-3} than the fish stocked at other densities (Fig. 6.1). There was a significant ($P < 0.05$) inverse relationship between plasma T_3 levels and stocking densities, in which total T_3 levels in the fish reared at 10.10, 12.98 and 15.86 kg m^{-3} were significantly higher ($P < 0.05$) than the fish reared at 18.75 kg m^{-3} (Fig. 6.2), but there were no differences in T_3 levels between 10.10, 12.98 and 15.86 kg m^{-3}.

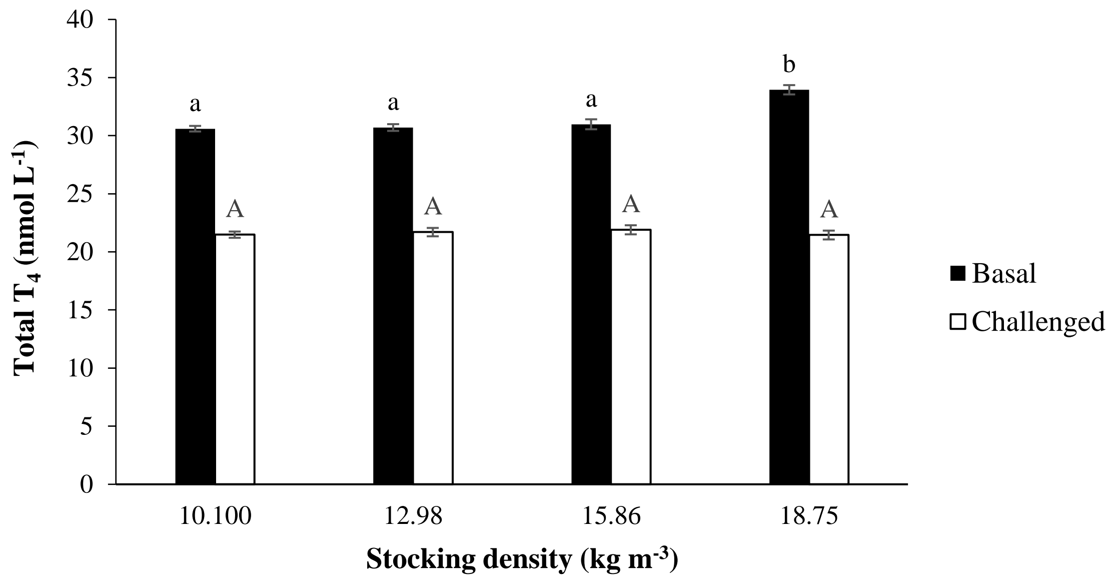


Figure 6.1. Total T4 concentration of blood plasma before and after an acute stressor. Data are shown as the mean ± SE.

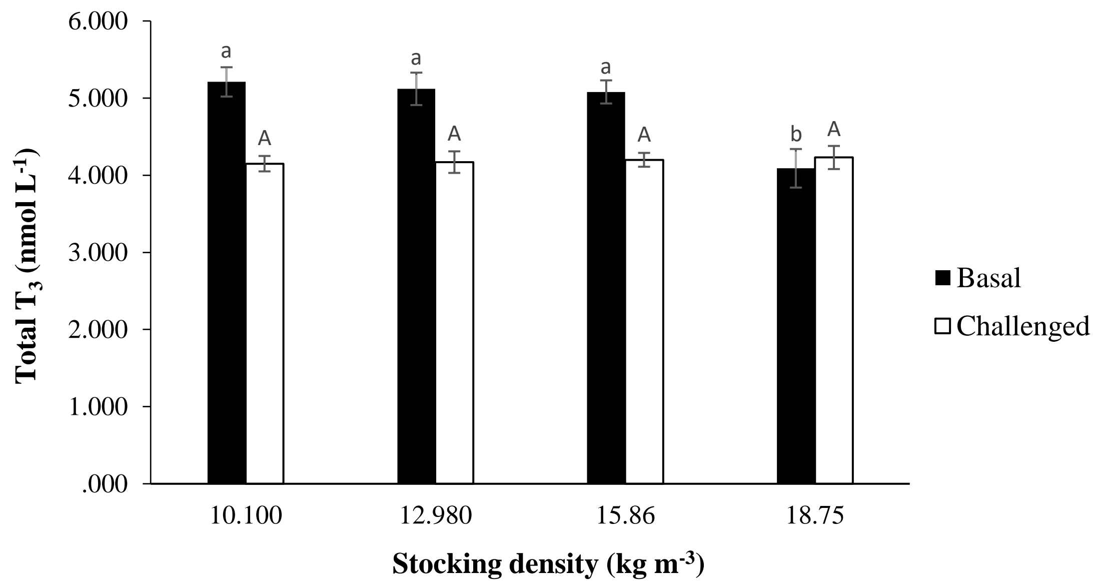


Figure 6.2. Total T_3 concentration of blood plasma before and after an acute stressor. Data are shown as the mean ± SE.

Basal plasma cortisol levels were significantly higher in the fish reared at SD_4 than at other densities (Fig. 6.3). Stocking density had significant effects on levels of plasma glucose (Fig. 6.4), and lactate (Fig. 6.5), for which the highest levels were observed in the fish reared at 18.75 kg m^{-3}. Similarly, whole-blood Hb contents significantly increased at 18.75 kg m^{-3} (Fig. 6.6). However, after acute stressor exposure, basal levels of total T_4 and T_3 decreased and basal levels of cortisol, glucose and lactate increased in fish stocked at all densities.

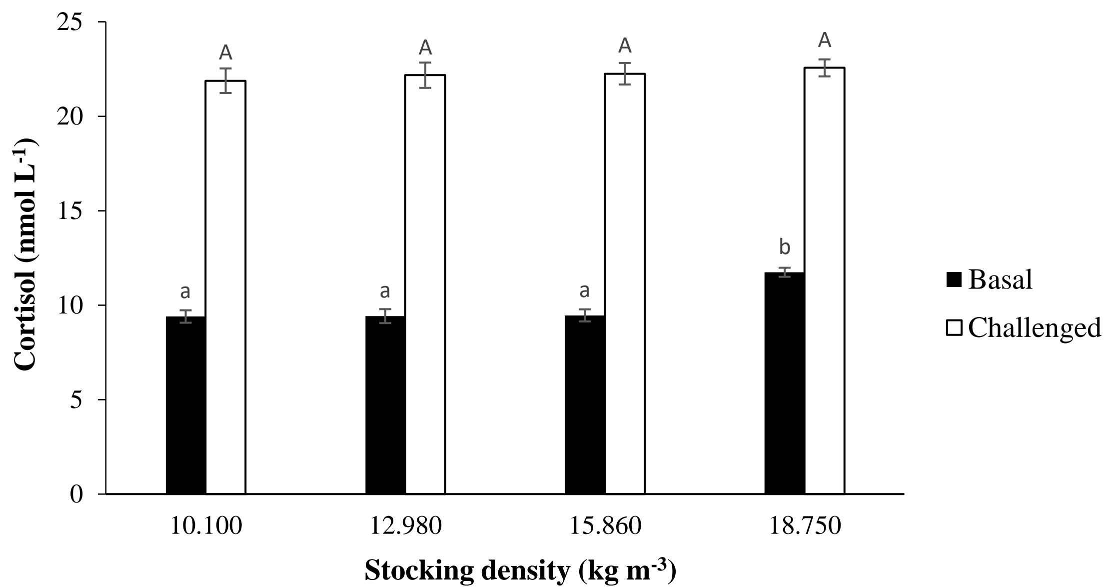


Figure 6.3. Cortisol contents of blood plasma before and after an acute stressor. Data are shown as the mean ± SE.

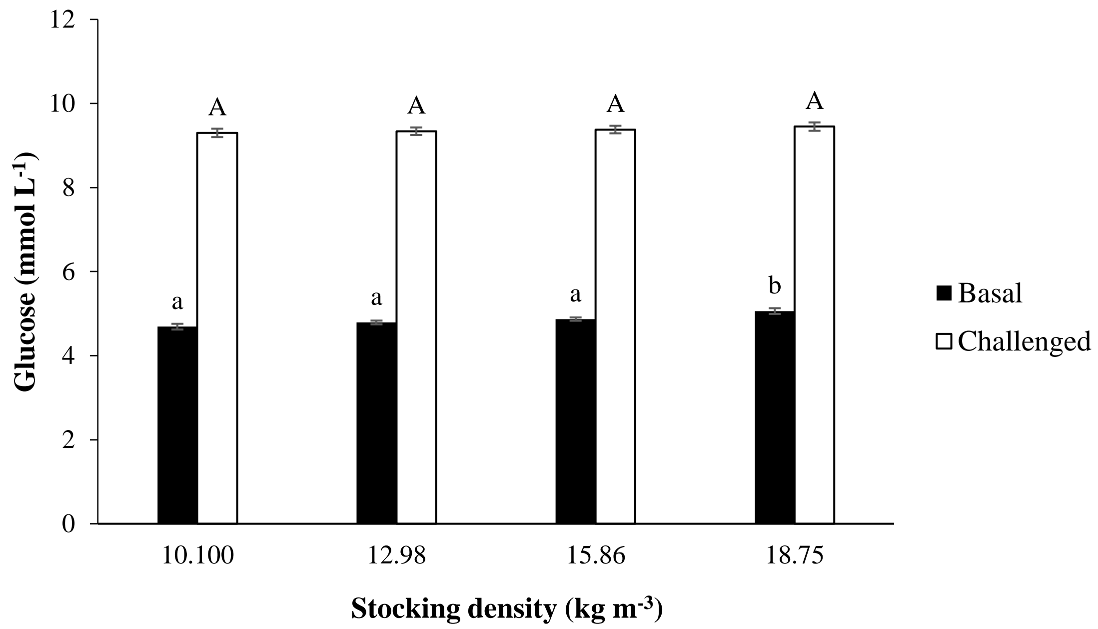


Figure 6.4. Glucose contents of blood plasma before and after an acute stressor. Data are shown as the mean ± SE.

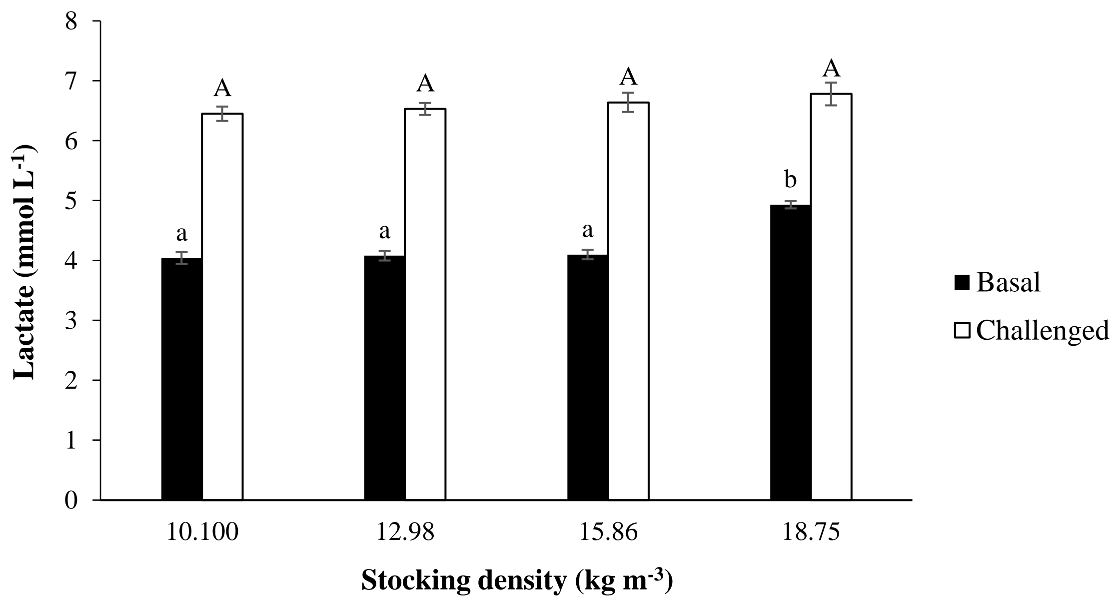


Figure 6.5. Lactate contents of blood plasma before and after an acute stressor. Data are shown as the mean ± SE.

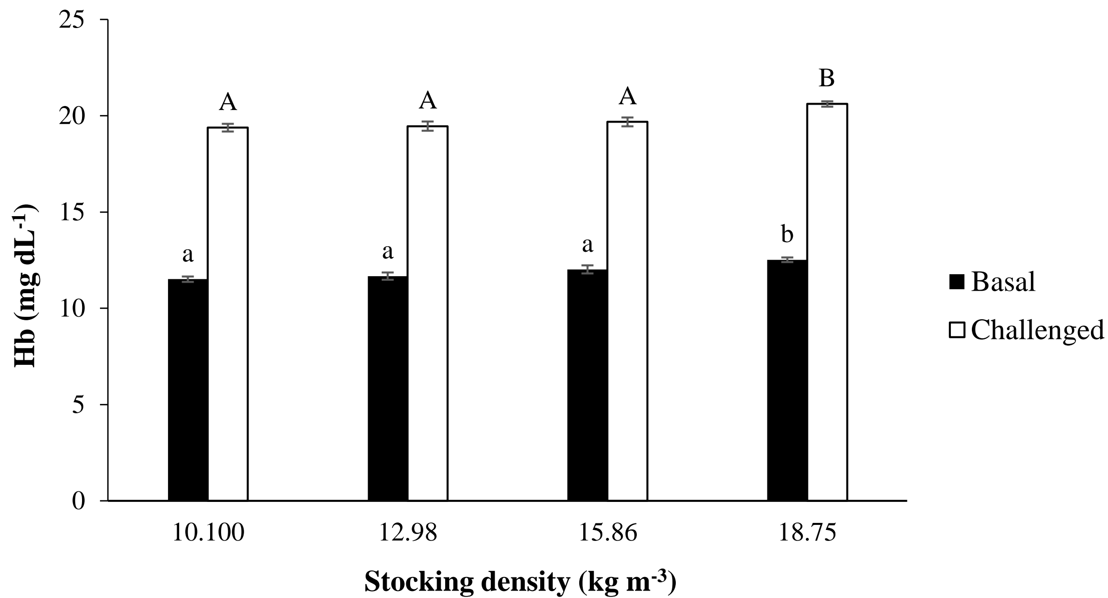


Figure 6.6. The Hb contents of whole blood before and after an acute stressor. Data are shown as the mean ± SE.

6.4 Discussion

In the present study, both the SGR and FCR of juvenile barramundi decreased at the highest stocking density of 18.75 kg m^{-3}. Higher stocking densities have been reported to inhibit the growth of European seabass (*Dicentrarchus labrax*) cultured in RASs (Sammouth et al., 2009) and other fish species cultured in various production systems (Montero et al., 1999; McKenzie et al., 2012). The deterioration of water quality associated with high stocking density is the main reason for this (Bjornsson and Olafsdottir, 2006; Person-Le Ruyet et al., 2008). Although the water quality parameters were maintained at the recommended levels for barramundi aquaculture (Cheong, 1989; Rimmer and Russel, 1998; Colt, 2006) in all densities throughout the 84-day trial, previous studies have reported that elevated nitrogen excretion associated with stocking density adversely affected fish growth in RASs (Björnsson and Ólafsdóttir, 2006) and flow-through systems (Tolussi et al., 2010). For example, high stocking densities increased the accumulation of nitrogenous wastes causing reductions in growth in Atlantic salmon (*Salmo salar*) in RASs (Wolters et al., 2009). The efficacy of *Lemna* spp. as a medium for absorbing nutrients has already been established (Jo et al., 2002; Ferdoushi et al., 2008). The elevated but acceptable nitrate and total nitrogen levels of 9.21 mg L^{-1} and 12.89 mg L^{-1}, respectively, at 18.75 kg m^{-3} in the current study may be one reason

for the activation of the thyroid gland, causing the release of T_4 hormone and resulting in increased metabolic rates in the fish.

In teleosts, T_3 and T_4 are involved in regulating the body's metabolic rate, heart rate and some digestive functions (Hulbert, 2000; Eales, 2006). Exposure to higher levels of nitrate and nitrite are known to disrupt the thyroid axis in multiple vertebrate species (Edwards et al., 2006; Hinther et al., 2012). As the thyroid hormone T_4 is the primary regulator of metabolic rate (Hollenberg and Forrest, 2008), higher T_4 levels cause the accelerated nitrogen excretion rate due to higher catabolism (Medda and Ray, 1979). As a consequence, the HPI axis is activated in order to stimulate the release of cortisol, a primary stress hormone (Geven et al., 2006).

The main response of cortisol to stressors includes redirection of energy towards the physiological functions that are required in order to restore homeostasis after exposure to a stressor (Wendelaar-Bonga, 1997; Geven et al., 2006), leading to growth retardation at a higher stocking density. Furthermore, in the current study, the increased total T_4 level of 33.71 nmol L^{-1} at the highest stocking density is much higher than has been reported for other species, such as juvenile tilapia, *Oreochromis niloticus* (4.11 ng mL^{-1} corresponds to 5.29 nmol L^{-1}) and crucian carp, *Carassius auratus* (11.08 ng mL^{-1} is equivalent to 14.26 nmol L^{-1}) (Li et al., 2008; Kpundeh et al., 2013).

In our study, the increase in stocking density of up to 18.75 kg m^{-3} associated with elevated levels of nitrogen metabolites (Table 6.1), resulted in a small but statistically significant elevation of plasma cortisol levels (Fig. 6.3). The mean plasma basal cortisol levels recorded for the juvenile barramundi at high stocking density was 11.07 nmol L^{-1}, which is higher than the resting level of <10 nmol L^{-1} as reported by Percival (1999). Cortisol, which is produced by the adrenal gland, has been reported to be a major factor in stimulating the conversion of T_4 to T_3 in the liver of brook trout (*Salvelinus fontinalis*) and Senegalese sole (*Solea senegalensis*) (Vijayan et al., 1988; Arjona et al., 2011). An increased cortisol level – a common endocrine response to a stressor – has been shown to interfere with thyroid functioning in fish (Walpita et al., 2007). Decreased levels of T_3 are associated with chronic stress resulting from higher stocking densities (Silberman et al., 2002). A reduction in blood plasma T_3 levels is caused by a drop in T_4 production or changes in peripheral thyroid metabolism (Power et al., 2001; Walpita et al., 2007). However, in this study, stocking densities significantly increased plasma T_4 levels in juvenile barramundi. The small but

significant increases in cortisol levels of the fish reared at high density suggest that the fish were under physiological stress, affecting the conversion of T_4 to T_3 – the most active form of thyroid hormone – by directly inhibiting the enzyme 5'-deiodinase, which is responsible for converting T_4 into active T_3, reducing T_3 levels (Vijayan et al., 1988; Brown et al., 1991). An elevated T_4 level could only play a role in regulating circulating T_3 levels under the conditions of crowding stress as the high stocking density does not influence the secretion of T_4, but rather decreases T_3 levels via altering the peripheral thyroid hormone metabolism in tissues, as seen in the current study.

The juvenile barramundi responded to high stocking density with small but statistically significant increases in plasma cortisol level, followed by elevated levels of glucose and lactate. The mean glucose and lactate levels at the highest density were 5.04 mmol L^{-1} and 4.83 mmol L^{-1}, respectively. These glucose and lactate values are higher than those reported by Wilkinson (2008) (4.17 mmol L^{-1}) and Percival (1999) (4 mmol L^{-1}) in barramundi. The increased blood plasma cortisol, glucose, and lactate levels are related to the decreased growth and increased food conversion efficiency of juvenile barramundi at higher stocking densities (Montero et al., 1999; Teles et al., 2007; Lupatsch et al., 2010).

Our results demonstrated that high stocking density caused endocrine alterations, including an elevation of blood plasma cortisol and glucose levels (Wedemeyer, 1997; Bayunova et al., 2002), which change the metabolic rates of fish. Cortisol can trigger a secondary response by increasing basal metabolic rate and thus the mobilisation the energy reserves (McDonald and Milligan, 1997; Wendelaar-Bonga, 1997) in the form of glucose. Glucose is an essential energy reserve that assists in fish metabolism in order to cope with the elevated energy demands caused by stress (Teles et al., 2007). Elevated blood plasma lactate may lead to hypoxia in fish tissues, which in turn activates the production of ATP through anaerobic glycolysis, using glycogen stored in the liver and muscles and forming lactate as a final product (Owen and Sunram-Lea, 2011). When the energy required for growth is deviated in order to provide for the increase in metabolic rate, this can then lead to a reduction in food assimilation (Tolussi et al., 2010), as reflected by a higher FCR and reduced growth rate.

The high stocking density also induced a significant increase in Hb concentrations in juvenile barramundi. Hb is also associated with stress responses, and the adrenalin produced during stress causes *in vivo* swelling of erythrocytes (Nikinma and Huestis, 1984), which then

triggers the erythrocytes to synthesise more Hb in order to maintain the optimum oxygen level in the blood (Nicula, 2004).

When the fish were exposed to the acute stressor in the form of unionised ammonia, decreased basal levels of total T_4 and T_3, coupled with increased basal levels of cortisol, glucose, lactate and Hb, were observed at all densities, demonstrating that, irrespective of any stocking density, unionised ammonia can induce significant changes in thyroid and other stress responses. Different results were obtained by Ruane et al. (2002), who reported elevated plasma cortisol levels in common carp (*Cyprinus carpio*) reared at high densities after challenge with net confinement compared with common carp reared at low densities. In line with the present study, Ramsay et al. (2006) reported that feeding and its interaction with stocking density caused elevated levels of cortisol, rather than density effects on the cortisol levels during the acute and prolonged stressor exposures in zebrafish (*Danio rerio*). Similarly, when African catfish (*Clarias gariepinus*) (Roques et al., 2015) and Nile tilapia (*O. niloticus*) (Metwally and Wafeek, 2014) were subjected to different concentrations of ammonium chloride, plasma cortisol, glucose, and lactate were elevated.

Table 6.3. Selected data on the effects of stocking density on growth of several important fish species in RAS.

Species	Recommended density	References
Cobia, *Rachycentron canadum*	≤ 30 kg m^{-3}	Riche et al., 2013.
Rainbow trout, *Oncorhynchus mykiss*	≤ 123 kg m^{-3}	Pickering and Pottinger, 1987.
Rainbow trout, *Oncorhynchus mykiss*	≤ 40 kg m^{-3}	Yarahmadi et al., 2014
Juvenile turbot, *Scopthalmus maximus*	> 14.16 kg m^{-3}	Li et al., 2013.
Atlantic salmon, *Salmo salar* L.	< 39 kg m-3 (Final density)	Liu et al., 2014.
Common carp, *Cyprinus carpio* L	> 70 kg m^{-3}	Sammouth et al., 2009
Juvenile European catfish, *Silurus glanis*	> 2.78 kg m^{-3}	Placinta et al., 2014
Juvenile nile tilapia, *Oreochromis niloticus*	≤ 37 kg m-^{3} (final density)	Gullian-Klanian & Aramburu-Adame, 2013
European seabass, *Dicentrarchus labrax*	< 60 kg m^{-3}	Lupatsch et al., 2010
Juvenile cod, *Gladhus morhua* L.	> 40 kg m^{-3}	Bjornsson and Olafsdottir, 2006
Juvenile cod, *Gladhus morhua* L.	≤ 40 kg m^{-3}	Lambert and Dutil, 2001
Juvenile red drum, *Sciaenops ocellatus*	≤ 30 kg m^{-3}	Wills et al., 2009.
Juvenile largemouth bass, *Micropterus salmoides*	< 75 kg m^{-3}	Park et al., 2015.

The stocking density of 18.75 kg m^{-3} in the present study is lower than that reported in RASs (Table 6.3). The possible explanation for this is the integration of duckweed into the RASs used to rear barramundi, which, although ideal for the reduction of nitrogen metabolites (Sipauba-Tavares et al., 2002; Ferdoushi et al., 2008), brings another biological entity into the equation by increasing the overall biomass. The integration of duckweed, due to its photorespiration activity, brings additional stress into the production system and hence reduces the carrying capacity of the target species, barramundi (Tolbert et al., 1995). The introduction of the macrophytes results in an increased level of nitrogen metabolism in the

RAS, resulting in the high consumption of oxygen during respiration. The DO in water fluctuates by up to 4 mg L^{-1} between day and night when an aquatic macrophyte is present (Perna and Demilio, 1986; Killgore et al., 1991). However, in this study, we did not measure the diurnal fluctuations in DO levels.

Stocking density is one of the factors that could potentially affect survival and production performance of aquatic organisms. Thus, the use of an appropriate density can increase the profitability of farming systems, by maximizing the utilization of water and the other resources in the rearing system (Fairchild and Howell, 2001). However, adequate stocking densities depend on a variety of factors such as the age of the system, fish size, dissolved oxygen levels, and the type and design of the rearing system (El-Sayed, 2006).

Our results demonstrated that high stocking density resulted in the elevation of nitrogenous waste excreted into the system. The carrying capacity of IRAS is strongly influenced by the ability of the system to remove nitrogenous waste, mainly in the form of ammonia and nitrate. The capacity of IRAS to eliminate the nitrogenous waste depends on the capacity of duckweed compartment, which in turn directly determines the carrying capacity of the IRAS. As stated by Helfrich and Libey (1990), the capacity of the biofilter is influenced by several factors, including the surface area of the biofilter compartment, hydraulic loading (gallons of water per day per media surface) and the turnover time. Our results also indicated that the optimum stocking density in the IRAS may be increased by increasing the capacity of the duckweed compartment.

6.5 Conclusions

This study suggests that a higher stocking density of 18.75 kg m^{-3} of juvenile barramundi in IRAS under normal aeration results in stressed fish, as reflected by small but significant increases in plasma cortisol. In addition, an increase in blood plasma total T_4, a reduction in blood plasma concentrations of total T_3 and the increase in FCR caused by high stocking density may be contributory factors in the growth suppression of the juvenile barramundi.

CHAPTER 7: The physiological responses of two–sized juvenile barramundi (*Lates calcarifer* Bloch) when duckweed (*Lemna minor* Linnaeus) as a biofilter medium is incorporated into the rearing system[5]

7.1 Introduction

Currently, around 85% of the world's fish stocks are fully exploited or overexploited. The Northeast Atlantic, Western Indian Ocean, and Northwest Pacific have the highest proportions of fully exploited stocks (FAO, 2012). Therefore, sustaining fish supplies from capture fisheries may not be able to meet the growing global demand for aquatic food. Meanwhile, during the past three decades, global aquaculture production has expanded at an average annual rate of more than 8%, from 5.2 million metric tons in 1981 to 62.7 million metric tons in 2011 (FAO, 2012). Aquaculture's contribution to total food fish supply grew from 9% in 1980 to 48% in 2011 (The Word Bank, 2013). However, intensification of aquaculture development is raising concerns about the environmental consequences and it'sustainability. All existing aquaculture systems produce waste nutrients, solids, and organic matter. These wastes are either dumped into the environment or treated on site (Tucker et al., 2008). This eventually leads to eutrophication and nitrification of effluent-receiving ecosystems and the pollution of water intended for other uses (Martinez-Porchas and Martinez-Cordova, 2012).

Increasing the recycling of resources such as water and nutrients through integrated multi-trophic aquaculture (IMTA) could solve aquaculture's environmental related problems; the nutrient waste from one system would become a resource for another. ITMA combines organisms from different trophic levels in order to increase the productivity of each organism (Chopin et al., 2008). The benefit of integrating the production of macroalgae with the fish is that waste nutrients can be recaptured (Zhou et al., 2006). For example, macroalgae, as primary producers, function as a bio-filter by removing CO_2 and nutrient wastes, like dissolved nitrogen and phosphorus, from the water. Thus nutrient discharge into the environment is reduced (Nobre et al., 2010). Additionally, the high nutrient uptake capacity of macroalgae stimulates macroalgae growth (Troell et al., 2006), generating protein-rich biomass that can be used as a fish diet of high protein content. Although macroalgae can be grown in proximity to open oceans or coastal fish farms, it is hard to verify the degree to which nutrients are being removed. Since the fish effluent is diluted by currents and the feed and extractive components are separated spatially, only a small portion of nutrients may be taken up by the macroalgae.

[5] This chapter has been ready to be submitted to Aquaculture International

A reduction in the volume of waste water is essential to enhance the sustainability of aquaculture; recirculation aquaculture systems (RAS) have been proposed for this purpose (Blancheton et al., 1996; Ramirez-Godinez et al., 2013). In these systems, water is mostly reused in the process after undergoing proper treatment, thereby reducing water usage and improving effluent quality. RAS reduce water dependence by 93% in comparison to the flow-through system (Ramirez-Godinez et al., 2013). Moreover, RAS eutrophication potential results in being 26%-38% lower than that of flow-through systems (Martins et al., 2010). However, some dissolved materials such as C, N, and P, cannot be effectively removed in RAS as these dissolved substances are dependent on the intensity of production. Higher C, N, and P concentrations are obtained in more intensive systems with higher feeding rates and less water exchanges (Leonard et al., 2002).

Although IMTA's contribution plays a significant role in coastal waste water filtration and bioaccumulation (Costa-Pierce et al., 2011; Klinger and Naylor, 2012), IMTA requires relevant research as it is highly site and species specific; one must evaluate factors that affect macroalgae growth and nutrient uptake capacity for extrapolation on a commercial scale. Most of the studies using macroalgae to treat fish effluents used macroalgae stocked at discharge channels or outflow ponds, with no recirculation back to the fish cultivation system (Nelson et al., 2001). Therefore, IMTA principles should be applied to the recirculation of fish effluent through macroalgae bio-filters in land-based intensive aquaculture farms. It can also be a more effective tool to increase recirculation practices and establish integrated recirculating aquaculture systems (IRAS) with all the known associated benefits (Robledoe et al., 2014). In IRAS, water treatment can be adequately controlled as nutrient availability to the macroalgae can be increased by adjustment of water flow rates and mixing (Buschmann et al., 2001; Schuenhoff et al., 2003). IRAS allow for the directing of the nutrients to an integrated recycling system by plants. Thus, IRAS offer a promising future because of their potential environmental and economic benefits.

As economic and resource conditions change in the short term, together with environmental pressures and the market for the cultivation of high-value species, the utilisation of RAS and IRAS are predicted to increase shortly. However, various strategies and procedures adopted can be a source of stress for the fish. Commercial scale production characterised by high stocking density is directly related to animal comfort and the productivity of fish culture. This could be the determining factor of the economic return on production (Braun et al., 2010), due to stocking

densities' profound effects on metabolism, growth and stress associated with fish rearing conditions (Chatterjee et al., 2006).

It is well known that physiological processes and metabolic rates are affected by, and depend on the body size. The same is also valid for fish species (Pickering and Pottinger, 1989; Pankhurst and Dedual, 1994; Carey and McCormick, 1998). The effect of body size with duration of noxious stimuli on the cortisol stress response, however, had not been studied extensively in fish species. Studies on Atlantic salmon (*S. salar*) have shown a higher plasma cortisol secretion in smolts than in smaller-sized parrs exposed to the same acute stressor (Carey and McCormick, 1998). In a previous preliminary study in European sea bass of two different sizes exposed to repeated stressors, smaller fish showed peak plasma cortisol concentration at 4 h, while larger fish at 8 h post-stress (Fanouraki, 2010).

As fish size is highly variable and, in general, not necessarily correlated with age, we posed the following question: what crucial role do the fish size and rearing systems play in physiological responses? Therefore, the study aimed to investigate the effects of the higher stocking density of the two sizes of juvenile barramundi on the growth performance and physiological responses, which were then used to compare the physiological status of the junveniles under two different rearing systems in order to validate the efficacy of IRAS.

7.2 Materials and methods

7.2.1 Fish and stress factors

The experiment was approved by the Animal Ethic Committee of Curtin University following the Australian Code for the Care and Use of Animals for Scientific Purposes (Approval number: AEC_2013_16). The study was conducted at Curtin Aquatic Research Laboratory, and the juvenile barramundi (*L. calcarifer* Bloch) used were provided by Challenger Marine Hatchery, Fremantle, Western Australia. Upon arrival, the juvenile barramundi used in this experiment were transferred to large storage tanks and reared under the temperature of 26°C until reaching an average weight of 13.34 g. The stress responses tested were a higher fish density and two rearing systems. In our previous study, the juvenile barramundi were stocked up to 18.75 kg m^{-3} in an integrated recirculating aquaculture systems (IRAS). The stocking density was increased to 21.63 kg m^{-3}. The effects of the higher fish density of two sizes reared in different rearing systems were evaluated for 48-day experimental period.

7.2.2 Experimental design

The experimental fish was divided into two different size groups; the fish with the average body weight of 13.34 g ± 0.12 g, and 26.67 ± 0.14 g. Each size group was randomly stocked at a density of 21.63 kg. m^{-3} in four experimental units of RAS and four experimental units of IRAS. Each unit of the experimental RAS consisted of a fish-rearing tank (400 L circular tank), a waste collection tank (100 L circular plastic drum), and a filtration system that used two external filters (Fluval 406, Hagen, Rome, Italy), whereas the experimental IRAS consisted of a fish-rearing tank (400 L circular tank), a waste collection tank (100 L circular plastic drum) and a bio-filter tank (100 L circular tank) that housed duckweed (*L. minor* Linnaeus) as the bio-filter media to replace the external filter.

Both groups of fish were hand fed twice daily (at 09.00 a.m. and 4.00 p.m.) at the same fixed rate of 2.5% of total body weight per day with a formulated diet. The amount of feed distributed to each rearing tank was recorded after each meal. The proximate composition of the formulated diet was crude protein 45%, crude fat 10%, crude ash 9.3%, and crude fibre 5.8%. Proximate composition of the feed ingredients was estimated using standard methods of AOAC (2000). Moisture was determined by oven drying at 105°C for 22 h. Crude protein was analysed by the Kjeldahl method while crude lipid was determined using chloroform-methanol extraction. The total ash content was determined by oven incineration at 550 °C for 24 h in a muffle furnace. In both rearing systems, the replacement water flow rate was adjusted weekly to the ingested food quantity and water quality conditions.

Water quality parameters were periodically monitored and maintained at a temperature of 26 - 28°C and a pH of 7.2 – 8.0, water flow rates of 4.1 L min^{-1}. Aeration kept DO levels above 6.0 mg L^{-1} with % saturation of > 90%. At the end of the experiment, and after 24 hours of fasting, the fish were weighed by groups and counted. The final mean bodyweight was calculated.

7.2.3 Growth performance

The total weight of the fish per unit was measured at the beginning and the end of the experimental period. The morphological growth parameters were calculated as follows: Weight gain (g) = final weight (g) - initial weight (g); Weight gain % = 100 [final weight (g) - initial weight (g)]/initial weight (g); Specific growth rate (SGR; % day^{-1}) = 100 [Ln final weight (g) - Ln initial weight (g)]/time (days); Feed conversion ratio (FCR) = feed intake/weight gain; Survival rate (%) = 100 [Final number of fish/initial number of fish]

7.2.4 Physiological response analyses

Blood sampling was conducted at the beginning (basal condition) and the end of the 48-day experimental period. 10 juvenile barramundi of each unit were anesthetised with Aqui-S (60 mg L^{-1}) and heparinised blood was drawn from the caudal vessel to determine haemoglobin (Hb) concentration. Plasma was separated by centrifugation and analysed immediately for cortisol, glucose, lactate, and total T_4 and T_3 concentrations. The remaining plasma was then stored frozen for determination of plasma ions.

Plasma cortisol was determined by indirect enzyme immunoassay (ELISA) validated for rainbow trout and gilthead sea bream (Tintos et al., 2006), and expressed as nmol L^{-1}. Plasma total T_4 and T_3 were measured following the manufacturer's instructions (Pointe Scientific, No T1007-96 and T1005-96, respectively. Lincoln Park, USA). Plasma glucose and lactate levels were measured by the enzymatic colorimetric method using the commercial kit (Pointe Scientific, No. G7521 and L7596 respectively, Lincoln Park, USA) and expressed as mmol L^{-1}. Haemoglobin (Hb) concentration was determined spectrophotometrically at 540 nm (UV-Visible Spectrophotometer, 1201, Shimadzu Co. Ltd., Japan), using the standard cyanmethemoglobin method recommended by Baker and Silverton (1976) and expressed as g dl^{-1}, whereas the concentrations of plasma chloride (Cl^-), potassium (K^+), and sodium (Na^+) were determined by Bayer Rapidlab 865 Blood Gas Analyser - direct ion-specific electrode (ISE) (Bayer Diagnostics, Australia).

7.3 Results

7.3.1 Physiological responses

High stocking density induced a significant ($P < 0.05$) increase in plasma cortisol, glucose, and lactate levels over the 48-day experimental period in both rearing systems (Fig 7.1, 7.2 and 7.3).

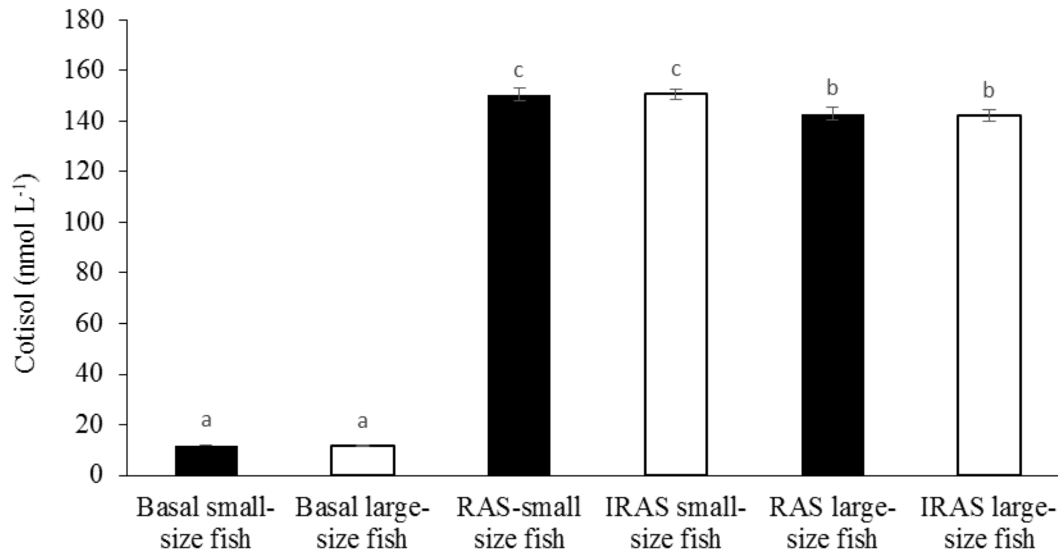


Figure 7.1. Cortisol concentrations of plasma blood in small and large-size fish after the 48-day experimental period. Data are shown as the mean ± SE.

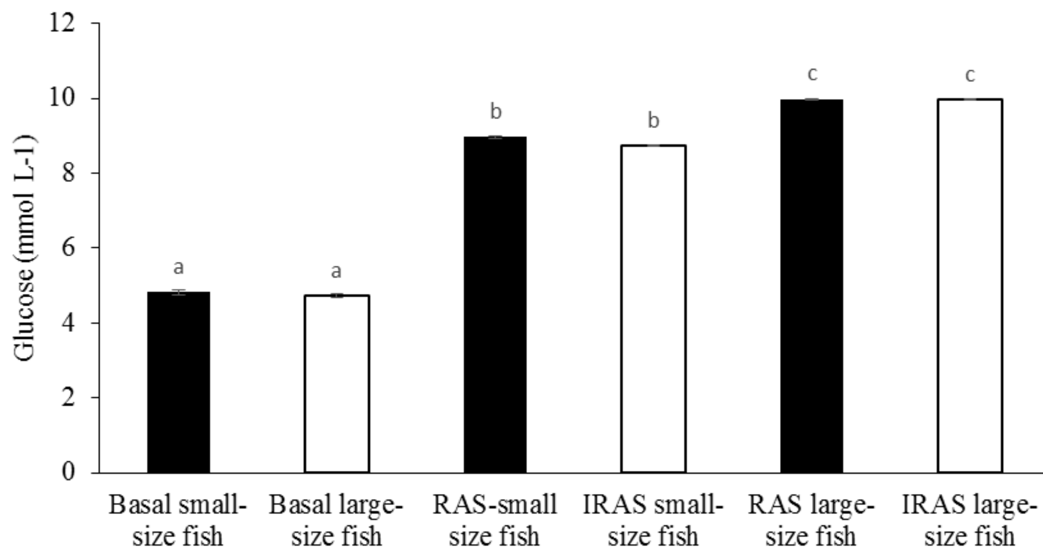


Figure 7.2. Glucose concentrations of plasma blood in small and large-size fish after the 48-day experimental period. Data are shown as the mean ± SE.

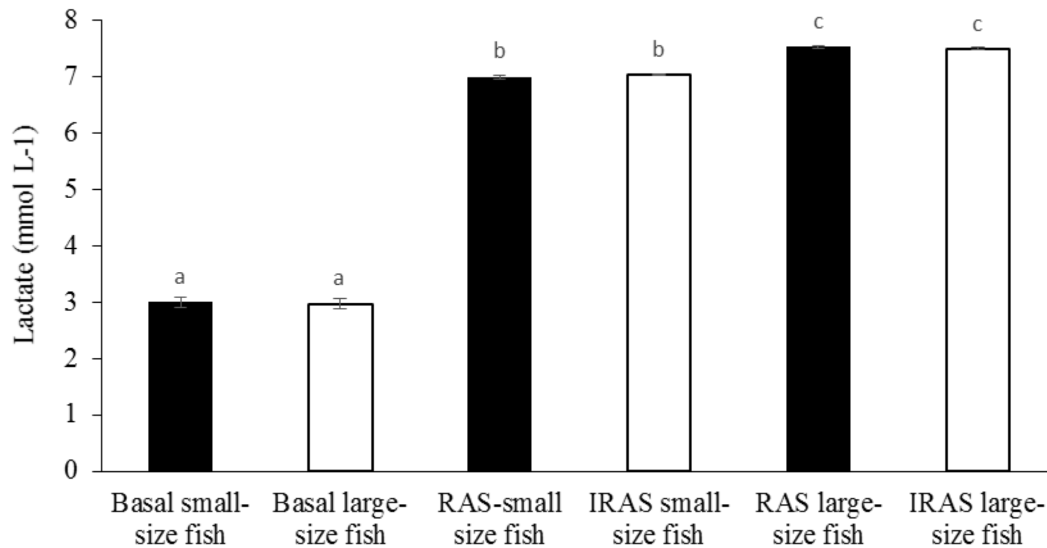


Figure 7.3. Lactate concentrations of plasma blood in small and large-size fish after the 48-day experimental period. Data are shown as the mean ± SE.

Differences in the fish size influenced the physiological responses to the stressful conditions, in which large-size fish had higher ($P \leq 0.05$) glucose and lactate levels but lower ($P \leq 0.05$) cortisol levels. However, no differences were observed as a result of different rearing systems. High stocking density also increased ($P \leq 0.05$) thyroxine (T_4) levels and decreased triiodothyronine (T_3), Hb and plasma sodium (Na^+) levels during the 48-day experimental period (Table 7.1), in which plasma T_4 levels were higher in large-size fish compared to small-size fish, whereas T_3 levels were significantly higher ($P \leq 0.05$) in small-size fish compared to large-size fish, but no differences were observed due to the rearing system ($P > 0.05$). High stocking density also decreased mean haemoglobin (Hb) and plasma sodium (Na^+) levels, in which the small-size fish had lower levels ($P \leq 0.05$) of Hb and Na^+ compared to the large-size fish (Table 1). Similarly, plasma chloride (Cl^+) and potassium (K^+) levels significantly decreased ($P \leq 0.05$) following the 48-day rearing period in both RAS and IRAS, but no differences were observed due to either fish size or the rearing systems (Table 7.1).

Table 7.1. Alterations in thyroid hormones and plasma ions in smaller and larger juvenile barramundi (*L. calcarifer*) when reared at two different rearing systems.

Blood parameters	Basal		RAS		IRAS	
	Smaller (3.5-month old)	Larger (8-month old)	Smaller (3.5-month old)	Larger (8-month old)	Smaller (3.5-month old)	Larger (8-month old)
T_4 (nmol L^{-1})	9.86 ± 0.09^a	10.00 ± 0.07^a	24.26 ± 0.22^b	26.09 ± 0.20^c	24.60 ± 0.21^b	26.21 ± 0.21^b
T_3 (nmol L^{-1})	5.13 ± 0.05^c	5.10 ± 0.06^c	4.86 ± 0.08^b	4.70 ± 0.07^a	4.86 ± 0.07^b	4.71 ± 0.08^a
Na (mmol L^{-1})	164.18 ± 0.78^c	165.96 ± 0.52^c	158.20 ± 1.40^a	161.73 ± 1.01^b	157.71 ± 1.34^a	161.47 ± 0.99^b
K (mmol L^{-1})	4.67 ± 0.07^a	4.66 ± 0.07^a	4.41 ± 0.07^b	4.40 ± 0.07^b	4.41 ± 0.08^b	4.38 ± 0.06^b
Cl (mmol L^{-1})	131.62 ± 0.34^a	132.60 ± 0.42^a	128.04 ± 0.66^b	127.89 ± 0.66^b	127.89 ± 0.64^b	127.91 ± 0.70^b
Hb (g dL^{-1})	9.51 ± 0.07^c	9.66 ± 0.05^c	8.22 ± 0.08^a	8.44 ± 0.07^b	8.22 ± 0.08^a	8.45 ± 0.06^b

[a.] Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$.

7.3.2 Water quality and growth performance

Data showed that there were no significant differences ($P > 0.05$) in water temperature, DO or pH among treatments of different densities (Table 7.2). Similarly, the mean TAN, nitrite-N levels, TP, and orthophosphate were similar for all rearing systems, whereas total nitrogen and nitrate-N levels were significantly higher ($P < 0.05$) in the water tank of the small-size fish reared in RAS and IRAS (Table 7.2).

Table 7.2. Summary of water quality assessment in *L. calcarifer* RAS and IRAS over the 48-day experimental period.

Water quality parameters	RAS		IRAS	
	Smaller (3.5-month old)	Larger (8-month old)	Smaller (3.5-month old)	Larger (8-month old)
Dissolved oxygen (mg L-1)	7.73±0.03	7.77±0.04	7.71±0.04	7.75±0.04
pH	7.51±0.01	7.53±0.03	7.49±0.02	7.52±0.04
Temperature (^{o}C)	26.77±0.22	26.74±0.32	26.78±0.34	26.75±0.26
TAN (mg L-1)	1.02±0.07	0.98±0.05	1.03±0.05	0.99±0.07
Nitrate nitrogen (mg L-1)	10.07±0.73[b]	9.98±0.68[ab]	10.02±0.69[ab]	9.93±0.70
Nitrite nitrogen (mg L-1)	0.25±0.04	0.23±0.04	0.25±0.03	0.24±0.03
Total nitrogen (mg L-1)	12.49±0.80	12.44±0.80	12.46±0.92	12.41±0.92
Orthophosphate (mg L-1)	1.40±0.11	1.37±0.12	1.41±0.14	1.37±0.14
Total phosphorus (mg L-1)	1.88±0.13	1.85±0.13	1.87±0.11	1.85±0.12

[a] Values in the same column with the same letter are not significantly different (*P*>0.05)

IHigh stocking density also influences the WG, SGR, and FCR of the experimental fish, in which the small-size fish had higher WG and SGR, and lower FCR (*P*>0.05) than the large-size fish (Table 7.3). However, no significant differences (*P*>0.05) in the mean WG, SGR, and FCR were observed due to the differences in the rearing systems. Similarly, no significant difference (*P*>0.05) was observed in the SR.

Table 7.3**.** Growth of juvenile barramundi (*L. calcarifer*) reared at two different rearing systems for 48 days.

Parameters	RAS		IRAS	
	Smaller	Larger	Smaller	Larger
WG	59.96±0.52[a]	58.96±0.47[b]	60.21±0.62[a]	59.21±1.27[b]
SGR	0.98±0.01[b]	0.94±0.01[a]	0.97±0.02[b]	0.94±0.01[a]
FCR	2.19±0.02[a]	2.31±0.01[b]	2.23±0.04[a]	2.31±0.01[b]
SR	100±0.00	100±0.00	99±0.01	99±0.01

[a.] Values in the same column with the same letter are not significantly different (P>0.05)

7.4 Discussion

When barramundi juveniles of different sizes and ages were reared at a higher stocking density of 21.63 kg m^{-3} in RAS and IRAS, differences in physiological stress responses and growth performances were observed. Rearing the juveniles at higher stocking density of 21.63 kg m^{-3} over the 48-day rearing period induced significant changes in endocrine (basal plasma cortisol) and metabolic indicators (basal plasma glucose and lactate), impaired hydromineral balances (basal plasma Na^+, K^+ and Cl^-), and affected growth performances (WG and SGR) of juvenile barramundi. However, differences in the rearing systems did not cause significant differences in endocrine, metabolic indicators, hydromineral balances, and growth performances of the smaller and larger juveniles.

Although baseline cortisol values for barramundi have not been established, our results showed that basal cortisol concentrations of the small and large-size juveniles were in agreement with that previously described by Pankhurst (2011) and Pickering and Pottinger (1989) in unstressed salmonid fish and most wild teleosts, respectively. After the 48-day experimental period, the elevation of plasma cortisol concentrations was observed in both IRAS and RAS. However, that the elevated cortisol concentrations found in this study are considered a mild stress level, as reported by Metz et al. (2005) that plasma cortisol concentrations between 138 and 160 nmol L^{-1} can be regarded as the mild stress level. Furthermore, the elevated concentrations of plasma cortisol observed in this study were near values previously reported by Rotlant et al. (1997) when reared red porgy (*Pagrus pagrus*) at 20 kg m^{-3} in RAS. Findings from European sea bass (*Dicentrarchus labrax*) showed similar responses in the fish reared at 40 kg m^{-3} in RAS (Roque-d'Orbcastel et al., 2010) and 100 kg m^{-3} in RAS (Sammouth et al., 2009). The value was very much higher than the cortisol concentrations reported on *D. labrax* (72.56 nmol L^{-1}, Tintos et al.,

2006) but the values were very much lower than the concentrations documented on *D. labrax* after exposure to the combination of trimethoprim and sulfamethoxazole (353 nmol L^{-1}, Yildiz and Altunay, 2011). Cortisol increase is typical after stress (Hosseini and Hoseini, 2012).

The current results showed that the high stocking density is a stress factor that activates the hypothalamic-pituitary-interrenal (HPI)-axis in the smaller and larger barramundi juvenile. Culturing of the juveniles of different sizes and ages under high stocking density induced significant differences in the cortisol stress responses. A similar trend was seen by Koakoski et al. (2012), in which the stress response as reflected by plasma cortisol lasts longer in 12- month-old jundia (*Rhamdia quelen*) than the 6-month juveniles. In another experiment on jundia, Barcellos et al. (2012) concluded that fish age, instead of weight and size, is the decisive factor for time course differences observed in cortisol stress responses. Rearing fish of different sizes and ages at higher stocking density may lead to a sustained increase in plasma cortisol concentrations (Wedemeyer, 1997), to which fish may adapt through a down-regulation of the HPI-axis that is induced by the negative feedback mechanism of cortisol (Procarione et al., 1999; Barton, 2002). The primary response comprises a neuroendocrine response, which involves the release of catecholamine and the activation of the HPI-axis. The corticotrophin-releasing factor (CRF) from the hypothalamus acts on the pituitary to synthesise and release adrenocorticotropic hormones, which subsequently stimulate the synthesis and mobilisation of glucocorticoid hormones (cortisol) from the interrenal tissue located in the head kidney (Huntingford et al., 2006; Ashley, 2007; Iwama, 2007). However, the effects of a lack of interaction on rearing systems and fish sizes and cortisol concentrations in this study might be caused by the acclamation of internal tissue to chronic stress induced by crowding over time (Pickering and Stewart, 1984). Another explanation may be that a significant source of energy derived from the fish feed ameliorates the crowding effects and enables the fish to cope with the stressor (Ramsay et al., 2006).

Both smaller and larger-size juveniles exhibited elevated glucose and lactate concentrations over the 48-day experimental period. Increased glucose levels of small and large juveniles barramundi in both rearing systems agreed with the previous study in juvenile tilapia reared at densities of up to 33 kg m^{-3} (8.62-10.77 mmol L^{-1}, Kpundeh et al., 2013). Changes in metabolic indicators, measured as plasma glucose and lactate, can also be used as general stress indicators in fish (Santos and Pacheco, 1996). The plasma glucose concentration in circulation is a function of its production versus absorption by tissues. Under stressful situations, glucose is generated to

provide energy substrates to tissues, in order to deal with the increased energy demand (Teles et al., 2007). Lactate has been widely used as a measure of anaerobic metabolism and rapid response to the depletion of tissue energy stores. Our results show that the size of the fish significantly affected the physiological stress response of the fish to high stocking density as indicated by the high levels of plasma glucose and lactate. This result confirms the previous findings in European sea bass (*D. labrax*), where fish size had significant effects on plasma glucose concentrations (Fatira et al., 2014), and in rainbow trout, where the larger fish showed higher lactate concentrations compared to the small-size fish (Goolish, 1989). However, in previous studies, the effect of treatment was only performed in a relatively short period (acute stressor). According to Yue et al. (2006), the effect of familiarity of the fish is stronger rather than size. Griffiths and Magurran (1997) showed the preference guppy (*Poecilia reticulate*) developed to familiar individuals after they had been placed together for 12 days.

Decreased concentrations of Hb after the 48-days rearing period may be related to the release of immature cells from the haemopoietic tissue into the blood as well as by disturbance of iron metabolism that leads to a decrease in Hb synthesis (Reddy and Bashanihideen, 1989). Additionally, reduction in Hb levels may be due to the alteration in the properties of Hb by lowering their affinity for oxygen, increasing the fragility of erythrocytes dan reducing deformability. Nussey et al. (1995) reported that a decrease in Hb concentration of tilapia indicates that the fish's ability to provide sufficient oxygen to the tissues is restricted considerably; this resulted in the reduction of physical activity. In our study, the reduction in Hb was accompanied by hyperglycemia (Tavares-Dias et al., 2001; Carneiro and Urbinati, 2001) due to cortisol release, which led to increased hepatic glycogenesis (Mommsen et al., 1999) that stimulated the production of glucose (Montero et al., 1999). A decreasing trend in Hb levels was also reported in cutthroat trout (*Oncorhynchus clarki*) stocked at 2998 fish m^{-3} (Wagner et al., 1997). When subjected to the stress of capture and handling, decreased Hb levels, increased plasma cortisol, and glucose levels were observed for ‘tambaqui’ (*Colossoma macropomum*) (Tavares-Dias et al., 2001).

Stress associated rises in plasma cortisol concentrations depressed thyroid activity (Walpita et al., 2007), increased basal metabolic rates, and reallocated energy away from immunity, growth, and reproduction (Wendelaar-Bonga, 1997). Previous studies have revealed the effects of cortisol on thyroid hormone metabolism. Cortisol stimulated the conversion of T_4 to T_3 in brook trout (*Salvelinus fontinalis*) liver in vitro by ORD (the outer ring deiodination, or 5-deiodination)

(Vijayan et al., 1988). However, in our experiment, significantly elevated cortisol concentrations resulted in elevated plasma T_4 concentrations and decreased plasma T_3. T_4 is a tyrosine compound with 4 atoms of iodine, whereas T_3 is a tyrosine compound with 3 atoms of iodine. Most of the thyroid hormones made are T_4 but T_3 is a more active form of thyroid hormones. Decreased plasma T_3 has been reported in Nile tilapia (*Oreochromis niloticus*) (Van der Geyten et al., 1998). Similarly, the hepatic conversion of T_4 to T_3 was also inhibited in rainbow trout (*Oncorhynchus mykiss*) (Brown et al., 1991), which could well represent a mechanism for adaptation by down-regulating energy expenditure away from growth and reproduction toward physiological functions required for coping with the stressor and to restore homeostasis (Schreck, 2000; Wandelaar-Bonga, 1997). Our results suggest that plasma T_4 and cortisol concentrations might follow similar patterns during exposure to the highest stocking density.

However, the negative correlations between plasma cortisol and T_3 concentrations could be due to the interaction of the hypothalamic-pituitary-adrenal (HPA)-axis and hypothalamic-pituitary-thyroid (HPT)-axis in the brain of the fish is impaired under stressful situations (Geven et al., 2006). Elevated cortisol concentrations cause the inhibition of thyroid hormone deiodination. The deiodination involves the enzymatic removal of an iodine atom from the outer (phenolic) ring and the inner (tyrosyl) ring of the iodothyronine molecule. The outer ring deiodination of T_4 is required to yield the most potently bioactive hormone T_3 (Moreno, 2008).

In teleost fish, glucocorticoids have mineralocorticoid properties, which are involved in the regulation of the water-salt metabolism (Evans et al., 2005). Thyroid hormones play an essential function in the making of ionic mechanisms and osmotic homeostasis in fish (Walpita et al., 2007; Arjona et al., 2011). The previous finding by Babitha and Peter (2010) revealed that elevated cortisol concentrations reduced plasma T_3 concentrations and altered the parameters of water-salt, carbohydrate and nitrogen balance in African catfish (*Clarias gariepinus*). This is also evident in our study; high cortisol concentrations decreased plasma T_3, plasma Na^+, K^+ and Cl^- after the 48-day experimental period. High stocking density induces osmotic disturbances that activate the HPI-axis and elevate plasma cortisol. Redding et al. (1991) reported that cortisol is an osmoregulatory hormone released from the interrenal tissue in response to the stress condition.

Responses to stress conditions are controlled by a complex neuro-endocrine system that releases catecholamines (epinephrine, noradrenaline) and cortisol (Urbinati and Carneiro, 2004), both of which are related to the control of ion regulation (McCormick, 2001) and the transport of ions in

freshwater fish (Evans, 2002). Stress causes a significant rise in cortisol, characteristic of the primary stress response, changed secondary and tertiary responses such as elevated plasma glucose levels, and suppressed thyroid hormones, growth hormones and plasma ions (Flodmark et al., 2002). The decreasing concentrations of the plasma ions may be due to the damaged epidermis and loss of the integument integrity, and hence there is an increase in the permeability with loss of the ions or being diffused from water into the fish body, potentially leading to the failure of osmoregulatory and disturbance of acid-base and electrolyte homeostasis (Tripathi et al., 2005). Plasma concentrations of Na^+ of the smaller juveniles were lower than the larger fish, a condition that indicates higher stress in the larger fish. Stress increases the blood flow in gills and the permeability of the epithelium, resulting in ionic losses in freshwater fish (Cech et al., 1996).

Juvenile barramundi of different sizes showed difference responses to higher stocking density. Growth rates and feed conversion ratio of the smaller juvenile were significantly better than the larger juveniles. The results agreed with the previous study of Tran-Duy et al. (2008) and Abdel-Tawwab et al. (2010) who found that the growth performance of the smaller tilapia *(O. niloticus)* was significantly higher than the larger tilapia. Significant alteration in fish growth and feed conversion rate due to differences in fish size or fish weight has been concluded in a number of studies. Booth et al. (2008) stated that growth of Australasian snapper (*Pagrus auratus*) was significantly influenced by fish size. Handeland et al. (2008) reported that fish size significantly affected growth rate and feed conversion rates of Atlantic salmon (*Salmo salar*). Policar et al. (2013) found that small-sized pikeperch (*Sander lucioperca* L) demonstrated higher SGR compared to the larger one. Sun and Chen (2014) confirmed growth rate and feed consumption of cobia (*Rachycentron canadum*) were significantly influenced by fish size. It is observed, survival of the juvenile barramundi was not affected by higher stocking density. In comparison with literature (North et al., 2006), the mortality rate was very low in both systems. Good water quality can partly explain those results. Throughout the experimental period, water quality parameters in IRAS and RAS were maintained within the acceptable limits for the indoor production of barramundi in RAS (Rimmer et al., 1994; Boyd, 1997). Therefore, even though the juvenile of different sizes had a different growth performance, the high survival observed in both systems confirmed the efficacy of duckweed incorporated into barramundi RAS.

7.5 Conclusions

The high stocking density of 21.63 kg m^{-3} is a stress factor for the small and large-sized barramundi juveniles reared in both RAS and IRAS. Differences in the juvenile size also impacted on growth and feed conversion ratio. However, there are no differences in stress responses and the growth performance between the juvenile reared in RAS or IRAS.

CHAPTER 8: Can duckweed (*Lemna minor* Linnaeus) harvested from IRAS be recycled into barramundi (*Lates calcarifer* Bloch) diets as a partial replacement of protein from the fishmeal?[6]

8.1 Introduction

Sustainable growth and intensification of aquaculture worldwide demand large quantities of a higher quality of protein feeds. Fishmeal is considered to be an ideal source of dietary proteins for fish, due to its high content of high-quality proteins, essential amino acids, high content of phospholipids, essential fatty acids, high level of digestibility and palatability (Barlow, 2003). However, the limiting factors in applying fishmeal in the production of aquafeed are high prices, limited resources and variable microbiological quality (Du and Niu, 2003). In the long term, the availability of fishmeal can be a potential threat to the sustainability of the target cultured species that require a high quantity of fishmeal in their feed. Furthermore, due to the increasing demand for fishmeal to be used in aquafeeds, there have been concerns over the reliance on capture fisheries for aquaculture led to overexploitation of certain types of fisheries (Naylor et al., 2000).

The time-series data show a downward trend in the catch of fish for feed since the 1980s (FAO, 2016). The accelerated usage for fishmeal, coupled with decreasing catches in world fisheries, have resulted in significant increases in the use of various alternative protein ingredients for aquafeeds (Tacon and Metian, 2008; Cheng et al., 2013). Due to the importance of low-cost and nutritionally balanced diets for fish, there is increasing research efforts to evaluate the nutritional value of different nonconventional feed resources, including the utilization of aquatic macrophytes or by recycling organic waste or plant by-product (Ansal et al., 2010; Jayathilakan et al., 2012).

In integrated recirculating aquaculture systems (IRAS), organic by-products may be reclaimed, recycled and re-used. This farming system has been proposed as an environmentally sustainable way of recycling unused by-products, especially those produced through the culture of high trophic level fish species which require the supply of exogenous energy (Buschmann, 2000). Reclamation, recycling, and re-use help to solve the problems of by-product disposal while relieving scarcity of resource materials. In this experimental study, duckweed (*Lemna minor* Linnaeus) was used as a producer and biofilter media, the target species, barramundi (*Lates calacrifer* Bloch) as a consumer, and aquatic or substrate attached microorganisms as

[6] This chapter has been ready to be submitted in Fatty Acids

decomposers which form a complete ecosystem (Hu and Zhou, 1989). Barramundi is a carnivorous species, and accepts most forms of frozen and live feed (Tubongbanua, 1987). Barramundi is a highly desired marine fish species for commercial culture due to its high market demand, fast growth, euryhaline nature, and its adaptability to various growing environments (Copland and Grey, 1986).

Plant proteins represent a good option for replacing fishmeal in formulated diets. Previous work has shown that the substitution of fishmeal with plant-based protein is feasible without compromising growth and performance of cultured barramundi (Nandakumar et al., 2017; Tantikitti et al., 2005). Duckweed has been successfully used as a complete feed for fish and shrimp (Bairagi et al., 2002; Flores-Miranda et al., 2015). Duckweed responds with linear increases in biomass yield and crude protein content when fertilized with biodigester effluent (Dang et al., 2011). Furthermore, a reduction of 30% in tilapia feeding costs was obtained by substituting duckweed meal for fishmeal for Nile tilapia (Oreochromis niloticus) (Mohedano et al., 2005). However, apart from previous reports (Flores-Miranda et al., 2015; Nouanthavong and Preston, 2011), there appears to be no information on the potential value of duckweed as a fish feed supplement when co-produced in an IRAS.Duckweed contains 28 to 43 % crude protein, 5 % fiber in dry weight, the high concentration of trace minerals, such as phosphorus and potassium, as well as xanthophylls and carotenes (Chaturvedi et al., 2003).

Duckweed also contains a higher amount of lysine (7.5%) and methionine (2.6%) than other plant feed stuff (Oron et al., 1984; Mishra, 2007). The composition of duckweeds is highly variable and influenced by the nutrient status of their growth media (Ansal et al., 2010). Duckweed grown in nutrient-poor media has lower protein content associated with high fibre, ash and carbohydrate content than that grown in nutrient-rich media (Ansal et al., 2010). However, the presence of fibre and anti-nutritional factors such as tannins and phytic acid in duckweed negatively affects their nutritional value and, consequently, the growth of the cultured species (Bairagi et al., 2002; Kumaraguru Vasagam et al., 2007). To this regard, processing duckweed materials through a simple and cheap method of fermentation may considerably enhance flavour and texture and decrease the anti- nutritional factors and crude fibre content thereby increasing the duckweeds' nutritional values and digestibility (Bairagi et al., 2002; Nout, 2009).

The origin of duckweed could come from various sources, but in this study, the duckweed was used as a by-product from IRAS culturing barramundi. The use of fermented duckweed as one of the ingredients in barramundi diets would reduce the cost of fish production. However, published data on the effects of fermented duckweed on the growth performance and feed utilization of the juvenile barramundi is very limited. Additionally, the relationship between dietary duckweed protein inclusion and fish physiological remains neglected (Patra, 2015; Mohapatra and Patra, 2013). Therefore, duckweed was fermented by a cellulose-degrading fish gut bacterium, *Bacillus* sp. and this study was designed to determine optimum inclusion level of the fermented duckweed in the formulated feed and to evaluate the growth performance, feed utilization, and physiological parameters of juvenile barramundi (*L. calcarifer* Bloch).

8. 2 Materials and methods

8.2.1 Animals

One thousand and two hundred barramundi juveniles with an average weight of 7.53 ± 0.21 g were used in the feeding trials for a period of 63 days in integrated recirculating aquaculture systems (IRAS) at Curtin Aquatic Research Laboratory, Perth, Western Australia. The experimental fish were maintained to ethical and welfare standards (the approval number of AEC-2013-16).

8.2.2. Fermentation of duckweed

Fermentation of duckweed was performed according to the method of Bairagi et al. (2002) with some modifications. *Bacillus sp* used for fermentation of duckweed was isolated from the intestine of marron (*Cherax canii*). Harvested duckweed obtained from the previous experiments with duckweed in IRAS were sundried. The dried duckweed were finely ground and passed through a fine meshed sieve to ensure homogeneity. A portion of sieved duckweed was moistened with 50% w/v liquid basal medium containing (g L^{-1}): KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_4$. $7H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO_4$. $7H_2O$, 0.004 and autoclaved for sterilization. *Bacillus sp* were inoculated at 1 x 10^8 CFU g^{-1} of the dried duckweed for 16 days at 37°C in an incubator.

8.2.3 Diet preparation

Experimental diets were formulated using fermented (F_1 to F_4) duckweed meals at 15%, 25%, 35% and 45% inclusion levels. A diet containing fishmeal as the main protein source was used as the control diet (CD). To each of the formulated diets, 1% chromic oxide was added as digestibility marker. All the diets were prepared in pelleted form using 0.5%

carboxymethylcellulose as a binder. The pellets were sun dried for a few days and crumbled prior to feeding.

8.2.4 Experimental design and rearing conditions

The experiment was conducted in 15 independent IRAS. Barramundi juveniles were transported to Curtin Aquatic Research Laboratory and acclimatized to the laboratory conditions for 21 days and fed with a commercial barramundi diet. The juvenile barramundi were randomly distributed at an optimum stocking density of 15.86 kg m^{-3} (Ardiansyah and Fotedar, 2016b) with three replicates for each treatment. All the fish were fed twice daily at 10.00 and 17.00 h at a fixed feeding rate of 2.5% body weight per day for 63 days. The feeding rate was adjusted every 10 days after weighing the fish. To determine the feed consumption, any left-over feed was collected 6 h after each feeding and weighed after oven drying. The faecal samples were regularly collected in the morning by siphoning, and following the ''immediate pipetting'' method described by Spyridakis et al. (1989), from every tank. The water quality parameters from each tank were monitored each week throughout the experimental period. Water quality parameters were measured in accordance with standard methods for the examination of water and wastewater (APHA, 2005).

8.2.5 Chemical analyses and data collection

Proximate analysis of the experimental diets and faecal samples was performed following the AOAC (2000) procedures as follows: moisture was determined after drying the samples in an oven at 105ºC for 24 h. Crude protein was determined by micro Kjeldahl digestion (N x 6.25) and distillation after acid digestion using a Kjeltec 1026 Distilling Unit together with a Tecator Digestion System (Tecator, Sweden). Ash was determined by incineration at 550ºC for 12 h in a Muffle furnace to constant weight. Lipid was determined by Soxhlet extraction with diethyl ether at 40-60ºC for 7–8 h while crude fibre content was determined as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH. Nitrogen-free extract (NFE) was computed by taking the sum of values for crude protein, crude lipid, crude fibre and moisture and subtracting this from 100 (Maynard et al., 1979). Chromic oxide in the diets and faecal samples was estimated following the method of Bolin et al. (1952).

At the end of the 63 day experiment, five individual fish from each tank were euthanised with 60 mg L^{-1} Aqui-S containing 50% isoeugenol (New Zealand Ltd., Lower Hutt, New Zealand), then oven-dried at 105ºC for 24 h, ground and stored at -20ºC for subsequent analysis. The

whole body was analysed for moisture, crude protein, crude lipid and ash following the aforementioned methods. Tannin concentration in both dried and fermented duckweed was determined using Folin–Denis reagent (Schanderi, 1970). Phytic acid concentration was determined using a spectrophotometric procedure following the method of Vaintraub and Lapteva (1988). The absorbance was measured at 830 nm against a blank. Result was calculated as mg phyctic acid/100 g dry sample using standard phyctic acid, whereas estimation of total free amino acids was conducted according to Moore and Stein (1948) using ninhydrin reagent dissolved in methyl cellosolve.

The fish were weighed at the beginning and at the end of the experiment. Weight gained (WG), specific growth rate (SGR, % day^{-1}), feed conversion ratio (FCR), and protein efficiency ratio (PER) were calculated according to Amaya et al. (2007), whereas apparent protein digestibility (APD, %) and apparent net protein utilization (ANPU, %) were calculated using standard methods (Steffens, 1989).

8.2.6 Physiological parameters

At the end of the 63 day feeding trial, five fish were randomly taken from each tank, anesthetized with 60 mg L^{-1} Aqui-S, containing 50% isoeugenol (New Zealand Ltd., Lower Hutt, New Zealand). The blood samples were collected by heparinised syringe from the caudal vein. The collected blood were immediately centrifuged at 3,000 x *g* for 10 min at 4ºC. Plasma samples were pooled and stored at -80ºC for subsequent analysis of physiological parameters. Plasma glucose, triglyceride, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), and activities of aminotransferase, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured by using commercially available kits (BioSino Bio-technology and Science, Inc., China). All the physiological parameters were determined by using an automatic biochemical analyser (Hitachi 7020, Japan).

8.3 Results

Water quality parameters monitored were similar in all treatments, and the ranges of water quality parameters were: temperature, 21-22ºC; pH, 7.5-8.0; dissolved oxygen, 7.2-7.8 mg L^{-1}; and total ammonia nitrogen, < 5 ppm.

The proximate compositions of feed ingredients and experimental diets are presented in Tables 8.1 and 8.2, respectively. Fermentation of duckweed resulted in a significant increase in crude

protein and a significant decrease in the levels of crude fibre and antinutritional factors, phyctic acid and tannin. A comparison of the proximate composition of the control diet, and duckweed incorporated diets indicated that the crude fibre level in the control diet was 10.82%, whereas, it ranged from 3.92-5.36% in diets containing fermented duckweed. In diets containing fermented duckweed, the tannin and phytic acid contents were below detection limit.

Table 8.1. Proximate composition of dried and fermented duckweed (% dry weight).

Recycled duckweed	**Moisture (%)**	**Crude protein (%)**	**Crude lipid (%)**	**Crude fiber (%)**	**Ash (%)**	**NFE (%)**	**Gross energy (K cal.g^{-1})**
Dried	3.2±0.12^{a}	29.86±0.07^{a}	3.80±0.14^{a}	13.22±0.21^{b}	14.00±0.15^{b}	41.24±0.12^{a}	3.32±0.04^{a}
Fermented	3.4±0.14^{b}	35.43±0.04^{b}	3.92±0.12^{b}	11.85±0.17^{a}	11.86±0.10^{a}	52.23±0.16^{b}	4.06±0.07^{b}

[a] Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$.

.

Table 8.2: Ingredient composition (% dry weight) and proximate analyses of fermented duckweed incorporated feeds (on dry matter basis).

Ingredients	**Experimental diets**				
	Control diet	**F1 (15%)**	**F2 (25%)**	**F3 (35%)**	**F4 (45%)**
Fishmeal	40	37	35	33	31
Duckweed meal	-	15	25	35	45
Soybean meal	22	25	20	15	10
Corn flake	34	19	16	13	10
Fish oil	2	2	2	2	2
Vitamin mineral mix	1	1	1	1	1
Chromic oxide	1	1	1	1	1
Total	100	100	100	100	100
Proximate composition					
Crude protein	35.45	34.63	33.74	32.92	32.17
Crude lipid	8.4	5.10	5.36	5.87	5.88
Crude fibre	11.82	3.92	4.24	4.85	5.36
Ash	10	10	12	12	14
Dry matter	98	96	98	96	95
NFE	32.50	47.30	46.90	40.50	38.70
Gross energy (K cal g^{-1})	4.5	4.2	4.1	3.9	3.8
Phyctic acid	ND	ND	ND	ND	ND
Tannins	ND	ND	ND	ND	ND
Chromic oxide	0.97	1.01	1.01	1.02	1.02

ND= Not detectable

The growth performance and feed utilization of barramundi juveniles in terms of WG, SGR, FCR, PER, ANPU and APD are presented in Table 8.3. The average weight of the juvenile barramundi increased in all the dietary treatments, in comparison to the control diet excepting in diet F4, which contained 45% fermented duckweed. The highest fish WG and SGR were observed in the group of fish reared on diet F2. Fish fed with F3 also showed good performance in terms of WG and SGR. PER was highest in fish fed F3 which was significantly different ($P<$ 0:05) from those reared with other diets. PER value was lowest with the control diet. The FCR value was lowest for fish fed F3 and highest for F4 diet.

Table 8.3. Growth and feed utilization efficiencies in juvenile barramundi fed experimental diets for 63 days.

Parameters	CD	Fermented duckweed meal			
		F1 (15%)	F2 (25%)	F3 (35%)	F4 (45%)
Initial weight (g)	7.53±0.02	7.53±0.02	7.53±0.01	7.53±0.01	7.53±0.02
Final weight (g)	18.24±0.06^{b}	18.68±0.08^{b}	18.84±0.03^{c}	18.82±0.04^{c}	18.01±0.28^{a}
WG (g)	10.71±0.06^{a}	11.15±0.09^{b}	11.31±0.03^{b}	11.28±0.05^{b}	10.58±0.30^{a}
SGR (% day^{-1})	1.40±0.01^{a}	1.44±0.01^{b}	1.46±0.01^{b}	1.45±0.01^{b}	1.39±0.03^{a}
FCR	2.45±0.01^{bc}	2.43±0.01^{b}	2.41±0.01^{a}	2.40±0.01^{a}	2.48±0.03^{c}
PER	1.17±0.01^{a}	1.19±0.01^{a}	1.23±0.01^{b}	1.26±0.01^{c}	1.24±0.01^{b}
APD (%)	80.85±0.01^{a}	83.01±0.02^{b}	85.27±0.01^{c}	86.17±0.02^{c}	85.52±0.01^{c}
ANPU (%)	33.82±0.05^{bc}	25.71±0.07^{b}	44.63±0.04^{c}	40.39±0.02^{c}	12.48±0.02^{a}

[a] Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$.

ANPU was highest in juvenile barramundi reared on diet F2 and lowest with diet F4. Apparent protein digestibility (APD) for all diets were, however, high, ranging from 82.25% to 86.17%. Juvenile barramundi fed diet F3 showed the highest APD value (86.17%). Poor protein digestibility was recorded in fish fed the control diet.

The carcass composition of juvenile barramundi before and after the experiment is presented in Table 8.4. Juvenile barramundi fed diet F4 had a slightly higher percentage of moisture than in fish fed other experimental diets. The deposition of protein in the carcass of juvenile barramundi increased over the initial value in all the dietary treatments. Although all the fish were fed isonitrogenous diets, the greatest accumulation of carcass protein was recorded in the group of fish reared on diet F2, containing 25% fermented duckweed meal. The carcass lipid content varied significantly ($P < 0:05$) among different dietary treatments, in which the highest tissue lipid accumulation occurred in fish fed the control diet. The ash content of fish carcass was highest ($P < 0:05$) in fish diet F3.

Table 8.4. Proximate carcass compositions (% wet weight) of juvenile barramundi at the start and end of the 63-day feeding trial.

Carcass composition	Initial	CD	F1	F2	F3	F4
Crude protein	13.21	16.45±0.15^{c}	15.62±0.04^{b}	17.32±0.03^{d}	16.83±0.12^{cd}	14.25±0.07^{a}
Crude lipid	2.43	2.76±0.11^{c}	2.34±0.04^{b}	2.60±0.04^{bc}	2.28±0.02^{a}	2.18±0.05^{a}
Moisture	76.19	75.31±0.04^{a}	75.29±0.02^{a}	75.87±0.03^{bc}	75.62±0.04^{b}	76.12±0.03^{c}
Ash	4.87	3.59±0.02^{a}	4.17±0.04^{ab}	4.59±0.03^{b}	5.01±0.03^{c}	4.87±0.05^{bc}

[a] Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$.

Glucose, cholesterol, LDL-C, and HDL-C levels were significantly influenced by the dietary treatments (Table 8.5).

Table 8.5. Glucose, cholesterol, LDL-C, HDL-C, ALT, AST and ALP changes of juvenile barramundi in response to dietary fermented duckweed meal inclusion.

Parameters	Control diet	Fermented duckweed inclusion level			
		F1 (15%)	F2 (25%)	F3 (35%)	F4 (45%)
Glucose (mmol L^{-1})	5.40±0.03^{a}	5.52±0.01^{a}	5.93±0.02^{b}	5.97±0.01^{b}	6.21±0.02^{c}
Cholesterol (mmol L^{-1})	5.89±0.02^{b}	6.04±0.03^{b}	4.71±0.01^{a}	4.60±0.03^{a}	4.65±0.01^{a}
Triglyceride (mmol L^{-1})	5.38±0.03	5.01±0.02	4.65±0.03	4.23±0.04	4.07±0.03
LDL-C (mmol L^{-1})	4.35±0.02^{c}	4.33±0.03^{c}	3.91±0.02^{b}	3.72±0.05^{a}	3.79±0.04^{a}
HDL-C (mmol L^{-1})	1.31±0.10^{a}	1.26±0.05^{a}	1.37±0.07^{a}	1.59±0.10^{b}	1.59±0.11^{b}
ALT (U L^{-1})	6.07±0.05^{c}	6.12±0.03^{c}	5.89±0.02^{b}	5.67±0.02^{a}	5.84±0.03^{b}
AST (U L^{-1})	7.98±0.03^{c}	7.91±0.05^{c}	7.35±0.10^{a}	7.39±0.07^{a}	7.71±0.12^{b}
ALP (U L^{-1})	172.43±0.03^{c}	161.52±0.05^{b}	160.82±0.02^{b}	150.24±0.02^{a}	149.57±0.02^{a}

[a] Values are expressed as mean ± SEM. Different letters in the same row indicate significant difference at $P < 0.05$.

Glucose concentration significantly ($P \leq 0.05$) increased as the dietary fermented duckweed meal inclusion increased above 15%. F1 diet, juvenile barramundi fed with F2, F3 and F4 diet had significantly ($P \leq 0.05$) higher glucose levels than the control. The cholesterol level of juvenile barramundi fed with the control diet and F1 diet was significantly ($P \leq 0.05$) higher than that of juvenile barramundi fed with other diets. Triglyceride level significantly ($P \leq 0.05$) decreased with increasing fermented duckweed meal inclusion in the diet. The LDL-C concentration decreased, but the HDL-C concentration gradually increased with increasing dietary fermented duckweed meal inclusion. The HDL-C concentration was significantly ($P \leq 0.05$) higher, whereas the LDL-C concentration was significantly ($P \leq 0.05$) lower in fish fed with F3 and F4 diet than that in fish fed with other experimental diets. Furthermore, activities of plasma ALT and AST of juvenile barramundi fed with the control diet and F1 diet were significantly ($P \leq 0.05$) higher than that of juvenile barramundi fed with other experimental diets. When the substitution level was above 25%, the activities of plasma ALP were significantly ($P \leq 0.05$) higher than the control diet.

8.4 Discussion

Fermented duckweed can be utilized as a feed ingredient into the formulated diets for juvenile barramundi effectively up to 35% level of inclusion without compromising growth performance. WG, SGR, and FCR of the juvenile barramundi were better with 25% and 35% fermented duckweed incorporated diet than other diets, while APD and ANPU of the juvenile barramundi fed with F2 and F3 were better than the control diet. Das and Ray (1989) demonstrated the possibility of incorporation of dried *Lemna polyrhiza* as a feed ingredient for the juvenile Indian major carp (*Labeo rohita*), and observed higher carbohydrate digestibility in relation to that of the control diet although the SGR and FCR in the fish fed 25% and 35% fermented duckweed were not different with the control diet. Shireman and Smith (1983) considered duckweed (*Lemna* sp.) as a highly nutritious feed ingredients for herbivorous fish such as grass carp because of its tenderness and high protein content compared to other aquatic plants. The protein content of dried and fermented duckweed was estimated to be 29.86 and 35.43%, respectively (Table 8.1). However, the presence of antinutritional factors in dried duckweed limits direct use of this floating plant as a dietary ingredient.

Dietary tannin as low as 0.5% causes growth depression in chickens (Vohra et al., 1996) and there are also reports on the toxicity of tannin to fish (Hossain and Jauncy, 1989; Krogdahl, 1989; Mukhopadhayay and Ray, 1999). Tannin and phytic acid could not be detected in

fermented duckweed incorporated diets. Apart from the presence of some antinutritional factors, fibre contents of plant ingredients could also be responsible for their poor digestibility (De Silva et al., 1990; Burel and Kaushik, 2008). High levels of fibre in the diet are known to reduce feed intake (NRC-NAS, 1977; Edwards et al., 1985), which can lead to the growth retardation (Edwards et al., 1985). A possible reason for poor performance of the juvenile barramundi on the control diet and 45% fermented duckweed meal diet may have been its relatively higher level of crude fibre compared to other fermented duckweed incorporated diets. Fibre is basically cellulose, which has less values in the nutrition of carnivorous fish. Fibre content should be restricted to less than 7% of fish diets (Robinson et al., 1981). In comparison to other vertebrates in the evolutionary process, carnivorous fish have rather simple, little-developed digestion system and as a consequence, they have reduced ability to digest carbohydrate as an energy source (Millward, 1989). As dietary fibre is part of the carbohydrate component of plant ingredients, most teleost fish cannot utilize it (Eusebio et al., 2004). Previous studies revealed that increasing the level of dietary lipids above 8% negatively affect growth performance and nutrient utilization, resulting in decreased protein accretion and slower growth rate (Borges et al., 2009).

During fermentation, nutrient losses may occur as a result of leaching, destruction by light, heat or oxygen or as a result of microbial activity (Jones, 1975). Nevertheless, loss of nutrients during fermentation is commonly negligible, and there may be an increase in the nutrient level through microbial synthesis (Wee, 1991). The protein utilization efficiency of fish fed 35% fermented duckweed meal incorporated diets was significantly better than those fed other diets. Edwards (1980) reported better growth of tilapia, *Oreochromis mossambicus*, fed diets containing composted water hyacinth than with conventional tilapia diet, although the latter contained more protein. Ray and Das (1992) also indicated the possibilities of incorporation of composted *Salvinia cuculata* in supplementary diets for the Indian major carp (*L. rohita*) substituting the conventional diet up to 20% level.

The better performance of juvenile barramundi in terms of percentage WG, SGR, FCR, APD, and ANPU was observed in fish fed the 25% fermented duckweed meal diet followed by the 35% fermented duckweed meal diet. Fish fed the diet with 45% fermented duckweed gave the poorest performance in terms of WG and SGR. Since all the diets were isonitrogenous, the reduced growth of the fish fed diets containing higher levels of fermented duckweed meal appeared to be due to increased fibre contents in the diets.

Biochemical variables of plasma blood can be used as indicators to evaluate the health condition of the fish (Rao, 2006; Çelik et al., 2012; Yılmaz and Ergün, 2012). The present study demonstrated that glucose level increased with an increasing dietary level of fermented duckweed meal above 15%. A similar result was observed in cobia (*Rachycentron canadum* L) (Ren et al., 2011). Although, there has been considerable debate about the limited ability of carnivorous teleost fish to utilize dietary carbohydrates efficiently. Altered dietary carbohydrate contents lead to prominent alterations in metabolic enzyme activities in the fish liver (Leung and Woo, 2012). Dietary carbohydrate impaired control of plasma glucose levels, leading to glucose intolerance in such species (Moon, 2001). Nutritional status is a factor that can have an effect on the glucose response. The intake of diets with different lipid and protein content resulted in different responses of blood glucose of the fish (Martínez-Porchas et al., 2009).

Variations in physiological parameters are an indicator of fish responses to their diet (Satheeshkumar et al., 2012). Thus, changes in cholesterol, triglyceride, LDL-C, and HDL-C are related to their composition of diet that it is used as energy for body activities. Cholesterol is a precursor of steroid hormones which has an essential role in the function of nerve fibres, the formation of bile salts, the maintenance of cell membrane structure. Meanwhile, triglycerides are as a reserve source of energy for body metabolism (Hoseini and Ghelichpour, 2012). Cholesterol and triglyceride levels can be used as main indicators of health status of fish because these two parameters are the indicator of liver function particularly lipid metabolism (Zhou et al., 2005; Gul et al., 2011). Plasma cholesterol and triglycerides were lower in the fish fed diets containing 35 and 45% fermented duckweed meal than in fish fed the control diet. Cholesterol and triglyceride levels in plasma were lower with the rising the inclusion of fermented duckweed meal. Romarheim et al. (2006) reported that the plasma cholesterol and triglyceride levels of rainbow trout were significantly reduced when 50% of the fishmeal was replaced by soybean meal. The result indicates that high proportion of fat in the chemical composition of the feed resulted in high cholesterol levels of *L. calcarifer* (Satheeshkumar et al., 2012).

Many plants proteins have been investigated to have cholesterol-lowering effects in fish (Dias et al., 2005; Lim and Lee, 2009). Decreased total cholesterol levels were reported in tiger puffer fed soybean diets (*Takifugu rubripes*) and seabream (*Diplodus vulgaris*) (Lim et al., 2011; Acar et al., 2013). This is due to several compounds in duckweed is capable of binding the bile acids, and inhibiting absorption of the bile acids in the distal intestine or preventing the conversion of the bile acids into secondary bile acids (Romarheim et al., 2006). Decreased triglyceride levels

were also reported in parrot fish (*Oplegnathus fasciatus*), common carp (*Cyprinus carpio*), and seabream (*Diplodus vulgaris*) ((Lim and Lee, 2009; Acar et al., 2013; Moradi et al., 2013). Many aquatic weeds including duckweed have been reported to contain a higher amount of flavonoid and phenolic compounds (Bright and Kanagappan, 2016). Flavonoid compounds are known as reduction factors of cholesterol, triglycerides, and LDL (Zern et al., 2003). Decreased cholesterol, triglyceride and LDL levels of the fish fed with fermented duckweed meals are most likely due to high flavonoid contents of duckweed. Since the liver is the most important site of lipid metabolism, reduction of triglyceride and LDL prevent from the fatty liver syndrome in fish (Hosseinifard et al., 2013).

The digestibility and utilization of nutrient mainly depend on the activities of digestive enzymes in the gastrointestinal tract. The activities of digestive enzymes including ALT and AST were lower in the fish fed with fermented duckweed meal than the control diet, which disagreed with the previously published results regarding digestibility of nutrients (Ajani et al., 2016). The result coincides with results for tilapia (*O. niloticus*) (Lin and Luo, 2011) and Atlantic cod (*Gadus morhua* L.) (Hansen et al., 2007). Elevation of plasma ALT and AST enzymes have been associated with increased cell damages, tissue necrosis, the risk of cardiovascular diseases and myocardial infarction (Alvarez and Mukerjee, 2011). Plasma AST can give information on the damage of organs and in particular of liver cells (Kumar et al., 2010). When liver cells are damaged, aminotransferases including ALT and AST leak into the blood (Gaudet et al., 1975). These results indicate that liver of the juvenile barramundi fed with fermented duckweed meal was healthier than that of the fish fed the control diet. Furthermore, ALP is considered indicative of enterocyte activity and a marker for the intensity of nutrient absorption of fish (Segner et al., 1989; Harpaz and Uni, 1999). In this study, activities of ALP decreased with increasing dietary fermented duckweed meal. These result similar to the findings of Dabrowski et al. (1989) and Krogdahl et al. (2003), who reported that activities of ALP of fish fed soybean meal diet were likely lower than fish fed fishmeal diets due to the lower content of phosphoproteins generally found in vegetable meals compared with fishmeal (Silva et al., 2010). All experimental diets tested resulted in an increase in carcass protein of the juvenile barramundi, while carcass lipid content reduced in F1, F3, and F4 diets.

8.5. Conclusions

The results suggested that fishmeal can be replaced with up to 35% fermented duckweed meal without adversely affecting growth, survival and physiological parameters of the juvenile barramundi. Decreased plasma ALP observed in the fish fed the fermented duckweed meal diets have been attributed to the healthier state of the plasma membranes of these fish compared to those in the control group. Therefore, duckweed besides acting as an efficient biological filter in IRAS can be recycled to remove the entire dependancy of fishmeal to barramundi cultured in the same IRAS.

CHAPTER 9: Summary and General Discussion

9.1 Introduction

The purpose of this chapter is to sum up the discussion that has already been provided in Chapters 3 to 8. The summary leads to main conclusions and finally the chapter provided some recommendations for the future research.

9.2 General discussion

9.2.1 Stocking density, water quality, stress, and growth interactions in barramundi IRAS

Data from the present study provide a comprehensive view of the interaction between physicochemical parameters of water quality, stocking density, biochemical parameters of the stress response, growth, and survival of the juvenile barramundi in IRAS. The results show the incorporation of duckweed into juvenile barramundi RAS is capable of reducing fluctuations in the physicochemical parameters of water quality. During the course of study, the mean concentrations of TAN and nitrite were maintained < 2 and 1 mg L^{-1}, respectively. These concentrations are not harmful to fish growth (Wheaton et al., 1994; Losordo et al., 1998), while the mean concentrations of nitrate were kept < 11 mg L^{-1}. Harvesting duckweed every four days maintained DO levels > 7 mg L^{-1} and generated the maximum TAN and TN uptake efficiency of 94.33 % 60.37%, respectively. Hence the physicochemical parameters of water quality are within the optimum range for the growth of juvenile barramundi (Cheong, 1989; Rimmer et al., 1994; Boyd, 1997; Colt, 2006). Even the water flows were similar to all stocking densities, no deterioration of water quality due to different stocking densities was observed. Oron et al. (1987) reported that even though biological nitrification-denitrification processes have several advantages concerning reliability and feasibility, the use of duckweed as a biofilter medium to remove nitrogenous waste from RAS culturing barramundi is less energy consuming and easier for commercial implementation.

No significant differences in physiological stress parameters were observed between stocking densities of 10.10 kg m^{-3}, 12.98 kg m^{-3}, and 15.86 kg m^{-3}. However, at stocking density up to 18.75 kg m^{-3} resulted in a small increase in cortisol levels, followed by elevated levels of glucose and lactate (Table 9.1). The increase in primary and secondary stress responses was due to the elevated, but acceptable nitrate and total nitrogen concentrations, which lead to changes in the metabolic rates of fish, reduction in food assimilation and retardation of growth rate. In contrast,

when fish of different sizes stocked at the density up to 21.63 kg m^{-3}, elevated cortisol levels were also observed, but considered a mild stress level (Metz et al., 2005). However, the higher stocking density did not suppress SGR of barramundi neither was the survival rate significantly reduced. The results indicated that when water quality parameters were maintained within the acceptable limits, SGR and FCR were also significantly influenced by fish size (Handeland et al., 2008; Policar et al., 2013; Sun and Chen, 2014). These findings agree with the studies of other species such as the Arctic charr (*Salvelinus alpinus*) (Jørgensen et al., 1993) and the rainbow trout (*Oncorhynchus mykiss*) (North et al., 2006) where an increase in SGR with increasing density of up to 120 kg m^{-3} was observed. In contrast, negative effects of stocking density on growth have been reported for European sea bass (*Dicentrarchus labrax*) (Saillant et al., 2003) and Amur sturgeon (*Acipenser schrenckii*) (Li et al., 2012). Apart from the varied responses to increased stocking density in juvenile barramundi, the effect of water quality associated with the higher density might be one of the reasons for the mild stress response. The results were in agreement with Person-Le Ruyet et al. (2008) that feed intake in rainbow trout was significantly affected by water quality, but not by stocking density. As stocking density is an essential factor affecting fish welfare in the farming of commercial fish species, particularly when high densities in confined environments are aimed to increase productivity (Ashley, 2007). The incorporation of duckweed in IRAS culturing barramundi can effectively reduce the fluctuation of water quality parameters, maintain the well-being of the targeted fish species, and reduce stress and the risk of diseases.

Table 9.1. Summary of stocking density, TAN (total ammonia nitrogen), cortisol and growth rate (SGR) of barramundi (*L. calcarifer*) in IRAS.

Stocking density (kg m^{-3})	Mean TAN concentration (mg L^{-1})	Mean nitrite concentration (mg L^{-1})	Cortisol levels (nmol L^{-1})	T_3 levels (nmol L^{-1})	SGR (% day^{-1})
10.10	0.83±0.03	0.23±0.02	8.00 - 9.50	5.00 – 5.15	0.78±0.01
12.98	0.83±0.03	0.25±0.02	8.00 - 9.50	4.93 – 5.14	0.78±0.01
15.86	0.83±0.03	0.26±0.02	8.00 – 9.50	4.91 – 5.14	0.78±0.01
18.75	0.91±0.06	0.26±0.02	9.00 – 11.50	3.95 – 4.19	0.70±0.01
21.63	0.99 – 1.03	0.23-0.25	11.50 – 12.50	4.70 – 5.13	0.94 – 0.98

[a] TAN: total ammonia nitrogen, SGR: specific growth rate, T_3: triiodithyronine

9.2.2 The use of duckweed (*Lemna minor*) harvested from IRAS as a partial replacement of fishmeal in the diets of barramundi (*Lates calcarifer*)

Considering that the treatment of barramundi farming effluent should be geared towards the effective reuse of nutrients, all these factors make duckweed used as biofilter medium is cost-effective for recycling as fish feed. Recycling of domestic sewage in barramundi farming is an effective form of pollution control, which can contribute to cost recovery and provides cheap protein food production. However, the presence of fiber and antinutritional factors in duckweed negatively affect its nutritional value (Flores-Miranda et al., 2015).

Duckweed that has been used as a biofilter medium in the previous experiment was harvested, sundried, and fermented. The results showed a significant increase in crude protein and a significant decrease in the levels of crude fibre and antinutritional factors. Previous studies have been conducted to evaluate the crude protein contents of various aquatic plants (Table 9.2).

Table 9.2. Selected data on crude protein contents of aquatic plants.

Aquatic plants	Crude protein content (DM)	References
Lilly *(Nymphaea lotus)*	20.28%	Shah et al. (2010)
	25.40%	Akmal et al. (2014)
	19.75%	Mohamad et al. (2013)
Water-fringe *(Nymphoides peltata)*	21.87%	Shah et al. (2010)
Water-thyme *(Hydrilla sp)*	17.10%	Shah et al. (2010)
	25.32%	Lal Murari and Pathak (1988)
Water hyacinth *(Eichhornia crassipes)*	11.85%	Akmal et al. (2014)
	15.27%	Okoye et al. (2002)
	16.50%	Mohapatra (2015)
Water spinach (*Ipomoea aquatic*)	17.67%	Umar et al. (2007)
	27.60%	Prak Kea et al. (2003)
	36.30%	Dong et al. (2006)
Phragmites *(Phragmites australis)*	20.12%	Akmal et al. (2014)
Water primrose *(Ludwigia peploides)*	12.11%	Akmal et al. (2014)
Hornwort (*Ceratophyllum sp*)	21.70%	Boyd (1968)
	18.30%	Bailey (1965)
Brazilian waterweed (*Elodea densa*)	20.50%	Boyd (1968)
	16.80%	Bailey (1965)

Hydrotrida caroliniana	9.70%	Boyd (1968)
Dwarf Hairgrass (*Eleocharis acicularis*)	12.50%	Boyd (1968)
Naiad *(Najas guadalupensis)*	22.80%	Boyd (1968)
	8.80% - 14.40%	Boyd and Blackburn (1970)
		Little and Henson (1967)
Water-milfoil (*Myriophyllum sp*)	14.11%	Boyd (1968)
	21.10%	Bailey (1965)
	9.80%	Boyd (1968)
Potamogeton diversifolius	17.30%	Boyd (1968)
Golden-club *(Orontium aquaticum)*	19.80%	Boyd (1968)
Duck-potato *(Sagittaria latifolia)*	17.10%	Boyd (1968)
	10.00%	Justice et al. (2005)
	20.00%	Fredrickson and Reid (1988)
Smartweed (*Polygonum sp)*	11.90%	Boyd (1968)
	24.00%	Marten and Andersen (1975)
Water canna *(Thalia dealbata)*	5.23% - 16.06%	Abarike et al. (2014)

El-Sayed (2003) reported that fermentation is required when 20% of water hyacinth (*Eichhornia crassipes*) is incorporated into Nile tilapia (*Orechromomis niloticus*) diets. Similarly, Bairagi et al. (2002) concluded that fermented duckweed meal was better than dried duckweed for the Indian carp (*Labeo rohita*). When the fermented duckweed was incorporated into the barramundi feed formulation, the results suggested that growth performances and physiological parameters of juvenile barramundi fed diets containing up to 35% fermented duckweed were comparable to that of the fish fed the fishmeal diet. The inclusion level of fermented duckweed into the formulated feed for the juvenile barramundi is greater than previous level reported in common carp (*Cyprinus carpio*) (Yilmaz et al., 2004; Mohapatra and Patra, 2013), in the Indian major carp (*Labeo rohita*) (Bairagi et al., 2002), and in the Pacific white shrimp (*Litopenaeus vannamei*) (Flores-Miranda et al., 2015). The study suggested that fishmeal could not be totally replaced with duckweed; however, partial replacement can be done by using fermented duckweed meal to reduce the cost without affecting growth rate. The present study revealed that 35% fermented duckweed meal can be incorporated into the formulated feed of barramundi. Further, duckweed based feeds are cheaper as compared to the conventional feeds, supplementation of duckweed in barramundi diets would also be economically viable.

However, optimal health and disease resistance of fish depend not only on the optimal balance of nutrients available, but also on the optimal functioning of the gastrointestinal tract and related organs (Hemre et al., 2009). Digestive apparatus is forced to adjust to the changing composition of the diet, and the mucosal defense system, which is provided by the gastrointestinal tract should protect the fish's body from harmful agents and at the same time establish oral tolerance to dietary antigens. Thus, the long-term implications dried or fermented duckweed may have on fish production, health, and product quality are largely unknown.

9.2.3 Duckweed (*L. minor*), heterotrophic, and phosphate solubilising bacteria interactions in IRAS

As a biofilter medium, duckweed was used as attachment substrates of a consortium of bacteria immobilized on the surface of the substrate. Different harvesting frequency of duckweed significantly influenced the abundance and diversity of microorganisms on the duckweed biofilter tank. A diverse microbial community that presents on the duckweed biofilter tank is responsible for heterotrophic nitrification and also phosphate solubilisation in IRAS. The

heterotrophic bacteria is critical for nitrogenous waste removal while PSB benefits for enhancing phosphate solubilisation in IRAS.

Data showed that genera *Bacillus* and *Pseudomonas* are the most common heterotrophic bacteria found in the biofilter tank. Genus Bacillus consisted of *Bacillus licheniformis* and *B. subtilis*, whereas *Pseudomonas* was represented by *Pseudomonas stutzeri* and *P. mendocina*. Previous findings reported that the heterotrophic bacterial community is basically dominated by Alphaproteobacteria and Gammaproteobacteria (Tal et al., 2003; Sugita et al., 2005; Wietz et al., 2009). A study was conducted on the experimental RAS concluded that the majority of the heterotrophic bacterial population that grew on the biofilters of RAS were from Bergey's group IV such as *Pseudomonas* (Leonard et al., 2000). Meanwhile, populations of heterotrophic denitrifying bacteria in a marine RAS contained a representative of the Firmicutes bacteria, such as genus *Bacillus* (Michaud et al., 2009). Among others bacteria found in biofilter media of freshwater RAS, *Pseudomonas, Acinetobacter, Flavobacterium, Flectobacillus,* and *Bacillus* dominated the microbial communities (Sugita et al., 2005; Itoi et al., 2007). Denitrifying *Pseudomonas* sp. appear to be the most abundant Gammaproteobacteria in marine RAS filters, with strains related to *P. stutzeri* and *P. fluorescence* (Leonard et al., 2000; Borges et al., 2008; Michaud et al., 2009), while *B. licheniformis* and *B. subtilis* have been used to maintain water quality parameters for intensive rearing of carp (*Cyprinus carpio* L) (Bocioc et al., 2015).

Different harvest frequency of duckweed also affected SGR and biomass harvest of duckweed in the biofilter tank. This is probably associated with the abundance and diversity of PSB that promotes duckweed growth by supplying it with soluble P in the biofilter tank (Yu et al., 2011). PSB may ensure availability of soluble phosphate by solubilising the insoluble phosphate, which is normally unavailable to this aquatic weed (Yadav et al., 2011). The present study showed that the PSB bacteria in the biofilter tank were dominated by the genera *Bacillus* and *Pseudomonas*. Genus *Bacillus* was represented by three species, *B. cereus, B. licheniformis*, and *B. subtilis*, while Pseudomonas consisted of two species, *P. fluorescence,* and *P. mendocina*. Rodriguez and Fraga (1999) stated that the insoluble inorganic compounds of phosphorus could be converted into plant usable forms. Acid production by certain microorganisms is indeed involved in phosphates solubilisation. The main strains that able to perform this conversion belong to the genera *Pseudomonas, Bacillus, Micrococcus, Mycobacterium, Achromobacter, Agrobacterium, Erwinia, Burkholderia, Flavobacterium, Rhizobium, Mesorhizobium, and Sinorhizobium*.

The results of the present study demonstrated that most microbial communities found on the duckweed biofilter not only get involved in heterotrophic nitrification, but also in phosphate solubilisation in IRAS (Table 9.3), indicating that several bacteria may take part in both processes, enhancing solubility of the phosphate as well as supporting the removal of nitrogenous metabolites from IRAS. Those bacteria are *B. licheniformis, B. subtilis, P. mendocina, Chrysobacterium indologenes, Azotobacter vinelandii, Acinetobacter calcoaceticus,* and *Achromobacter xylosoxidans*. The results of the present study suggested that PSB and heterotrophic bacteria may have synergistic effects on the growth of duckweed. The PSB may have a direct consequence on the growth of duckweed other than the mechanism of phosphate solubilisation, for example through the production of phytohormones (IAA), biological nitrogen fixation, enhance the availability of other trace elements, increased iron nutrition through iron-chelating siderophores, and volatile compounds that affects the plant signalling pathways (Gyaneshwar et al., 2002; Hariprasad and Niranjana, 2009).

The improvement in growth and biomass of duckweed by using PSB and heterotrophic bacteria may be because the PSB could change the composition of duckweed root secretion and plasticity, which in turn may affect the colonization and development of other bacteria including heterotrophic bacteria. The mix of PSB and heterotrophic bacteria provide more balanced nutrition for duckweed with the additional supply of N and P to duckweed in addition to growth-promoting substances generated by these organisms. Another reason is the interactions between PSB and heterotrophic bacteria result in a synergistic effect that allows for the exploitation of poorly soluble P sources (Yu et al., 2011).

Table 9.3. Activities associated with the duckweed biofilters and participating microorganisms.

Process	Microorganisms
Heterotrophic nitrification	1. *Bacillus licheniformis* 2. *Pseudomonas stutzeri* 3. *Aeromonas salmonicida* 4. *Chryseobacterium indologenes* 5. *Bacillus subtilis* 6. *Pseudomonas mendocina* 7. *Azotobacter vinelandii* 8. *Achromobacter xylosoxidans* 9. *Acinetobacter calcoaceticus*
Phosphate solubilisation	*1. Bacillus cereus* *2. B. licheniformis* *3. B. subtilis* *4. Chrysobacterium indologenes* *5. Pseudomonas fluorescence* *6. P. mendocina* *7. Micrococcus luteus* *8. Azotobacter vinelandii,* *9. Acinetobacter calcoaceticus* *10. Achromobacter xylosoxidans*.
Heterotrophic nitrification and Phosphate solubilisation	*1. Bacillus licheniformis* *2. Bacillus subtilis* *3. Pseudomonas mendocina* *4. Chrysobacterium indologenes* *5. Azotobacter vinelandii* *6. Acinetobacter calcoaceticus* *7. Achromobacter xylosoxidans*

This study revealed that different harvesting frequency of duckweed not only affects the abundance and diversity of both heterotrophic and phosphate solubilising bacteria that play essential roles in removing nitrogen and phosphorus from the fish rearing tanks, but also support the growth rate and biomass increase of duckweed, which in turn enhances nutrient uptake rates by duckweed from barramundi IRAS.

9.3 Conclusions

The conclusions have been summarised and stated in the previous chapter from chapter 3 to 9. However, the following list is the summary of conclusions drawn:

1. Duckweed's integration into barramundi juvenile RAS can maintain optimum water quality for barramundi growth and survival due to its high bioremediation efficiency and assimilative capacity.

2. Harvesting duckweed every four days resulted in higher TAN removal efficiency from barramundi juvenile RAS.
3. Duckweed biofilters are suitable substrates for the attachment, survival, and growth of PSB and heterotrophic bacteria.
4. Some bacteria may involve in both heterotrophic nitrification and phosphate solubilisation.
5. A 4-day harvest frequency showed stronger correlations between duckweed SGR and biomass harvest with the PSB and heterotrophic bacteria diversity in the biofilter tank.
6. A 4-day harvest frequency maintained an optimum biomass of duckweed.
7. The carrying capacity of barramundi IRAS may be affected by the capacity of duckweed compartment.
8. Under normal aeration, the stocking density of up to 18.75 kg m^{-3} resulted in stressed fish, as indicated by the increase of plasma cortisol and total T_4, and the decrease of total T_3.
9. A higher stocking density of 18.75 kg m^{-3} may be contributory factors in the increase of FCR and the growth suppression of the target species.
10. The stocking density of 21.63 kg m^{-3} affected the physiological stress responses in a differential manner according to fish size. However, no differences were resulted from different rearing systems, RAS and IRAS.
11. Fish meal can be replaced with up to 35% fermented duckweed meal without compensating growth, survival and adverse physiological responses of the juvenile barramundi.

9.4 Limitations of IRAS

The following are some limitations of the study:

1. This research was conducted on a small scale, making it hard to extrapolate the size of the barramundi. Therefore, a higher scale (commercial) research should be conducted in order to evaluate the value of integrating duckweed with barramundi in IRAS.
2. The possibility of infection; high nitrogenous waste products excreted from the cultured species through gill diffusion, as well as urine, and feces, may result in the potential exists for a serious disease outbreak. The bacterial fish pathogens may attach and subsist in duckweed. When duckweed is harvested and used as an ingredient for the formulated feed, the bacterial pathogens transmitted from duckweed to the fish.

9.5 Recommendations

Based on current findings, following recommendations are made regarding further research and usage of duckweed in the fish farming system:

1. To determine the effectiveness of using duckweed as biofilter media in juvenile barramundi RAS, water quality parameters should be monitored for 24 hours throughout the study.
2. Quantification of nitrogen removal rates of heterotrophic bacteria should be conducted in order to evaluate duckweed efficiency as substrates for attachment and growth of the heterotrophic bacteria.
3. Further study should be conducted to see the ability of some bacteria in both nitrogen removal and phosphate solubilisation in the RAS and IRAS.
4. A comparative assessment of IRAS and RAS should be evaluated at the same time and incorporated all parameters such as growth indices, immune competence, and antioxidant enzymes activity.
5. Physiological stress responses of small size barramundi are different with large-size barramundi.
6. The stocking density of juvenile barramundi in IRAS should be less than 18.75 kg m^{-3} However, rearing juvenile barramundi in IRAS is as effective as RAS.
7. 35% fermented duckweed meals in the diet should be supplemented to maintain higher growth performance and health of juvenile barramundi.

References

Abarike, E.D., E. Amenyogbe, E.H. Alhassan, D.N. Akongyuure, S.A. Atindaana, A. Ampofo-Yeboah, and S.M. Abobi. 2014. Nutritional composition of Thalia dealbata in Banka dam in the tolon district of the northern region of Ghana. Proceedings of the 32nd biennial conference of the Ghana Animal Science Association.

Abdel-Tawwab, M., M.H. Ahmad, Y.A.E. Khattab, and A.M.E. Shalaby. 2010. Effect of dietary protein level, initial body weight, and their interaction on the growth, feed utilization, and physiological alterations of Nile tilapia (*Oreochromis niloticus* L.). *Aquaculture* 298:267-274.

Acar, U., A. Türker, M. Bulut, Ö. Yıldırım, S. Yılmaz., and O.S. Kesbiç. 2013. The effect of dietary soybean meal on growth, nutrient utilization, body composition and some serum biochemistry variables of two banded seabream, *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817). *Iranian J. Fisheries Sci*. 12(4): 749-758.

Afzal, A., and A. Bano. 2008. Rhizobium and phosphate solubilising bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum*). *Int. J. Agri. Biol. 10*: 85-88.

Ahamad, M.U., M.R.S., Swapon, T.V. Yeasmin, M.S. Raham, and M.S. Ali. 2003. Repalcement of sesame oiul cake by duckweed (*Lemna minor*) in broiler diet. *Pak. J. Bio. Sci.* 6: 1450-1453.

Ahemad, M., and M.S. Khan. 2012. Effect of fungicides on plant growth promoting activities of phosphate solubilising *Pseudomonas putida* isolated from mustard (*Brassica campestris*) rhizosphere. *Chemosphere* 86: 945-950.

Ahemad, M., A. Zaidi, M.S. Khan, and M. Oves. 2009. Biological importance of phosphorus and phosphate solubilising microorganisms-an overview. In: Khan, M.S., Zaidi, A. (eds). *Phosphate solubilising microbes for crop improvement*. Nova, New York, pp 1-4.

Ajani, E.K., O. Orisasona, B.O. Omitoyin, and E.F. Osho. 2016. Total Replacement of Fishmeal by Soybean Meal with or Without Methionine Fortification in the Diets of Nile Tilapia, Oreochromis niloticus. *J. Fish Aquatic Sci.* 11: 238-243.

Akinbile, C.O., and M.S. Yusoff. 2011. Water hyacinth (*Eichhornia crassipes*) and lettuce (*Pistia stratiotes*) effectiveness in aquaculture wastewater treatment. *Int. J. Phytorem.* 14: 201-211.

Akiyama, D.M., and A.M. Anggawati. 1999 Polyculture of shrimp and tilapia in East Java. American Sybean Association (ASA), Technical Bulletin AQ 47.

Akmal, M., M. Hafeez-ur-rehman, S. Ullah, N. Younus, K.J. Khan, and M. Qayyum. 2014. Nutritive value of aquatic plants of Head Baloki on Ravi River, Pakistan. *Int. J. Biosciences*, 115-122.

Alam, S., S. Khalil, N. Ayub, and M. Rashid. 2002. In vitro solubilization of inorganic phosphate by phosphate solubilising microorganism (PSM) from maize rhizosphere. *Int J Agric Biol* 4: 454-458.

Alaerts, G.J., M.D.R. Mahbubar, and P. Kelderman. 1996. Performance analysis of a full-scale duckweed-covered sewage lagoon. *Water Res*. 30: 843-852.

Alex-Díaz, C., G. Víctor-Atencio, and C. Sandra-Pardo. 2010. Assessment of an artificial free-flow wetland system with water hyacinth (*Eichhornia crassipes*) for treating fish farming effluents. *Rev. Colomb. Cienc Pecu.* 27: 202-210.

Al-Kindi, A.Y.A., J.A. Brown, C.P. Waring, and J.E. Collins. 1996. Endocrine, osmoregulatory, respiratoty and hematological parameters in flounder exposed to water solublefraction of crude oil. *J. Fish Biol.* 49: 1291-1305.

Allen, K.O. 1974. Effects of stocking density and water exchange rate on growth and survival of channel catfish *Ictalurus punctatus* (Rafinesque) in circular tanks. *Aquaculture* 4: 29-39.

Al-Nozaily, F., G. Alaerts, and S. Veenstra. 2000. Performance of duckweed-covered sewage lagoon. 2. Nitrogen and phosphorus balance and plant productivity. *Water Res.* 34: 2734-2741.

Alvarez, A.M., and D. Mukerjee. 2011. Liver abnormalities in cardiac diseases and heart failure. *Int J. Angiol.* 20 (3): 135-42.

Amirkolaie, A.K. 2011. Reduction in the environmental impact of waste discharged by fish farms through feed and feeding. *Rev Aquacult*. 3: 19-26.

Anand, K., B. Kumar, and M.A. Mallick. 2016. Phosphate solubilising microbes: an effective and alternative approach as biofertilizers. *Int J Pharm Pharm Sci* 8: 37-40.

Anderson, K.E., Z. Lowman, A.M. Stomp, and J. Chang. 2011. Duckweed as a feed ingredient in laying hen diets and its effect on egg production and composition. Int. *J. Poult. Sci.* 10: 4-7.

Ansal, M.D., A. Dhawan, and V.I. Kaur. 2010. Duckweed based bio-remediation of village ponds: An ecologically and economically viable integrated approach for rural development through aquaculture. *Livest. Res. Rural Dev.* 22, 7.

AOAC. 2000. Official Methods of Analysis, 17th ed. AOAC International. Gaithersburg, MD.

Aoi, Y., T. Miyoshi, T. Okamoto, S. Tsuneda, A. Hirata, A. Kitayama, and T. Nagamune. 2000. Microbial ecology of nitrifying bacteria in wastewater treatment process examined by Fluorescence In Situ Hybridization. J. Biosci. Bioeng. 90: 234-240.

APHA (American Public Health Association). 2005. Standard Methods for the Examination of Water and Wastewater. Washington, DC.

Aquilanti, L., F. Favili, and F. Clementi. 2004. Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. *Soil Biol Biochem* 36: 1475-1483.

Ardiansyah, and R. Fotedar. 2016a. The abundance and diversity of heterotrophic bacteria as a function of harvesting frequency of duckweed (*Lemna minor* L.) in recirculating aquaculture systems. *Lett. Appl. Microbiol*. 63: 53-59.

Ardiansyah, and R. Fotedar. 2016b. Water quality, growth and stress responses of juvenile barramundi (*Lates calcarifer* Bloch), reared at four different densities in integrated recirculating aquaculture systems. *Aquaculture* 458: 113-120.

Arjona, F.J., L. Vargas-Chacoff, M.P.M. Del-Rio, G. Flik, J.M. Mancera, and P.H.M. Klaren. 2011. Effects of cortisol and thyroid hormone on peripheral outer ring deiodination and osmoregulatory parameters in the Senegalese sole (*Solea senegalensis*). J. Endocrinol. 208: 323-330.

Ashley, P. 2007. Fish welfare: current issues in aquaculture. *Appl. Anim. Behav. Sci.* 104: 199-235.

Avnimelech, Y., S. Mokacly, and G.L. Schoroder. 1989. Circulated ponds as efficient bioreactors for single cell protein production. *Isr. J Aquac.-Bamidgeh* 41: 58- 66.

Awuah, E., M. Oppong-Peprah, H.J. Lubberding, and H.J. Gijzen. 2004. Comparative performance studies of water lettuce, duckweed and algal-based stabilization ponds using low-strength sewage. *J. Toxicol. Environ. Health-Part A*. 67: 1727-1739.

Azza, N.G.T., F. Kansiime, M. Nalubega, and P. Denny. 2000. Differential permeability of papyrus and Miscanthidium root mat in Nakivubo swamp, Uganda. *Aquat. Bot.* 67: 169-178.

Azcón-Aguilar, C., and J.M. Barea. 1995. Saprophytic growth of arbuscular–mycorrhizal fungi. In: Hock B, Varma A, eds. Mycorrhiza structure, function, molecular biology and biotechnology. Heidelberg, Germany: Springer-Verlag, 391–407.

Babitha, G.S., and M.C.S. Peter. 2010. Cortisol promotes and integrates the osmotic competence of the organs in North African catfish (Clarias gariepinus Burchell): evidence from in vivo and in situ approaches. *Gen. Comp. Endocrinol*. 168: 14-21.

Badiola, M., D. Mendiola, and J. Bostock. 2012. Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. *Aquacult Eng*. 51: 26-35.

Bagyaraj, D.J., P.U. Krishnaraj, and S.P.S. Khanuja. 2000. Mineral phosphate solubilization: agronomic implication, mechanism and molecular genetics. *Proc. Indian natn. Sci. Acad.* 66: 69-82.

Bailey, T.A. 1965. Commercial possibilities of dehydrated aquatic plants. Proc. Annu. Meet. South. *Weed. Sci. Soc.* 18:543–51.

Bais, H.P., R. Fall, and J.M. Vivanco. 2004. Biocontrol of Bacillus subtilis against infection of Arabidopsis roots by Pseudomonas syringae is facilitated by biofilm formation and surfactant production. *Plant Physiol* 134: 307-319.

Bairagi, A., K. Sarkar Ghosh, S.K. Sen, and A.K. Ray. 2002. Duckweed (*Lemna polyrhiza*) leaf meal as a source of feedstuff in formulated diets for rohu (*Labeo rohita* Ham.) fingerlings after fermentation with a fish intestinal bacterium. *Bioresour Technol* 85: 17-24.

Baker, F.J., and R.E. Silverton. 1976. Introduction to Medical Laboratory Technology Butterworth London, UK.

Balm, P.H.M., J.D.G. Lambert, and S.E. Wendelaar- Bonga. 1999. Corticosteroid biosynthesis in the inerrenal cells of the teleost fish, *Onchyromis mossambicus*. *Gen. Compar. Endocrinol.* 76: 53-62.

Barak, Y., and J. Van Rijn. 2000. Biological phosphate removal in a prototype recirculating aquaculture treatment system. *Aquacult Eng* 22: 121-136.

Barcellos, L.J.G., S. Nicolaiewsky, S.M.G. de Souza, and F. Lulhier. 1999. Plasmatic levels of cortisol in response to acute stress in Nile tilapia, previously exposed to chonic stress. *Aquacult. Res.* 30: 437-444.

Barcellos, L.J.G., L.C. Kreutza, R.M. Quevedob, I. Fiorezec, L. Cericatod, A.B. Sosod, M. Fagundesd, J. Conradd, R.K. Baldisserad, A. Bruschid, and F. Ritterd. 2004. Nursery rearing of jundiá, Rhamdia quelen (Quoy & Gaimard) in cages: cage type, stocking density and stress response to confinement. *Aquaculture* 232: 383-394.

Barcellos, L.J.G., L.C. Kreutz, G. Koakoski, T.A. Oliveira, J.G. Santos da Rosa, and M. Fagundes. 2012. Fish age, instead of weight and size, as a determining factor for time course differences in cortisol response to stress. *Physiol. Behavior* 107: 397–400.

Barg, U.C. 1992. Guidelines for the promotion of environmental management of coastal aquaculture development. FAO FisheriesTechnical Paper No. 328. Rome, FAO. 122p.

Barik, S.K. and C.S. Purushothaman. 1998. Proceedings of National Symposium on Frontiers in Applied Environmental Microbiology, 11-13 Dec, Cochin, 1998, pp. 165-170.

Barlow, S.M. 2003. Encyclopedia of food sciences and nutrition (second edition). Fish meal. Food Technology and Nutrition, Academic Press, pp. 2486-2491.

Barry, T.P., A.F. Lapp, T.B. Kayes, and J.A. Malison. 1993. Validation of microtitre plate ELISA for measuring cortisolin fish and comparision of stress response of rainbow rout, *Oncorhynchus mykiss* and lake trout, *Salvelinus namaycush*. *Aquaculture* 117: 351-363.

Barton, B.A. 1988. Endocrine and metabolic responses of fish to stress. In: Proc. 19th Annual Conference of International Association of Aquatic Animal Medicine, Orlando, Florida. 19: 41-55.

Barton, B.A., and G.K. Iwama. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish. Dis.* 1: 3-36.

Barton, B.A. 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol* 42: 517-525.

Bayunova, L., I. Barannikova, and T. Semenkova. 2002. Sturgeon stress reactions in aquaculture. *J. Appl. Ichthyol*. 18: 397-404.

Begg, K., and N.W. Pankhurst. 2004. Endocrine and metabolic responses to stress in a laboratory population of the tropical damselfish Acanthochromis polyacanthus. *J. Fish Biol.* 64, 133-145.

Benjawan, L., and T. Koottatep. 2007. Nitrogen removes in recirculation duckweed ponds system. *Water Sci. Technol*. 55 (11): 103–110.

Bergmann, B.A., J. Cheng, J. Classen, and A.M. Stomp. 2000. Nutrient removal from swine lagoon effluent by duckweed. *Trans. ASAE.* 42: 263-269.

Bjerknes, V. 2007. Vannkvalitet og smoltproduksjon (first ed.). Norway: Juul forlag.

Björnsson, B., and S.R. Ólafsdóttir. 2006. Effects of water quality and stocking density on growth performance of juvenile cod (*Gadus morhua* L). *ICES J. Mar. Sci.* 63: 326-334.

Blancheton, J.P., C. De la Pomelie, and M. Vincent. 1996. Potential gains through new rearing technologies: culture in recirculation systems. Seabass and seabream culture: problems and prospects. In: Chatain, B., Saroglia, M., Sweetman, J., Lavens, P. (Eds.), International Workshop on Seabass and Seabream Culture, Verona, Italy, October 16-18, 1996. European Aquaculture Society, Oostende, pp. 189-205.

Blancheton, J.P., K.J.K. Atramadal, L. Michaud, E. Roque d'Orbcastel, and O. Vadstein. 2013. Insight into bacterial population in aquaculture systems and its implication. A*quacult. Eng*. 53: 30-39.

Blanton, M.L., and J.L. Specker. 2007. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. *Crit. Rev. Toxicol.* 37: 97-115.

Bocioc, E., V. Cristea, N. Patriche, I. Grecu, S. Placintă, M.C. Mocanu, I. Vasilean. 2015. The effect of temperature on carp (*Cyprinus Carpio*, L., 1758) microbiota, reared in intensive conditions, by using the bioplus® 2b probiotic. *Anim. Sci. Biotechnol*. 48 (1): 234-238.

Bolin, D.W., R.P. King, and E.W. Klosterman. 1952. A simplified method for the determination of chromic oxide (Cr_2O_3) when used as a index substance. *Science* 116, 634–635.

Booth, M.A., B.J. Tucker, G.L. Allan, and D. Stewart-Fielder. 2008. Effect of feeding regime and fish size on weight gain, feed intake and gastric evacuation in juvenile Australian snapper (*Pagrus auratus*). *Aquaculture* 282:104-110.

Borges, M.T., A. Sousa, P. De Marco, A. Matos, P. Honigova, and P.M. Castro. 2008. Aerobic and anoxic growth and nitrate removal capacity of a marine denitrifying bacterium isolated from a recirculation aquaculture system. *Microb Ecol* 55: 107-118.

Borges, P., B. Oliveira, S. Casal, J. Dias, and L. Conceição. 2009. Dietary lipid level affects growth performance and nutrient utilisation of Senegalese sole (*Solea senegalensis*) juveniles. *Br. J. Nutr*. 102: 1007-1014.

Botella, M.A., A. Cerda, V. Martinez, and S.H. Lips. 1994. Nitrate and ammonium uptake by wheat seedlings as affected by salinity and light. *J. Plant Nutr.* 17: 839-850.

Boyd C. E. 1997. Practical aspects of chemistry in pond aquaculture. *Prog. Fish Cult.* 59 (2): 85-93.

Boyd, C.E., and R.D. Blackburn. 1970. Seasonal changes in the proximate composition of some common aquatic weeds. *Hyacinth Control J.* (In press).

Boyd, C.E. 1968. Fresh water plants: a potential source of protein. *Econ. Bot*. 22:359–368.

Braun, N., R.L. Lima, B. Baldisserotto, A.L. Dafre, and A.P.O. Nuñer. 2010. Growth, biochemical and physiological responses of Salminus brasiliensis with different stocking densities and handling. *Aquaculture* 301: 22-30.

Briggs, M.R.P., and S.J. Funge-Smith. 1994. A nutrient budget of some intensive marine shrimp ponds in Thailand. *Aquacult. Fish Manage.* 5: 89-811.
Bright, K., and M. Kanagappan. 2016. In vitro antioxidant activity of selected aquatic weeds of kanyakumari district of South India. *World J. Pharma. Pharmaceutical Sci.* 5 (6): 1090-1108.
Brown, S.B., D.L. MacLatchy, T.J. Hara, and J.G. Eales. 1991. Effects of cortisol on aspects of 3,5,3-triiodo-L-thyronine metabolism in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol*. 81: 207-216.
Brown, W.Y., M. Choct, J.R. Pluske. 2013. Duckweed (*Landoltia punctata*) in dog diets decreases digestibility but improves stool consistency. *Anim. Prod. Sci.* 53 (11): 1188-1194.
Brzeski, V., and G. Newkirk. 1997. Integrated coastal food production systems - a review of current literature. *Ocean Coast. Manage.* 34: 66-71.
Burel, C., and S.J. Kaushik. 2008. Use of rapeseed/canola in diets of aquaculture species. In: Lim C, Webster CD, Lee C, eds. Alternative protein sources in aquaculture diets. New York: Haworth Press, Taylor & Francis Group, pp 343-403.
Burford, M.A., P.J. Thompson, R.P. McIntosh, and D.C. Pearson. 2003. Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. *Aquaculture* 219 (1-4): 393-411.
Buschamann, A.H., O.A. Mora, P. Gomez, M. Bottger, S. Buitana, C. Retamales, P.A. Vergara, and A. Guitterez. 1994. Gracilaria tank cultivation in Chile: use of land based salmon culture effluents. *Aquac. Eng.* 13: 283-300.
Buschmann, A.H., M. Troell, N. Kautsky, and L. Kautsky. 1996. Integrated tank cultivation of salmonids and *Gracilaria chilensis* (Gracilariales Rhodophyta). Hydrobiologia 326/327: 75-82.
Buschmann, A.H. 2000. Integrated cultivation of salmon and seaweed: Productive, Environmental and Economic issues. *Aquaculture Canada* 2000. Abstracts.
Buschmann, A.H., D. López, and M. González. 2000. Cultivo integrado de moluscos y macroalgas en líneas flotantes y en estanques. In: Faranda, F.M., Albertini, R., Correa, J.A. (Eds.), Manejo Sustentable de los Recursos Marinos Bentónicos en Chile Centro-Sur: Segundo Informe de Avance. Pontificia Universidad Católica de Chile, Santiago, pp. 7–16.
Buschmann, A., M. Troell, and N. Kautsky. 2001. Integrated algal farming: a review. *Cah Biol Mar* 43:615–655.
Caicedo, J.R., N.P. Van Der Steen, O. Arce, and H.J. Gijzen. 2000. Effect of total ammonia nitrogen concentration and pH on growth rates of duckweed (*Spirodella polyrrhiza*). *Water Res* 34: 3829-3835.
Campbell, T.W. 2004. Blood biochemistry in lower vertebrates. In: 55th Annual Meeting of the American College of Veterinary Pathologists (ACVP) and 39th Annual Meeting of American Society of Clinical Pathology (ASVCP), ACVP and ASVPC (eds.).
Carey, J.B., and S.D. McCormick. 1998. Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr. *Aquaculture* 168:237-253.
Carneiro, P.C.F., and C. Urbinati. 2001. Salt as a stress response mitigator of matrinxã *Brycon cephalus* (Teleostei: Characidae) during transport. *Aquacult. Res*. 32: 297-304.
Carrasco, G.A., and L.D. Van de Kar. 2003. Neuroendocrine pharmacology of stress. *Eur J Pharmacol*. 463: 235-72.
Carter, C.G., B. Glencross, R. Katersky, and M. Bermudes. 2010. Chapter 14. The Snooks (Family Centropomidae).
Carvalho, G., P.C. Lemos, A. Oehmen, and M.A.M. Reis. 2007. Denitrifying

Phosphorus Removal: Linking the process performance with the microbial community structure. *Water Res.* 41: 4383-4396.

Cech, J.J., S.D. Bartholow, P.S. Young, and T.E. Hopkins. 1996. Striped bass exercise and handling stress in freshwater: Physiological responses to recovery environment. *Trans. Am. Fish. Soc.* 125: 308-320.

Cedergen, N., and T.V. Madsen. 2002. Nitrogen uptake by the floating macrophyte *Lemna minor*. *New Phytol.* 155: 285-292.

Çelik, E., H. Kaya, and S. Yılmaz. 2012. Changes in hematological and biochemical parameters of European chub (*Squalius cephalus* L.) in unpolluted reservoir and polluted creek. Kafkas Universitesi Veteriner Fakültesi Dergisi 18: 413-418.

Chaiprapat, S., J.J. Cheng, J.J. Classen, and S.K. Liehr. 2005. Role of internal nutrient storage in duckweed growth for swine wastewater treatment. *Trans. ASAE.* 48: 2247–2258.

Chan, D.K.O., and N.Y.S. Woo. 1978. Effect of cortisol on the metabolism of the eel, Anguillia japonica. *Gen. Comp. Endocrinol*. 35: 205-215.

Chatterjee, N., A.K. Pal, T. Das, M. Mohammed, K. Sarma, G. Venkateshwarlu, and S.C. Mukherjee. 2006. Secondary stress responses in Indian major carps Labeo rohita (Hamilton), *Catla catla* (Hamilton) and *Cirrhinus mrigala* (Hamilton) fry to increasing packing densities. *Aquacult Res* 37: 472-476, 2006.

Chen, P., Li, J., X.L. Qing, Y.C. Wang, S.P. Lid, T.C. Ren, and L.G. Wang. 2012a. Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium Rhodococcus sp. CPZ24. *Bioresour Technol* 116: 266-270.

Chen, W.M., Y.Q. Tang, K. Mori, and X.L. Wu. 2012b. Distribution of culturable endophytic bacteria in aquatic plants and their potential for bioremediation in polluted waters. *Aquat Biol* 15: 99-110.

Chen, Y.P., P.D. Rekha, A.B. Arun, F.T. Shen, W.A. Lai, and C.C. Young. 2006. Phosphate solubilising bacteria from subtropical soil and their tricalcium phosphate solubilising abilities. *Appl Soil Ecol* 34: 33-41.

Cheng, J., B.A. Bergmann, J.J. Classen, A.M. Stomp, and J.W. Howard. 2002. Nutrient recovery from swine lagoon water by *Spirodela punctata*. *Bioresour Technol* 81: 81–85.

Cheng, W., C.S. Chiu, Y.K. Guu, S.T. Tsai, and C.H. Liu. 2013. Expression of recombinant phytase of Bacillus subtilis E20 in *Escherichia coli* HMS174 and improving the growth performance of white shrimp, *Litopenaeus vannamei*, juveniles by using phytase-pretreated soy-bean meal-containing diet. *Aquacult. Nutr.* 19: 117-127.

Cheng, J., B.A. Bergman, J.J. Classen, A.M. Stomp, and J.W. Howard. 2002a. Nutrient recovery from swine lagoon water by *Spirodela punctata*. *Bioresour. Technol.* 81: 81-85.

Cheng, J., L. Landesman, B.A. Bergman, J.J. Classen, J.W. Howard, and Y.T. Yamamoto. 2002b. Nutrient removal from swine lagoon liquid by *Lemna minor*. *Am. Soc. Agric. Eng.* 45: 1003-1010.

Cheong, L. 1989. Status of knowledge on farming of Seabass (*Lates calcarifer*) in South East Asia. In: Barret, J. (Eds.), Advances in Tropical Aquaculture. Proceedings of a workshop, AQUACOP IFREMER Actes de Colloque 9, Tahiti, pp. 421-428.

Chien, Y.H. 1992. Water quality requirements and management for marine shrimp Culture. Proceedings of the Special Session on Shrimp Farming. World Aquaculture Society, p. 144.

Chien, Y.H., and W.S. Tsai. 1985. Integrated pond culture: a type of spatially sequential polyculture. *J. World Maricult. Soc.* 16: 429-436.

Chowdhury, M.M.R., M. Shahjahan, M.S. Rahman, and M. Sadiqul Islam. 2008. Duckweed (*Lemna minor*) as supplementary feed in monoculture of Nile tilapia, *Oreochromis niloticus*. *J. Fisheries Aquatic Sci.* 3: 54-59.

Chopin, T., A.H. Buschmann, C. Halling, M. Troell, N. Kautsky, A. Neori, G. Kraemer, J. Zertuche-Gonzalez, C. Yarish, and C. Neefus. 2001. Integrating seaweeds into aquaculture systems: a key towards sustainability. *J. Phycol.* 37: 975-986.

Chopin, T., S.M.C. Robinson, M. Troell, A. Neori, A.H. Buschmann. 2008. Multitrophic integration for sustainable marine aquaculture. In: Jørgensen SE, Fath B (eds) Encyclopedia of ecology. Elsevier, Oxford, pp 2463–2475.

Chow, F., J. Macchiavello, S. Cruz, E. Fonck, and J. Oliviare. 2001. Utilization of Gracilariaceae as biofilter in the depuration of effluents from tank cultures of fish, oysters and sea urchins. *J. World Aquac Soc.* 32: 215-220.

Cohen, I., and A. Neori. 1991. *Ulva lactuca* biofilters for marine fishpond effluents. I. Ammonia uptake kinetics and nitrogen content. *Bot. Mar.* 34: 475-482.

Colombe, L., A. Fostier, N. Bury, F. Pakdel, and Y. Guiguen. 2000. A mineralocorticoid like receptor in the rainbow trout, *Oncorhynchus mykiss*: cloning and characterization of its steroid binding domain. *Steroids* 65: 319-328.

Colt, J. 2006. Water quality requirements for reuse systems. *Aquacult. Engineer.* 34: 143-156.

Comin, F.A., J.A. Romero, V. Astorga, and C.Garcia. 1997. *Water Sci. Technol*. 35: 255-261.

Conte, F.S. 2004. Stress and the welfare of cultured fish. *Appl. Anim. Behav. Sci.* 86: 205-223.

Copertino, M., T. Tormena, and U. Seeliger. 2009. Biofiltering efficiency, uptake and assimilation rates of *Ulva clathrata* (Roth) J. Agardh (Clorophyceae) cultivated in shrimp aquaculture waste water. *J. Appl. Phycol*. 21(1): 31-45.

Copland, J.W., and D.L. Grey. 1986. Management of wild and culture sea bass/barramundi (*Lates calcarifer*): Proceedings of an international workshop held at Darwin, N.T., Australia, 24-30 September 1986. ACIAR Proceedings No. 20, Australian Centre for International Agricultural Research, Canberra, Australia, 210 pp.

Correll, D.S., and H.B. Correll. 1972. Aquatic and wetland plants of southwestern United States. E. P. A. U. S. Gov. Printing Office, Washington, D. C. 1777 pp.

Costa-Pierce, B.A. 2002. Ecological Aquaculture. Blackwell Science, Oxford, UK.

Costa-Pierce, B.A., D.M. Bartley, M. Hasan, F. Yusoff, S.J. Kaushik, K. Rana, D. Lemos, P. Bueno, and A. Yakupitiyage. 2011. Responsible use of resources for sustainable aquaculture. Global Conference on Aquaculture 2010, Sept. 22-25, 2010, Phuket, Thailand. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Cottee, S., and P. Petersan. 2009. Animal Welfare and Organic Aquaculture in Open Systems. *J. Agricult. Environ. Ethics* 22: 437-461.

Cowey, C.B., and J.R. Sargent. 1979. Nutrition. In: Fish Physiol. Vol. 8 (Hoar, W.S., Randall, D.J. and Brett. J.R. eds.), Academia Press, New York. pp. 1-69.

Crab, R., Y. Avnimelech, T. Defoirdt, P. Bossier, and W. Verstraete. 2007. Nitrogen removal in aquaculture towards sustainable production. *Aquaculture* 270: 1-14.

Cripps, S.J. 1994. Minimizing outputs: treatment. *J. Appl. Ichthyology* 10 (4): 284-294

Cripps, S. J., and A. Bergheim. 2000. Solids management and removal for intensive landbased aquaculture production systems. *Aquacult. Eng.* 22 (1-2): 33-56.

Cross, J.W. 2002. The charms of duckweed. http://www.mobot.org/jwcross/duckweed/duckweed.htm

Chrousos, G.P., and P.W. Gold. 1992. The concepts of stress and stress system disorders. Overview of physical and behavioral hmeostasis. *JAMA*. 267: 1244-1252.

Cruz-Suárez, L.E., M. Tapia-Salazar, D. Villareal-Cavazos, J. Beltran-Rocha, M.G. Nieto-López, A. Lemme, and D. Ricque-Marie. 2009. Apparent dry matter, energy, protein and amino acid digestibility of four soybean ingredients in white shrimp Litopenaeus vannamei juveniles. *Aquaculture* 292: 87–94.

Culley, D.D., E. Rejmankova, J.B. Kvet, and Fry. 1981. Production, chemical quality, and use of duckweed (*Lemnaceae*) in aquaculture, waste management, and animal feeds. *J. World Maric. Soc*. 12: 27-49.

Cuvin-Aralar, M.L.A., E. Aralar, M. Laron, and W. Rosario. 2007. Culture of *Macrobrachium rosenbergii* (De Man 1879) in experimental cages in a freshwater eutrophic lake at different stocking densities. *Aquacult. Res*. 38: 288-294.

Dabrowski, K., P. Poczyczynski, G. Kock, and B. Berger. 1989. Effect of partially or totally replacing fish-meal protein by soybean-meal protein on growth, food utilization and proteolytic-enzyme activities in rainbow-trout (Salmo-Gairdneri) - new invivo test for exocrine pancreatic-secretio. *Aquaculture* 77: 29-49.

Dang, T.M.T., T.K.D. Nguyen, and T.R. Preston. 2011. Effect on duckweed composition of different levels of biodigester effluent in the growth medium and of transferring nutrient-rich duckweed to nutrient-free water. Workshop on Reducing Greenhouse Gas Emissions from Livestock and Soils (Editors: Reg Preston and Sisomphone Southavong). National Institute of Animal Science, Hanoi, 14-15 November 2011.

Das, I., and A.K. Ray. 1989. Growth performance of the Indian major carp *Labeo rohita* (Ham.) on duckweed incorporated pelleted feed: a preliminary study. *J. Inland Fish. Soc. Ind*. 21 (1): 1-6.

Dastager, S., D. Deepa, and A. Pandey. 2010. Isolation and characterization of novel plant growth promoting *Micrococcus* sp. NII-0909 and its interaction with cowpea. *Plant Phisiol. Biochem* 48: 987-992.

Davey, M.E., and G.A. O'Toole. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev*. 64: 847-867.

David, M., R. Shivakumar, S.B. Mushigeri, and R.C. Kuri. 2005. Blood glucose and glycogen levels of stress in the fresh water fish, Labeo rohita under fenvalerate intoxication. *J. Ecotoxicol. Env. Monitor.* 15: 1-5.

Davis, K.B., and N.C. Parker. 1990. Physiological stress in striped bass: effect of acclimation temperature. *Aquaculture* 91: 349-358.

Davies, P.J. 1995. Plant Hormones Physiology, Biochemistry and Molecular Biology, 2nd ed. Dordrecht: Kluwer Academic Publisher.

De-Bashan, L.E., and Y. Bashan. 2004. Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997-2003). *Water Res* 38: 4222-4246.

De Freitas, J.R., M.R. Banerjee, and J.J. Germida. 1997. Phosphate solubilising rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (Brassica napus L.). *Biol Fertil Soils* 24: 358-364.

Demirezen, D., A. Aksoy, and K. Uruç. 2007. Effect of population density on growth, biomass and nickel accumulation capacity of *Lemna gibba* (Lemnaceae). Chemosphere 66 (3): 553-557.

DPI (Department of Primary Industries). 2008. Best Practice Environmental Guidelines for Recirculating Aquaculture Systems. Fisheries Victoria Management Report Series No. 37.

De Silva, S.S., K.F. Shim, and A.K. Ong. 1990. An evaluation of the method used in digestibility estimations of a dietary ingredient and comparisons on external and internal markers and time of faeces collection in digestibility studies in the fish Oreochromis aureus (Steindachner). *Reprod. Nutr. Dev*. 30: 215-226.

De Souza, B.D.MJ., S. Nair, and D. Chandramohan. 2000. Phosphate solubilising bacteria around Indian peninsula. *Indian J. Mar. Sci.* 29: 48-51.

Devraj, K.V., G.Y. Keshavappa, and J.K. Manissery. 1986. Growth of grass carp (*Ctenoparyngodon idella*) fed on two terrestrial todder plants. *Aquacult. Fish. Manage.* 17: 123-128.

Deubel, A., A. Gransee, and W. Merbach. 2000. Transformation of organic rhizodeposits by rhizoplane bacteria and its influence on the availability of tertiary calcium phosphate. *J Plant Nutr Soil Sci* 163: 387-392.

Diana, J.S. 2009. Aquaculture production and biodiversity conservation. *BioScience* 59: 27-38.

Dias, J., M.J. Alvarez, J. Arzel, G. Corraze, A. Diez, J.M. Bautista, and S.J. Kaushik. 2005. Dietary Protein Source Affects Lipid Metabolism in the European Seabass (*Dicentrarchus labrax*). *Comp. Biochem. Physiol.* Part A 142:19-31.

Dobbelaere, S., J. Vanderleyden, and Y. Okon. 2003. Plant growth promoting effects of diazotrophs in the rhizosphere. *Crit Rev Plant Sci* 22: 107-149.

Dong, N.T.K., N.V. Thu, B. Ogle, and T.R. Preston. 2006. Effect of supplementation level of water spinach (*Ipomoea aquatic*) leaves in diets based on para grass (*Brachiara mutica*) on intake, nutrient utilization, growth rate, and economic returns of crossbred rabbits in the Mekong Delta of Vietnam. Workshop on Forages for Pigs and rabbits. MEKARN-CelAgrid.

Driever, S.M., E.H. van Nes, and R.M.M. Roijackers. 2005. Growth limitation of *Lemna minor* due to high plant density. *Aquat. Bot.* 81: 245-251.

Du, L., and C.J. Niu. 2003. Effects of dietary substitution of soy bean meal for fish meal on consumption, growth, and metabolism of juvenile giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquacult. Nutr.* 9: 139-143.

Eales, J.G. 2006. Modes of action and physiological effects of thyroid hormones in fish. In: Reinecke, M., Zaccone, G., Kapoor, B.G., (Eds.), Fish Endocrinology. Science Publishers, New Hampshire, pp. 767-808.

Ebeling, J.M., S.R. Ogden, P.L. Sibrell, and K.L. Rishel. 2004. Application of chemical coagulation aids for the removal of suspended solids (TSS) and phosphorus from the microscreen effluent discharge of an intensive recirculating aquaculture system. *N AM J Aquacul.* 66: 198–207.

Edwards, P. 1980. Food potential of aquatic macrophytes. ICLARM Stud. *Rev.* 5, 51.

Edwards, P., M. Kamal, and K.L. Wee. 1985. Incorporation of composted and dried water hyacinth in pelleted feed for the tilapia *Oreochromis niloticus* (Peters). *Aquacult Fish. Manage*. 1: 233–248.

Edwards, P., R.S.V. Pullin, and J.A. Gartner. 1988. Research and education for the development of integrated crop-livestock-fish farming systems in the tropics. Manila: ICLARM.

Edwards, T.M., K.A. McCoy, T. Barbeau, M.W. McCoy, J.M. Thro, and L.J.Jr. Guillette. 2006. Environmental context determines nitrate toxicity in Southern toad (*Bufo terrestris*) tadpoles. *Aquat. Toxicol*. 78: 50-58.

Eiler, A., S. Langenheder, S. Bertilsson, and L.J. Tranvik. 2003. Heterotrophic bacterial growth efficiency and community structure at different natural organic carbon concentrations. *Appl. Environ Microbiol.* 69: 3701–3709.

Ekin, Z. 2010. Performance of phosphate solubilising bacteria for improving and yield of sunflower (*Helianthus annuus* L) in the presence of phosphorus fertilizer. Afr. J. Biotechnol. 9 (25): 3794-3800.

Elasri, M.O., and R.V. Miller. 1999. Study of the response of a biofilm bacterial community to UV radiation. *Appl. Environ. Microbiol* 65: 2025-2031.

El-Kheir, W.A., G. Ismail, A. El-Nour, T. Tawfik, and D. Hammad. 2007. Assessment of the efficiency of duckweed (*Lemna gibba*) in wastewater treatment. *Int. J. Agricult. Biol* 5: 681-689.

Ellis, T., B. North, A.P. Scott, N.R. Bromage, M. Porter, and D. Gadd. 2002. The relationships between stocking density and welfare in farmed rainbow trout. *J. Fish Biol.* 61: 493-531.

El-Sayed, A.F.M. 2006. Tilapia Culture, first ed. CABI Publishing, Wallingford, UK.
El-Sayed, A. F. M., 2003. Effects of fermentation methods on the nutritive value of water hyacinth for Nile tilapia (*Oreochromis niloticus* L.) fingerlings. *Aquaculture* 218: 471-478.
El-Shafai, S.A., H.J. Gijzen, F.A. Nasr, and El-Gohary. 2004. Microbial quality oftilapia reared in fecal-contaminated ponds. *Environ. Res*. 95: 231-238.
El-Shafai, S.A., F.A. El-Gohary, F.A. Nasr, N.P. Van Der Steen, and H.J. Gijzen. 2007. Nutrient recovery from domestic wastewater using a UASB-duckweed ponds system. *Bioresource Technol.* 98, 798-807.
Endut, A., A. Jusoh, and A. Nora'aini. 2011. Nutrient removal from aquaculture wastewater by vegetable production in aquaponics recirculation system. *Desalin. Water Treat.* 32: 422-430.
Erol Nalbur, B., L. Akca, and H. Bayhan. 2003. Nitrogen removal during secondary treatment by aquatic systems. *Water Sci. Technol*. 48: 355-361.
Eusebio, P.S., R.M. Coloso, and R.E. Mamauag. 2004. Apparent digestibility of selected ingredients in diets for juvenile grouper, *Epinephelus coioides* (Hamilton). *Aquacult. Res*. 35: 1261-1269.
Evans, D.H. 2002. Cell signaling and ion transport across the fish gill epithelium. *J Exp Zool.* 293 (3): 336-47.
Evans, D.H., P.M. Piermarini, and K.P. Choe. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, andexcretion of nitrogenous waste. *Physiol. Rev*. 85: 97-177.
Fairchild, E.A., and W.H. Howell. 2001. Optimal stocking density for juvenile winter flounder *Pseudopleuronectes americanus*. *J. World Aquac Soc*. 32: 300-308.
Faisal, M., A.E. Eissa, and E.E. Elsayed. 2007. Isolation of *Aeromonas salmonicida* from sea lamprey (*Petromyzon marinus*) with furuncle-like lesions in Lake Ontario. *J. Wildl Dis.* 43: 618–622.
Fan, B., X.H. Chen, A. Budiharjo, W. Bleiss, J. Vater, and R. Borriss. 2011. Efficient colonization of plant roots by the plant growth promoting bacterium Bacillus amyloliquefaciens FZB42, engineered to express green fluorescent protein. *J. Biotechnol.* 151: 303–311.
Fang, Y.Y., O. Babourina, Z. Rengel, X.E. Yang, P.M. Pu. 2007. Ammonium and Nitrate Uptake by the Floating Plant *Landoltia punctate*. *Ann. Bot*. 99: 365-370.
Fankem, H., D. Nwaga, A. Deubel, L. Dieng, W. Merbach, and F.X. Etoa. 2006. Occurrence and functioning of phosphate solubilising microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. *Afr. J. Biotechnol*. 5: 2450-2460.
Fanouraki, E. 2010. Investigation of stress response and development and evaluation of a non-invasive method for the determination of free cortisol release into seawater in several Mediterranean fish species, with emphasis on the European sea bass, Dicentrarchus labrax. University of Crete, Dissertation (in Greek).
FAO. 2006. State of world aquaculture. FAO Technical Paper No. 500. Rome. 134 pp.
FAO. 2007. Cage Aquaculture. Regional reviews and global overview. FAO Fihseries Technical Paper 498. Rome.
FAO. 2011. FAO yearbook. Fishery and Aquaculture statistic. 2009. FAO Fisheries and Aquaculture Department.
FAO. 2012. FishStatJ-software for fishery statistical time series, Rome.
FAO. 2016. In brief - The state of world fisheries and aquaculture. Contributing to food security and nutrition for all. FAO, Rome.

Farbridge, K.J., and J.F. Leatherland. 1988. Interaction between ovaine growth hormone and triiodo-L-thyronine on metabolic reserves of rainbow trout, *Salmo gairdneri*. *Fish Physiol. Biochem.* 5: 141-151.

Fatira, E., N. Papandroulakis, and M. Pavlidis. 2014. Diel changes in plasma cortisol and effects of size and stress duration on the cortisol response in European sea bass (*Dicentrarchus labrax*). *Fish Physiol. Biochem.* 40:911–919.

Ferdoushi, Z., F. Haque, S. Khan, and M. Haque. 2008. The effects of two aquatic floating macrophytes (*Lemna* and *Azolla*) as biofilters of nitrogen and phosphate in fish ponds. *Turkish J. Fish. Aqua. Sci.* 8: 253-258.

Fei, X.G. 2004. Solving the coastal eiutrophication problem by large scale seaweed cultivation. *Hydrobiologia* 512: 145-151.

Ferreira, J.G., A.J.S. Hawkins, and S.B. Bricker. 2007. Management of productivity, environmental effects and profitability of shellfish aquaculture — the Farm Aquaculture Resource Management (FARM) model. *Aquaculture* 264: 160-174.

Flimlin, G., J. Buttner, and D. Webster. 2008. Aquaculture systems for the northeast. NRAC Publication No. 104-2008.

Flodmark, L.E.W., H.A. Urke, J.H. Halleraker, J.V. Arnekleiv, L.A. Vøllestad, and A.B.S. Poléo. 2002. Cortisol and glucose responses in juvenile brown trout subjected to a fluctuating flow regime in an artificial stream. *J. Fish Biol.* 60: 238-248.

Flores-Miranda, Ma.dC, A. Luna-González, D. Cortés-Espinosa, P. Álvarez-Ruiz, E. Cortés-Jacinto, F. Valdez-González, R. Escamilla-Montes, and H. González-Ocampo. 2015. Effects of diets with fermented duckweed (*Lemna* sp) on growth performance and gene expression in the Pacific white shrimp, *Litopenaeus vannamei*. *Aquacult. Int.* 23(2): 547-561.

Forbes, B.A., D.F. Sahm, and A.S. Weissfeld. 1998. Bailey & Scott's Diagnostic Microbiology, 10th ed. St. Louis. Mosby Elsevier.

Fox, L.J., P.C. Struik, B.L. Appleton, and J.H. Rule. 2008. Nitrogen phytoremediation by water hyacinth *Eichhornia crassipes* (Mart) Solms. *Water Air Soil Pollut.* 194: 199-207.

Frankic, A., and C. Hershner. 2003. Sustainable aquaculture: developing the promise of aquaculture. *Aquac Int.* 11: 517-530.

Frederic, M., L. Samir, M. Louise, and A. Abdelkrim. 2006. Comprehensive modelling of mat density effect on duckweed (*Lemna minor*) growth under controlled eutrophication. *Water Res.* 40: 2901-2910.

Fredrickson, L.H., and F.A. Reid. 1988. Nutritional values of waterfowl foods. Waterfowl management handbook. Fish and Wildlife Leaflet 13.1.1.

Gamboa-Delgado, J., M.G. Rojas-Casas, M.G. Nieto-López, and L.E. Cruz-Suárez. 2013. Simultaneous estimation of the nutritional contribution of fish meal, soy protein isolate and corn gluten to the growth of Pacific white shrimp (*Litopenaeus vannamei*) using dual stable isotope analysis. *Aquaculture* 380-383:33-40. doi: 10.1016/j.aquaculture.2012.11.028.

Gamperl, A.K., M.M. Vijayan, and R.G. Boutilier. 1994. Experimental control of stress hormone levels in fishes: techniques and applications. *Rev. Fish Biol. Fisheries* 4: 215-255.

Gannam, A.L., and R.T. Lovell. 1991. Effects of feeding 17α- methyltestosterone, 11-ketotestosterone, 17β- estradiol and 3, 5, 3'-triiodo-thyronine to channel catfish, *Ictalurus punctatu*. *Aquaculture* 92: 377-388.

Garr, A.L., H. Lopez, R. Pierce, and M. Davis. 2011. The effect of stocking density and diet on the growth and survival of cultured Florida apple snails, *Pomacea paludosa*. *Aquaculture* 311: 139–145.

Gaudet, M., J.G. Racicot, and C. Leray. 1975. Enzyme activities of plasma and selected tissues in rainbow trout Salmo gairdneri Richardson. *J. Fish Biol*. 7: 505-512.

Gautier, D., J. Amador, and F. Newmark. 2001. The use of mangrove wetland as a biofilter to treat shrimp pond effluents: preliminary results of an experiment on the Caribbean coast of Colombia. *Aquac. Res*. 32: 787-799.

Geven, E.J.W., F. Verkaar, G. Flik, and P.H.M. Klaren. 2006. Experimental hyperthyroidism and central mediators of stress axis and thyroid axis activity in common carp (*Cyprinus carpio* L.). *J. Mol. Endocrinol.* 37: 443-452.

Gillberg, L., H. Hansen, and I. Karlsson. 2003. About Water Treatment. Kemira Kemwater. ISBN: 91-631-4344-5.

Glencross, B., N. Wade, and K. Morton. 2013. Chapter 8. *Lates calcarifer* nutrition and feeding practices. Biology and Culture of Asian Seabass *Lates Calcarifer*, 178-228. CRC Press.

Glick, B.R. 2005. Modulation of plant ethylene levels by the enzyme ACC deaminase. *FEMS Microbiol Lett* 251: 1-7.

Gokcek, C.K., and I. Akyurt. 2007. The effect of stocking density on yield, growth, and feed efficiency of himri barbel (*Barbus luteus*) nursed in cages. *Isr. J. Aquacult.-Bamid*. 59: 99-103.

Goldman, J.C., R.K. Tenore, H.J. Ryther, and N. Corwin. 1974a. Inorganic nitrogen removal in a conbined tertiary treatment-marine aquaculture system. I. Removal efficiencies. *Water Res*. 8:45-54.

Goldman, J.C., K.R. Tenore, and H.I. Stanley. 1974b. Inorganic nitrogen removal in a combined tertiary treatment-marine aquaculture system. II. Algal bioassays. *Water Res*. 8: 55-59.

Goldstein, A.H. 1986. Bacterial solubilization of mineral phosphates: historical perspective and future prospects. *Am J Altern. Agric.* 1: 51-57.

Goldstein, A.H. 1994. Involvement of the quinoprotein glucose dehydrogenase in the solubilisations of exogenous phosphates by gram-negative bacteria. In: Torriani-Gorini A, Yagil E, Silver S (edi). Phosphate in microorganisms.

Gonçalves, J.F.M., B.G. Da Silva Turini, and R.O. De Almeida Ozório. 2010. Performance of juvenile turbot (*Scophthalmus maximus*) fed varying dietary L-carnitine levels at different stocking densities. *Sci. Agric.* 67: 151-157.

Goolish, E.M. 1989. The scaling of aerobic and anaerobic muscle power in rainbow trout (*Salmo gairdneri*). *J Exp. Biol.* 147: 493–505.

Gornati, R., E. Papis, S. Rimoldi, G. Terova, M. Saroglia, and G. Bernardini. 2004. Rearing density influences the expression of stress-related genes in sea bass (*Dicentrarchus labrax* L). *Gene* 341: 111-118.

Goss, G.G., and C.M. Wood. 1988. The effects of acid and acid/ aluminium exposure on circulating plasma cortisol levels and other blood parameters in the rainbow trout, *Salmo gairdnei*. *J. Fish Biol*. 32: 63-76.

Graber, A., and R. Junge. 2009. Aquaponic systems: nutrient recycling from fish wastewater by vegetable production. *Desalination* 246: 147–156.

Greenway, M., and A. Woolley. 2000. Changes in Plant Biomass and Nutrient Removal over 3 Years in a Constructed Free Water Surface Flow Wetland in Cairns Australia, Proceedings of the Seventh International Conference on Wetland Systems for Water Pollution Control, International Water Association, Lake Buena Vista, Florida, pp. 707–718.

Griffiths, S.W., and A.E. Magurran. 1997. Familiarity in schooling fish: how long does it take to acquire. *Anim Behav* 53: 945–949.

Gul, Y., Z.X. Gao, X.Q. Qian, and W.M. Wang. 2011. Haematological and serum biochemical characterization and comparison of wild and cultured northern snakehead (*Channa argus* Cantor, 1842). *J Appl. Ichthyol*. 27(1): 122-128.

Gull, M., F.Y. Hafeez, M. Saleem, and K.A. Malik. 2004. Phosphorus uptake and growth promotion of chickpea by co-inoculation of mineral phosphate solubilising bacteria and a mixed rhizobial culture. *Aust. J. Exp. Agric.* 44: 623-628.

Gullian-Klanian, M., and C. Arámburu-Adame. 2013. Performance of nile tilapia (*Oreochromis niloticus*) fingerlings in a hyper-intensive recirculating aquaculture system with low water exchange. *Lat. Am. J. Aquat. Res.* 41: 150-162.

Gürtekin, E., and N. Şekerdağ. 2008. The role of duckweed (Lemna minor L.) in secondary clarifier tanks. Report. Firat University, Turkey. 4 p.

Guru, Sandhya, R., and A.K. Patra. 2007. Evaluation of Nutritional Impact of water weed-based feeds-on the growth-rate of Labeo rohita. M.Phil Thesis 2007, Utkal University, Bhubaneswar.

Gyaneshwar, P., G.N. Kumar, L.J. Parekh, and P.S. Poole. 2002. Role of soil microorganisms in improving P nutrition of plants. *Plant Soil* 245: 83-93.

Hagopian, D.S., and J.G. Riley. 1998. A closer look at the bacteriology of nitrification. *Aquacul. Eng*. 18: 223-224.

Hammouda, O., A. Gaber, and M.S. Abdel-Hameed. 1995. Assessment of the effectiveness of treatment of wastewater-contaminated aquatic systems with *Lemna gibba*. *Enz. Micro. Technol.* 17: 317-323.

Handeland, S.O., A.K. Imsland, and S.O. Stefansson. 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture* 283:36-42.

Hansen, B., I. Karlsson, S. Cassidy, and L. Petterson. 2000. Operational experience from a sludge recovery plant. *Water Sci. Tech.* 41: 23-30.

Hansen, A.C., G. Rosenlund, Ø. Karlsen, W. Koppe, and G.I. Hemre. 2007. Total replacement of fish meal with plant proteins indiets for Atlantic cod (*Gadus* morhua L.) I: Effects on growthand protein retention. *Aquaculture* 272: 599-611

Hargreaves, J.A. 2013. Biofloc production systems for aquaculture. SRAC Publication No. 4503, 12 pp.

Hari, B., M.B. Kurup, J.T. Varghese, J.W. Sharma, and M.C.J. Verdegem. 2004. Effects of carbohydrate addition on production in extensive shrimp culture systems. *Aquaculture* 241: 179-194.

Hariprasad, P., and S.R. Niranjana. 2009. Isolation and characterization of phosphate solubilising rhizobacteria to improve plant health of tomato. *Plant Soil.* 316:13-24.

Harpaz, S., and Z. Uni. 1999. Activity of intestinal mucosal brush border membrane enzymes in relation to the feeding habits of three aquaculture fish species. *Comp. Biochem. Physiol*. 124: 155-160.

Harvey, R.M., and J.L. Fox. 1973. Nutrient removal using *Lemna minor*. *J WPFC* 45: 1928-1938.

Hattingh, J. 1997. Blood sugar as an indicator of stress in the freshwater fish, Labeo capinsis (Smith). *J. Fish Biol*. 10: 191-195.

Haukenes, A.H., B.A. Barton, and H. Bollings. 2008. Cortisol response of pallid sturgeon and yellow perch following challenge with lipopolysaccharide. *J. Fish Biol*. 780-784.

He, S., X.F. Liang, L. Li, J. Sun, and D. Shen. 2013. Differential gut growth, gene expression and digestive enzyme activities in young grass carp (*Ctenopharyngodon idella*) fed with plant and animal diets. *Aquaculture* 410-411: 18-24.

Hecht, T., L. Oellermann, and L. Verheust. 1996. Perspectives on clariid catfish culture in Africa. *Aquat. Living Resour*. 9: 197-206.

Heidenwang, I., U. Langheinrich, and V. Luderitz. 2001. Self-purification in upland and lowland streams. *Acta Hydroch. Hydrobiol*. 29: 22-33.
Helfrich, L.A., and G. Libey. 1990. Fish Farming in Recirculating Aquaculture Systems (RAS). Department of Fisheries and Wildlife. Sciences, Virginia Tech, New York.
Hemre, G.I., H. Amlund, M. Aursand, A.M. Bakke, R.E. Olsen, E. Ringo, and B. Svihus. 2009. Criteria for safe use of plant ingerdients in diets for aquacultured fish. Norwegian Scientific Committee for Food Safety. VKM.
Henry-Silva, G.G., and A.F. Monteiro-Camargo. 2006. Efficiency of aquatic macrophytes to treat Nile tilapia pond effluents. *Scientia Agricola* 3: 433-438.
Henze, M., Harremoes, P., Arvin, E., Cour Jansen, J., 1996. Waste Water Treatment. Lyngby, Springer Verlag, Berlin.
Henze, M., P. Harremoës, J.I.C. Jansen, and E. Arvin. 2002. Wastewater treatment: Biological and chemical processes. Springer Verlag, Berlin Heidelberg.
Higgs, D.A., U.H.M. Fagerlund, and J.R. Mcbride. 1979. Influence of orally administered L Thyroxine of 3, 5, 3'- triiodo- L-thyronine on growth, food consumption and food conversion of underyearlying coho salmon, *Oncohynchus kisutch*. *Can. J. Zoo.* 57: 1974-1979.
Higuera, M., and O. Cardenas. 1986. Hormonal effects of gluconeogenesis from (U 14C) glutamate in rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol*. 58B: 517-521.
Hillman, W.S., and D.D. Culley. 1978. The uses of duckweed. *Amer. Scien.* 66: 442-51.
Himic, B.A., and J.G. Eales. 1990. Acute correlated changes in plasma T4 and glucose in physically disturbed cannulated rainbow trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol.* 97: 165-167.
Hinther, A., T.M. Edwards, L.J. Guillette, and C.C. Helbing. 2012. Influence of nitrate and nitrite on thyroid hormone responsive and stress-associated gene expression in cultured *Rana catesbeiana* tadpole tail fin tissue. *Front Genet*. 3: 1-7.
Hochheimer, J.N., and F. Wheaton. 1998. Biological Filters: Trickling and RBC Design.
Holt, J.G., N.R. Krieg, H.A. Sneath, J.T. Staley, and S.T. Wiliam. 1994. Bergey's Manual of Determinative Bacteriology 9th Ed. William & Wilkins, Baltimore.
Hollenberg, A.N., and D. Forrest. 2008. The thyroid and metabolism: the action continues. *Cell Metab*. 8: 10-2.
Hontela, A., P. Dumont, D. Duclos, and R. Fortin. 1995. Endocrine and metabolic dysfunction in yellow perch, *Perca flavescens*, exposed to organic contaminants and heavy metals in the St. Lawrence River. *Environ. Toxicol. Chem.* 14: 725-731.
Hossain, M.A., and K. Jauncy. 1989. Nutritional evaluation of some Bangladeshi oilseed meals as partial substitutes for fish meal in the diet of common carp, *Cyprinus carpio* L. *Aquacult. Fish Manage*. 20: 255-268.
Hosseini, S.A., and S.M. Hoseini. 2012. Effect of acute crowding stress on subsequent osmotic challenge and recovery in juvenile common carp Cyprinus carpio (Linnaeus). *Comp. Clin. Pathol*. 21: 583.
Hosseinifard, S.M., Sh. Ghobadi, E. Khodabakhsh, and M. Razeghi Mansour. 2013. The effect of different levels of soybean meals and avizyme enzyme supplement on hematological and biochemical parameters of serum in rainbow trout. *Iranian Vet. J.* 9: 43-53.
Hoseini, S.M., and M. Ghelichpour. 2012. Efficacy of clove solution on blood sampling and hematological study in Beluga, *Huso huso*. *Fish Physiol. Biochem.* 38: 493-498. http://dx.doi.org/10.1007/s10695-011-9529-5.
Hovanec, T., and E. DeLong. 1996. Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria. *Appl. Environ Microbiol.* 62: 2888–2896.

Hu, B., and E. Zhou. 1989. Review on development of integrated fish farming in China. Selected papers of freshwater fisheries research, Asian-Pacific Regional Research and Training centre for integrated fish farming, p.89-110.
Huang, X.F., J.M. Chaparro, K.F. Reardon, R. Zhang, Q. Shen, and J.M. Vivanco. 2014. Rhizosphere interactions: root exudates, microbes, and microbial communities. Botany 92: 267-275. dx.doi.org/10.1139/cjb-2013-0225
Hughes, J.S., M.M. Alexander, and K. Balu. 1988. In: Adams, W.J., Chapman, G.A., Landis, W.G. (Eds.), 1988. An evaluation of appropriate expressions of toxicity in aquatic plant bioassays as demonstrated by the effects of atrazine on algae and duckweed. Aquatic Toxicology and Hazard Assessment, vol. 10. American Soc. for Testing and Materials, Philadelphia, pp. 531e547. ASTM STP 971.
Hulbert, A. J. 2000. Thyroid hormones and their effects: a new perspective. *Biol. Rev.* 75: 519-631.
Hultman, B., E. Levlin, and K. Stark. 2001. Phosphorus recovery from sewage sludges: Research and experiences in Nordic countries, Scope Newsletter, Scientific committee on Phosphates in Europe, 41.
Huntingford, F.A., C. Adams, V.A. Braithwaite, S. Kadri, T.G. Pottinger, P. Sandoe, and J.F. Turnbull. 2006. Current issues in fish welfare. *J. Fish Biol*. 68: 332-372.
Iguchi, K.I., K. Ogawa, M. Nagae, and F. Ito. 2003. The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis*). *Aquaculture* 220: 515-523.
Ikeda-Ohtsubo, W., M. Miyahara, S.W. Kim, T. Yamada, M. Matsuoka, A. Watanabe, S. Fushinobu, T. Wakagi, H. Shoun, K. Miyauchi, and G. Endo. 2013. Bioaugmentation of a wastewater bioreactor system with the nitrous oxide-reducing denitrifier *Pseudomonas stutzeri* strain TR2. *J. Biosci Bioeng.* 115: 37-42.
Illmer, P., and F. Schinner. 1992. Solubilization of inorganic phosphates by microorganisms isolated from forest soil. *Soil Biol. Biochem*. 24:389–395
Illmer, P., A. Barbato, and F. Schinner. 1995. Solubilization of hardly-soluble $AlPO_4$ with P-solubilising microorganisms. *Soil Biol. Biochem.* 27: 265-270.
Ingersoll, T., and L.A. Baker. 1998. Nitrate removal in wetland microcosms. *Water Res*. 32: 677-684.
Intriago, P. 2012. Marine Microorganisms: perspectives for getting involved in cellulosic ethanol. *AMB Express* 2: 46.
Itoi, S., A. Niki, and H. Sugita. 2006. Changes in microbial communities associated with the conditioning of filter material in recirculating aquaculture systems of the pufferfish *Takifugu rubripes*. *Aquaculture* 256: 287-295.
Itoi, S., N. Ebihara, S. Washio, and H. Sugita. 2007. Nitrite-oxidizing bacteria, Nitrospira, distribution in the outer layer of the biofilm from filter materials of a recirculating water system for the goldfish *Carassius auratus*. *Aquaculture* 264 (1-4): 297-308.
IWA. 2000. Constructed wetlands for pollution control: processes, performance, design and operation. In. R.H. Kadlec, R.L. Knight, J. Vymazal, H. Brix, P. Cooper and R. Haberl (eds), IWA specialist group on the use of macrophytes in water pollution control, Scientific and Technical. Report No. 8, IWA Publishing, London, UK.
Iwama G.K. 2007. The welfare of fish. *Dis. Aquat. Org.* 75, 155–158.
Jayathilakan, K., K. Khudsia-Sultana, Radhakrishna, and A.S. Bawa. 2012. Utilization of byproducts and waste materials from meat, poultry and fish processing industries: a review. *J. Food Sci. Technol*. 49 (3): 278-293.
Jeong, S., H.S. Moon, K. Nam, J.Y. Kim, and T.S. Kim. 2012. Application of phosphate-solubilising bacteria for enhancing bioavailability and phytoextraction of cadmium (Cd) from polluted soil. *Chemosphere* 88: 204-210.

Jha, P., and A. Kumar. 2009. Characterization of novel plant growth promoting endophytic bacterium *Achromobacter xylosoxidans* from wheat plant. *Microb. Ecol.* 58: 179-188.
Jo, J.Y., J.S. Ma, and I.B. Kim. 2002. Comparisons of four commonly used aquatic plants for removing nitrogen nutrients in the intensive bioproduction Korean (IBK) recirculating aquaculture system. Proceedings of the third International Conference on Recirculating Aquaculture, Roanoke VA.
Jones, A.B., W.C. Dennison, and N.P. Preston. 2001. Integrated treatment of shrimp effluent by sedimentation, oyster filtration and macroalgal absorption: a laboratory scale study. *Aquaculture* 193 (1-2):155-178.
Jones, A.B., N.P. Preston, and W.C. Dennison. 2002. The efficiency and condition of oysters and macroalgae used as biological filters of shrimp pond effluent. *Aquacult Res* 33: 1-19.
Jones, I.D. 1975. Effect of processing by fermentation of nutrients. In: Harris, R.S., Karmas, E. (Eds.), Nutritional Evaluation of Food Processing. Avi. Publ. Co., Inc, Westport, Connecticut, p. 324.
Jones, R.D. 2002. Phosphorus Cycling. In Manual of Environmental Microbiology (Editor in chief Hurst, C.J.). ASM Press, Washington DC, pp 450-453.
Jørgensen, E.H., J.S. Christiansen, and M. Jobling. 1993. Effects of stocking density on food intake, growth performance and oxygen consumption in Arctic charr (*Salvelinus alpinus*) *Aquaculture* 110: 191-204.
Journey, T., P. Skillicorn, and B. Spira. 1991. Duckweed aquaculture: A new aquatic farming system for developing countries. Emena Technical Department, World Bank.
Jupsin, H., H. Richard, and J.L. Vasel. 2004. Contribution of floating macrophytes (*Lemna* sp.) in pond modelization. 6th International Conference on Waste Stabilisation Ponds, September 28th - October 1st, 2004, Avignon, France, 387-393.
Justice, W.S., C.R. Bell, and A.H. Lindsey. 2005. Wild Flowers of North Carolina (2ed.). Chapel Hill, NC: Univ. of North Carolina Press. p. 255.
Kabir, A.N.M.A., M.A. Hossain, and M.S. Rahman. 2009. Use of duckweed as feed for fishes in polyculture. *J. Agric Rural Dev*. 7: 157-160.
Kang, Sc., C.G. Hat, T.G. Lee, and D.K. Maheshwari. 2002. Solubilization of inorganic phosphates by a soil-inhabiting fungus *Fomitopsis* sp. PS 102. *Curr. Sci.* 82: 439-442.
Kemp, M.J., and W.K. Dodds. 2002. The influence of ammonium, nitrate, and dissolved oxygen concentrations on uptake, nitrification, and denitrification rates associated with prairie stream substrata. *Limnol. Oceanogr*. 47: 1380-1393.
Khan, M.S., A. Zaidi, and P.A. Wani. 2007. Role of phosphate-solubilising microorganisms in sustainable agriculture - a review. Agron. *Sustain Dev.* 27:29-43.
Khan, M.S.A. Zaidi, and E. Ahmad. 2014. Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate-Solubilising Microorganisms. In: MS Khan. Eds. Springer publishers Switzerland, pp. 31-62.
Khatune-Jannat, M., M.M. Rahman, M.A. Bashar, M.N. Hasan, and F. Ahamed. 2012. Effects of stocking density on survival, growth and production of Thai Climbing Perch (*Anabas testudineus*) under fed ponds. *Sains Malaysiana* 41: 1205-1210.
Khellaf, K., and M. Zerdaoui. 2010. Growth response of the duckweed *Lemna gibba* L. to copper and nickel phytoaccumulation. *Ecotoxicology* 19: 1363-1368.
Killgore, K.J., J.J. Hoover, and R.P. Morgan. 1991. Habitat value of aquatic plants for fishes. Aquatic Plant Control Research Program, Tech. Rep. A-91-5. Department of the Army, Vicksburg, USA.
Klinger, D., and R. Naylor. 2012. Searching for solutions in aquaculture: Charting a sustainable course. *Annu. Rev. Environ. Resour*. 37: 247–276.

Koakoski, G., T.A. Oliveira, J.G.S. Rosa, M. Fagundes, L.C. Kreutz, and L.J.G. Barcellos. 2012. Divergent time course of cortisol response to stress in fish of different ages. *Physiol. Behavior* 106: 129-132.

Kone, D. 2002. Lagunage à microphytes et à macrophytes (Pistia stratiotes) en Afrique de l'Ouest et du Centre: Etat des lieux, performances épuratoires et critères de dimensionnement. Thèse de doctorat : Ecole Polytechnique Fédérale de Lausanne: Environnment naturel - Architectural and Construction Faculty (Suisse).

Korner, S., and J.E. Vermaat. 1998. The relative importance of *Lemna gibba* L. bacteria and algae for the nitrogen and phosphorus removal in duckweed covered domestic wastewater. *Water Res*. 32: 3651-3661.

Korner, S., G.B. Lyatuu, and J.E. Vermaat. 1998. The influence of *Lemna gibba* L. on the degredation of organic material in duckweed covered domestic wastewater. *Water Res.* 32: 3092-3098.

Korner, S., J.E. Vermaat, and S. Veenstra. 2003. The capacity of duckweed to treat wastewater. *J. Environ. Qual*. 32: 1583-1590.

Kpundeh, M.D., P. Xu, H. Yang, J. Qiang, and J. He. 2013. Stocking densities and chronic zero culture water exchange stress' effects on biological performances, hematological and serum biochemical indices of GIFT tilapia juveniles (*Oreochromis niloticus*). *J. Aquac. Res. Dev*. 4: 1-5.

Krogdahl, A. 1989. Alternative protein sources from plants containing anti-nutrients affecting digestion in Salmonids. In: Takeda, M., Watanabe, T. (Eds.), Proceedings of the Third International Symposium in Feeding and Nutrition in Fish. Tokyo University of Fisheries, Tokyo, pp. 253–261.

Krogdahl, A., A.M.B. McKellep, and G. Baeverfjord. 2003. Effects of graded levels of 539 standard soybean meal on intestinal structure, mucosal enzyme activities, and 540 pancreatic response in Atlantic salmon (Salmo salar L.). *Aquacult. Nutr.* 9: 361–371.

Kristiansen, T.S., A. Fernö, J.C. Holm, L. Privitera, S. Bakke, J.E. Fosseidengen. 2004. Swimming behaviour as an indicator of low growth rate and impaired welfare in Atlantic halibut (*Hippoglossus hippoglossus* L.) reared at three stocking densities. *Aquaculture* 230: 137-151.

Krogdahl, A., A.M.B. McKellep, and G. Baeverfjord. 2003. Effects of graded levels of 539 standard soybean meal on intestinal structure, mucosal enzyme activities, and 540 pancreatic response in Atlantic salmon (Salmo salar L.). *Aquacult. Nutr.* 9: 361–371.

Kroglund, F., B. Finstad, S.O. Stefansson, T.O. Nilsen, T. Kristensen, B.O. Rosseland, and B. Salbu. 2007. Exposure to moderate acid water and aluminum reduces Atlantic salmon post-smolt survival. *Aquaculture 273* (2-3): 360-373.

Kucey, R.M.N., H.H. Jenzen, and M.E. Leggett. 1989. Microbially mediated increases in plant available phosphorus. *Adv. Agron.* 42: 199-228.

Kumar, V., H.P.S. Makkar, W. Amselgruber, and K. Becker. 2010. Physiological, haematological and histopathological responses incommon carp (*Cyprinus carpio* L.) fingerlings fed with differentlydetoxifiedJatropha curcaskernel meal. *Food Chem. Toxicol*. 48: 2063-2072.

Kumaraguru-Vasagam, K.P., T. Balasubramanian, and R. Venkatesan. 2007. Apparent digestibility of differently processed grain legumes, cow pea and mung bean in black tiger shrimp, *Penaeus monodon* Fabricius and associated histological anomalies in hepatopancreas and midgut. *Anim. Feed Sci. Technol*. 132 (3–4): 250-266.

Kundu, P., A. Pramanik, S. Mitra, J. Debabrata, Choudhury, J. Mukherjee, and S. Mukherjee. 2012. Heterotrophic nitrification by *Achromobacter xylosoxidans* S18 isolated from a small-scale slaughterhouse wastewater. *Biopro. Biosyst. Eng.* 35: 721-728.

Kundu, P., A. Pramanik, A. Dasgupta, J. Mukherjee, and S. Mukherjee. 2014. Simultaneous Heterotrophic Nitrification and Aerobic Denitrification by *Chryseobacterium* sp. R31 Isolated from Abattoir Wastewater. *BioMed. Res. Int.* 2014: 1-12.

Kutty, S.R.M., S.N.I. Ngatenah, M.H. Isa, and A. Malakahmad. 2009. Nutrients removal from municipal wastewater treatment plant effluent using *Eichhornia crassipes*. *World Acad. Sci. Eng. Technol.* 60: 826-831.

Kyambadde, J., F. Kansiime, and G. Dalhammar. 2005. Nitrogen and phosphorus removal in substrate-free pilot constructed wetlands with horizontal surface flow in Uganda. *Water Air Soil Poll.* 165: 37-59,

Lal Murari, N.N., and Pathak. 1988. Aquatic weeds (*Lemna* and *Hydrilla*) as livestock feed. *Indian J. Anim. Nutr.* 5:329-332.

Lalucat, J., A. BenQam, R. Bosch, E. García-Valdés, and N.J. Palleroni. 2006. Biology of *Pseudomonas stutzeri*. *Microbiol Mol Biol Rev* 70: 510–547.

Lambert, Y., and J.D. Dutil. 2001. Food intake and growth of adult Atlantic cod, *Gadus morhua* L., reared under different conditions of stocking density, feeding frequency and size-grading. *Aquaculture* 192: 233-247.

Landesman, L., C. Fedler, and R. Duan. 2000. Plant nutrient phytoremediation using duckweed. Eutrophication: causes, consequences and control: 341-354.

Landolt, E. 1986. *The Family of Lemnacae - A Monographic Study vol 1. Part of the series: Biosystematic Investigations in the family of duckweeds (Lemnacae)*, vol 2. Veroffentlichungen Des Geobotanischen (pub.)

Landolt, E., and R. Kandeler. 1987. *The family of Lemnaceae - a monographic study: phytochemistry, physiology, application and bibliography*. Veroffentlichungen des Geobotanisches Institut der Edg. Tech. Hochschule, Stiftung Ruebel, Zurich.

Lasfar, S., F. Monette, L. Millette, and A. Azzouz. 2007. Intrinsic growth rate: A new approach to evaluate the effects of temperature, photoperiod and phosphorus-nitrogen concentrations on duckweed growth under controlled eutrophication. *Water Res.* 41: 2333-2340.

Leatherland, J.F., J.W. Hilton, and S.J. Singer. 1987. Effects of thyroid hormones supplementation of canola meal- based diets on growth and interenal and thyroid gland physiology of rainbow trout, Salmo gairdneri. *Fish Physiol. Biochem.* 3: 730-782.

Leatherland, J.F., and C.Y. Cho. 1985. Effect of rearing density on thyroid and interrenal gland activity and plasma and hepatic metabolite levels in rainbow trout, *Salmo gairdneri* Richardson. *J Fish Biol.* 27: 583-592.

Lechevallier, M.W., W. Schulz, and R.G. Lee. 1991. Bacterial nutrients in drinking-water. *Appl Environ. Microbiol*. 57: 857-862.

Lefrancois, C., G. Claireaux, C. Mercier, and J. Aubin. 2001. Effect of density on the routine metabolic expenditure of farmed rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 195: 269-277.

Leng, R.A. 1999. Duckweed, a tiny aquatic plant with enormous potential for agriculture and environment. FAO. Tran Phu Printing Co.

Leonard, N., J.P. Blancheton, and J.P. Guiraud. 2000. Populations of heterotrophic bacteria in an experimental recirculating aquaculture system. *Aquacult Eng.* 22: 109-120.

Leonard, N., J.P. Guiraud, E. Gasset, J.P. Cailleres, and J.P. Blancheton. 2002. Bacteria and nutrients – nitrogen and carbon – in a recirculating system for sea bass production. *Aquacult. Eng.* 26: 111–127.

Leung, L.Y., and N.Y.S. Woo. 2012. Influence of dietary carbohydrate level on endocrine status and hepatic carbohydrate metabolism in the marine fish. Sparus sarba. *Fish Physiol. Biochem.* 38: 543-554.

Lewis, W.M., and M. Bender. 1961. Effect of cover of duckweeds and alga Pithophora upon the

dissolved oxygen and free carbon dioxide of small ponds. *Ecology* 42: 602-603.
Li, D., P. Xie, and X. Zhang. 2008. Changes in plasma thyroid hormones and cortisol levels in crucian carp (*Carassius auratus*) exposed to the extracted microcystins. *Chemosphere* 74: 13-18.
Li, D., Z. Liu, and C. Xie. 2012. Effect of stocking density on growth and serum concentrations of thyroid hormones and cortisol in Amur sturgeon, *Acipenser schrenckii*. *Fish Physiol. Biochem.* 38 (2): 511-520.
Li, H., Y. Chen, and Y.G. Gu. 2007. The effect of propionic to acetic ratio on anaerobic - aerobic (low dissolved oxygen) biological phosphorus and nitrogen removal. *Bioresour. Technol.* 99: 4400-4407.
Li, S.F. 2003. Aquaculture research and its relation to development in China, agricultural development and the opportunities for aquatic resources research in China. *World Fish Center Conference Proceedings* 65: 17-28, Shanghai, China.
Li, X., Y. Liu, and J.P. Blancheton. 2013. Effect of stocking density on performances of juvenile turbot (*Scophthalmus maximus*) in recirculating aquaculture systems. Chinese *J. Oceanol. Limnol.* 31: 514-522.
Liberti, L., D. Petruzzelli, and L. De Florio. 2001. Rem nut ion exchange plus struvite precipitation process. *Environ. Technol* 22: 1313-1324
Lim, S.J., S.S. Kim, G.Y. Ko, J.W. Song, D.H. Oh, J.D. Kim, J.U.Kim, and K.J. Lee. 2011. Fish meal replacement by soybean meal in diets for Tiger puffer (*Takifugu rubripes*). *Aquaculture* 313: 165-170.
Lim, S., and K. Lee. 2009. Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. *Aquaculture* 290: 283-289.
Lin, C.K., P. Ruamthaveesub, and P. Wanuchsoontorn. 1993. Culture of the green mussel (Perna viridis) in wastewater from an intensive shrimp pond: concept and practice. *World Aquac.* 24: 68–73.
Lin, C.K. 1995. Progression of intensive marine shrimp culture in Thailand. In: CL Browdy and JS Hopkins (editors), swimming through troubled water, pp. 13-23. Proceedings of the Special Session on Shrimp Farming. World Aquaculture Society, Baton Rouge, USA.
Lin, S., and L. Luo. 2011. Effects of different levels of soybean mealinclusion in replacement for fish meal on growth, digestiveenzymes and transaminase activities in practical diets for juveniletilapia, *Oreochromis niloticus* vs *O. aureus*. *Anim. Feed Sci. Technol.* 168: 80-87.
Lin, T.F., H.I. Huang, F.T. Shen, and C.C. Young. 2006. The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-Al74. *Bioresource Technol.* 97: 957–960.
Little, E.C.S., and I.E. Henson. 1967. The water content of some waste lagoons. M.S. Thesis, Louisiana State Univ. 74 p. important tropical water weeds. *Pest Artic. News Sum. J.* 13: 223-227.
Liu, B., Y. Liu, Z. Liu, D. Qiu, G. Sun, and X. Li. 2014. Influence of stocking density on growth, body composition and energy budget of Atlantic salmon *Salmo salar* L. in recirculating aquaculture systems. *Chinese J. Ocean. Limno*. 32: 982-990.
Liu, J.W., and S.L. Dong. 2001. Comparative studies on utilizing nitrogen capacity between two macroalgae *Gracilaria tenuistipitata* var. liui (rhodophyta) and *Ulva pertusa* (chlorophyta) I. Nitrogen storage under enrichment and starvation. *J. Env. Sci.* 13: 318-22.
Liu, J.X., and Q. Cai. 1998. Integrated aquaculture in Chinese lakes and paddy fields. *Ecol. Eng.* 11: 48–59.

Liu, X., H. Zhao, and S. Chen. 2006. Colonization of maize and rice plants by strain Bacillus megaterium C4. *Curr. Microbiol*. 52: 186-190.
Liu, Y., Y. Chen, and Q. Zhou. 2007. Effect of initial pH control on enhanced biological phosphorus removal from wastewater containing acetic and propionic acids. *J Chemos* 66: 123-129.
Losordo, T.M., M.R. Masser, and J. Rakocy. 1998. Recirculating Aquaculture Tank Production Systems: An Overview of Critical Considerations. Southern Regional Aquaculture Center. Publication No. 451, 6 pp.
Lowe, C.J., and W. Davidson. 2005. Plasma osmolarity, glucose concentration and erythrocyte responses of two Antarctic fishes to acute and chronic thermal change. *J. Fish Biol.* 67: 752-766.
Lu, J., Z. Fu, and Z. Yin. 2008. Performance of a water hyacinth (*Eichhornia crassipes*) system in the treatment of wastewater from a duck farm and the effects of using water hyacinth as duck feed. *J. Environ. Sci*. 20: 513-519.
Lu, Y.F., Y.R. Zhou, S. Nakai, M. Hosomi, H.L. Zhang, H.J. Kronzucker, W.M. Shi. 2014. Stimulation of nitrogen removal in the rhizosphere of aquatic duckweed by root exudate components. *Planta* 239:591-603.
Lupatsch, I., G.A. Santos, J.W. Schrama, and J.A.J. Verreth. 2010. Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax. Aquaculture* 298: 245-250.
Luthen, H., M. Claussen, and M. Bottger. 1999. Growth: progress in auxin research. *Prog. Bot.* 60: 315-340.
Lyerly, N.C. 2004. Swine Wastewater Treatment in an Integrated System of Anaerobic Digestion and Duckweed Nutrient Removal: Pilot Study. Dissertation. North Carolina State University, United States.
Ma, Y., M. Rajkumar, and H. Freitas. 2009. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by Brassica juncea. *J. Environ. Manage.* 90: 831-837.
Maclatchy, D.L., and J.G. Eales. 1990. Growth hormone stimulates thyroxine 5' monodeiodinase activity and 3, 5, 3'-triiodo-thyronine levels in rainbow trout, Salmo gairdneri. *Gen. Comp. Endocrinol.* 78: 164-172.
Maguire, G.B. B. Wisely, and M.E. Skeel. 1981. Cultivation of the Sydney rock oyster Crassostrea commercialis (Iredale and Roughley) in prawn farming ponds. Aquaculture 24: 63-75.
Maitra, N., S.K. Manna, S. Samanta, K. Sarkar, D. Debnath, C. Bandopadhyay, S.K. Sahu, and A.P. Sharma. 2014. Ecological significance and phosphorus release potential of phosphate solubilising bacteria in freshwater ecosystems. *Hydrobiologia* 745: 69-83.
Malboobi, M.A., P. Owlia, M. Behbahani, E. Sarokhani, S. Moradi, B. Yakhchali, A. Deljou, K. Morabbi-Heravi. 2009. Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates. *World J Microbiol Biotechnol*. 25 (8): 1471-1477.
Mamta, P. Rahi, V. Pathania, A. Gulati, B. Singh, R.K. Bhanwra, and R. Tewari. 2010. Stimulatory effect of phosphate-solubilising bacteria on plant growth, stevioside and rebaudioside-A contents of Stevia rebaudiana Bertoni. *Appl. Soil Ecol*. 46:222–229.
Mara, D. D., and S. Cairncross. 1989. Guidelines for the safe use of wastewater and excreta in agriculture and aquaculture: Measures for public health protection. Geneva, Switzerland: World Health Organization.
Maria Guineth, T.B., S.A. Valencia Plata, J.B. Castillo, and P.M. Nieto. 2000. Isolation of *Enterobacteria*, *Azotobacter* sp and *Pseudomonas* sp producers of indole- 3-acetic acid and siderophores from Colombian rice rizosphere. *Rev. Latinoam Microbiol.* 42: 171-176.

Marinho-Soriano, E., C. Morales, and W.S.C. Moreira. 2002. Cultivation of Gracilaria (Rhodophyta) in shrimp pond effluents in Brazil. *Aquacult. Res*. 33: 1081-1086.
Marten, G.C., and R.N. Andersen. 1975. Forage nutritive value and palatability of 12 common annual weeds. *Crop Sci.* 15:821-827.
Martinez-Cordova L.R., and M. Martinez-Porchas. 2006 Polyculture of Pacific white shrimp, *Litopenaeus vannamei*, giant oyster, Crassostrea gigas and black clam, *Chione fluctifraga* in ponds in Sonora, Mexico. *Aquaculture* 258: 321-326.
Martínez-Porchas, M., L.R. Martínez-Córdova, M.A. Porchas-Cornejo, and J.A. López-Elías. 2010. Shrimp polyculture: a potentially profitable, sustainable, but uncommon aquacultural practice. *Rev Aquacult*. 2: 73-85.
Martinez-Porchas, M., and L.R. Martinez-Cordova. 2012. World aquaculture: environmental impacts and troubleshooting alternatives." *The Scientific World Journal 2012*, 9.
Martins, C.I.M., E.H. Eding, C. Schneider, R. Rasmussen, B. Olesen, L. Plesner, J.A.J. Verreth. 2005. Recirculation Aquaculture Systems in Europe. CONSENSUS. Oostende, Belgium, Consensus working Group, European Aquaculture Society, 31.
Martins, C.I.M., M.G. Pistrin, S.S.W. Ende, E.H. Eding, and J.A.J. Verreth. 2009. The accumulation of substances in Recirculating Aquaculture Systems (RAS) affects embryonic and larval development in common carp *Cyprinus carpio*. *Aquaculture* 291: 65-73.
Martins, C.I.M., E.H. Eding, M.C.J. Verdegem, L.T.N. Heinsbroek, O. Schneider, J.P. Blancheton, E. Roque d' Orbcastel, and J.A.J. Verreth. 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacult. Eng.* 43: 83-93.
Mathew, G. 2009. Taxonomy, identification and biology of Seabass (*Lates calcarifer*). National Training on 'Cage Culture of Seabass' held at CMFRI, Kochi. Central Marine Fisheries Research Institute, India.
Matos, J., S. Costa, A. Rodrigues, R. Pereira, and I. Sousa. 2006. Experimental integrated aquaculture of fish and red seaweeds in northern Portugal. *Aquaculture* 252: 31-42.
Maule, A.G., C.B. Shreck, and C. Sharp. 1993. Seasonal changes in cortisol sensitivity and glucocorticoid receptor affinity and number in leukocytes of Coho salmon. *Fish Physiol. Biochem.* 10: 497-506.
Maynard, L., J. Loosli, H. Hintz, R. Warner. 1979. In: Zappa, C.R. (Ed.), Animal Nutrition, seventh ed. McGraw-Hill, New York, pp. 13–14.
Mazeaud, M.M., F. Mazeaud, and E.M. Donaldson. 1977. Primary and secondary effects of stress in fish: some new data with a general review. *Trans. Am. Fish. Soc.* 106: 201-212.
Mazlum, Y. 2007. Stocking density affects the growth, survival, and cheliped injuries of third instars of narrow-clawed crayfish, *Astacus leptodactylus* Eschscholtz, 1823 juveniles. *Crustaceana* 80: 803-815.
McDonald, D.G., M.D. Goldstein, and C. Mitton. 1993. Responses of hatchery- reared brook trout, lake trout and splake to transport stress. *Trans. American Fish Soc.* 122: 1127-1138.
McDonald, G., and L. Milligan. 1997. Ionic, osmotic and acid-base regulation in stress. In: Iwama, G.W., Pickering, A.D., Sumpter, J.P., Schreck, C.B (Eds.), Fish Stress and Health in Aquaculture. Cambridge University Press, New York, pp. 119-144.
McGraw, W.J. 2002. Utilization of heterotrophic and autotrophic bacteria in aquaculture. *Global Aquacult. Advoc*. 5: 82-83.
McIntosh, R.P., 1999. Changing paradigms in shrimp farming: I. General description. *Global Aquacult Adv*. 2: 40-47.

McKenzie, D.J., E. Höglund, A. Dupont-Prinet, B.K. Larsen, P.V. Skov, P.B. Pedersen, and A. Jokumsen. 2012. Effects of stocking density and sustained aerobic exercise on growth, energetics and welfare of rainbow trout. *Aquaculture* 338-341: 216-222.

McLay, C. L. 1976. The effect of ph on the population growth of three species of duckweed: *Spirodella oligorrhiza, Lemna minor* and *Wolffia arrhiza*. *Freshwater Biol.* 6: 125-36.

Medda, A.K., and A.K. Ray. 1979. Effect of thyroxine and analogs on protein and nucleic acid contents of liver and muscle of lata fish (*Ophicephalus punctatus*). *Gen. Comp. Endocrinol*. 9: 473-480.

Mehta, P., A. Walia, A. Chauhan, and C.K. Shirkot. 2013. Plant growth promoting traits of phosphate-solubilising rhizobacteria isolated from apple trees in trans Himalayan region of Himachal Pradesh. *Arch. Microbiol.* 195 (5): 357-369.

Mehta, S., and C.S. Nautiyal. 2001. An efficient method for qualitative screening of phosphate-solubilising bacteria. *Curr. Microbiol.* 43: 51-56.

Melotti, P., A. Roncarati, E. Garella, O. Garnerval, G. Mosconi, and A. Polzonetti- Magni. 1992. Effects of handling and capture stress on plasma glucose, cortisol and androgen levels in brown trout, *Salmo trutta* morpha fario. *J. Appl. Icthyol*. 8: 234-239.

Merino, G.E., R.H. Piedrahita, and D.E. Conklin. 2007. Settling characteristics of solids settled in a recirculating system for California halibut (*Paralichthys californicus*) culture. *Aquacult. Eng*. 37: 79-88.

Metaxa, E., G. Deviller, P. Pagand, C. Alliaume, C. Casellas, and J.P. Blancheton. 2006. High rate algal pond treatment for water reuse in a marine fish recirculation system: water purification and fish health. *Aquaculture* 252: 92-101.

Metwally, M.A.A., and M. Wafeek. 2014. Effect of ammonia toxicity on carbohydrate metabolism in nile tilapia (*Oreochromis niloticus*). *World J. Fish Mar. Sci.* 6: 252-261.

Metz, J.R., E.J.W. Geven, E.H. Van den Burg, and G. Flik. 2005. ACTH, alpha-MSH and control of cortisol release: cloning, sequencing and functional expression of the melanocortin-2 and melanocortin-5 receptor in *Cyprinus carpio*. *Am. J. Physiol.* 289: 814–826.

Michaud, L., J.P. Blancheton, V. Bruni, and R. Piedrahita. 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. *Aquacult Eng*. 34: 224-233.

Michaud, L., Lo Giudice, A., Troussellier, M., Smedile, F., Bruni, V., and Blancheton, J.P. 2009. Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system. *J. Appl Microbiol.* 107: 1935-1946.

Midlen, A., and T.A. Redding. 1998. Environmental Management for Aquaculture. Kluwer Academic Publishers, London: 215pp.

Millward, D.J. 1989. The nutritional regulation of muscle growth and protein turnover. *Aquaculture* 79: 1-28.

Mishra, B. 2007. On duckweed, the multi-use aquatic plant. *Fishing Chimes* 27 (5): 11-13.

Mohamad, H.A., U.N. Uka, Y.A.B. Yauri. 2013. Evaluation of nutritional composition of water lily (*Nymphaea lotus* Linn.) from Tatabu Flood Plain, north-central, Nigeria. *J. Fish Aquat Sci* 8: 261-264.

Mohapatra, S., T. Chakraborty, V. Kumar, G. Deboeck, and K.N. Mohanta. 2012. Aquaculture and stress management: a review of probiotic intervention. *J Anim Physiol Anim Nutr.* 2012: 1–26.

Mohapatra, S.B., and A.K. Patra. 2013. Effect of partial replacement of fishmeal with duckweed (*Lemna minor*) feed on the growth performance of *Cyprinus carpio* Fry. *IOSR-JAVS* 4: 34-37.
Mohapatra, S.B. 2015. Utilization of water hyacinth (Eichhornia crassipes) meal as partial fish protein Replacement in the diet of *Cyprinus carpio* fry. *Eur. J. Exper. Biol.* 5 (5): 31-36.
Mohedano, R.A., R.H.R. Costa, F.A. Tavares, and P. Belli. 2012. High nutrient removal rate from swine wastes and protein biomass production by full-scale duckweed ponds. *Bioresource Technol.* 112: 98-104.
Molleda, M.I. 2007. Water quality in recirculating aquaculture systems for arctic charr (*Salvelinus alpinus* L.) Culture, Project report-Fisheries Training Programme. The United Nations University.
Mommsen, T.P., M.M. Vijayan, and T.W. Moon. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Fish Biol. Fish*. 9: 211-268.
Montero, D., M.S. Izquierdo, L. Tort, L. Robaina, J.M. Vergara. 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiol. Biochem*. 20: 53-60.
Montero, D., L.E. Robaina, J. Socorro, J.M. Vergara, L. Tort, and M.S. Izquierdo. 2001. Alteration of liver and muscle fatty acid composition in gilthead sea bream (Sparus aurata) juveniles held at high stocking density and fed an essential fatty acid deficient diet. *Fish Physiol. Biochem.* 24: 63-72.
Moon, T.W. 2001. Glucose intolerance in teleost fish: fact or fiction?. *Comp. Biochem. Physiol.* B 129: 243-249.
Moore, S., and W.H. Stein. 1948. In: Colowick, S.P., Kaplan, N.D. (Eds.). Methods in Enzymology. Academic Press, New York, p. 468.
Moradi, N., M. Imanpoor, and V. Taghizadeh. 2013. Hematological and Biochemical Changes Induced by Replacing Fish Meal with Plant Protein in the *Cyprinus carpio* Linnaeus (1785). *Global Veterinaria* 11: 233-237.
Moss, S.M. 2000. Dietary importance of microbes and detritus in penaeid shrimp aquaculture. In: Lee, C.-S., O'Bryen, P. (Eds.), Microbial Approaches to Aquatic Nutrition within Environmentally Sound Aquaculture Production Systems. The World Aquaculture Society, Baton Rouge, Louisiana, USA.
Msuya, F.E., M.S. Kyewalyanga, and D. Salum. 2006. The performance of the seaweed *Ulva reticulata* as a biofilter in a low-tech, low-cost, gravity generated water flow regime in Zanzibar, Tanzania. *Aquaculture* 254: 284–292.
Muangkeow, B., K. Ikejima, S. Powtongsook, and Y. Yi. 2007. Effects of white shrimp, *Litopenaeus vannamei* (Boone), and Nile tilapia, *Oreochromis niloticus* L., stocking density on growth, nutrient conversion rate and economic return in integrated closed recirculation system. *Aquaculture* 269 (1-4): 363-376.
Muangkeow, B., K. Ikejima, S. Powtongsook, and W. Gallardo. 2010. Growth and nutrient conversion of white shrimp *Litopenaeus vannamei* (Boone) and Nile tilapia *Oreochromis niloticus* L. in an integrated closed recirculating system. *Aquacult. Res.* 42: 1246-1260.
Muangkeow, B., K. Ikejima, S. Powtongsook, and W. Gallardo. 2011. Growth and nutrient conversion of white shrimp *Litopenaeus vannamei* (Boone) and Nile tilapia *Oreochromis niloticus* L. in an integrated closed recirculating system. *Aquac. Res.* 42: 1246-1260.
Mukherjee, A.K., P. Kalita, B.G. Unni, S.B. Wann, D. Saikia, and P.K. Mukhopadhyay. 2010. Fatty acid composition of four potential aquatic weeds and their possible use as fish-feed nutraceuticals. *Food Chem.* 123: 1252-1254.

Mukhopadhyay, N., and A.K. Ray. 1999. Effect of fermentation on the nutritive value of sesame seed meal in the diets for roha, *Labeo rohita* (Hamilton), fingerlings. *Aquacult. Nutr*. 5: 229-236.

Murat, J.C., and A. Serfaty. 1970. Au subject d'un effect hypoglycemiant de la thyroxione chez la carpe, *Cyprinus carpio* L.C. *Séance Sac. Biol.* 164: 1842-1845.

Nabti, E.H., N. Mokrane, M. Ghoul, H. Manyani, M. Dary, and M.G. Megias. 2013. Isolation and characterization of two halophilic Bacillus (*B. licheniformis* and *Bacillus* sp) with antifungal activity. *J Eco Heal Env* 1: 13-17.

Naik, V.M., J. D'Souza, and A. Araujo. 1982. Phosphorus solubilising yeasts in estuarine environment. *Ind J. Mar Sci.* 11: 197-198.

Naher, U.A., O. Radziah, Z.H. Shamsuddin, M.S. Halimi, and I. Mohd Razi. 2009. Isolation of diazotrophs from different soils of Tanjong Karang Rice growing area in Malaysia. *Int J Agric. Biol.* 11: 547-552.

Nahlik, A.M., and W.J. Mitsch. 2006. Tropical treatment wetlands dominated by free-floating macrophytes for water quality improvement in Costa Rica. *Ecol. Eng.* 28: 246-257.

Nandakumar, S., K. Ambasankar, S.S.R. Ali, J. Syamadayal, K. Vasagam. 2017. Replacement of fish meal with corn gluten meal in feeds for Asian seabass (*Lates calcarifer*). Aquacult Int 25 (4): 1495-1505.

Nautiyal, C.S. 1999. An efficient microbiological growth medium for screening phosphate solubilising microorganisms. *FEMS Microbiol. Lett*. 170:265–270.

Naylor, R.L., R.J. Goldburg, J.H. Primavera, N. Kautsky, M.C.M. Beveridge, J. Claym, C. Folke, J. Lubchenco, H. Mooney, and M. Troell. 2000. Effect of aquaculture on world fish supplies. *Nature* 405: 1017-1024.

Nekoubin, H., and M. Sudagar. 2013. Effect of different types of plants (*Lemna sp*., *Azolla filiculoides* and *Alfalfa*) and artificial diet (with two protein levels) on growth performance, survival rate, biochemical parameters and body composition of grass carp (*Ctenopharyngodon idella*). *J. Aquac. Res. Development* 4, 2.

Nelson, S.G., B.D. Smith, and B.R. Best. 1980. Nitrogen uptake by tropical freshwater macrophytes. Technical Report by Water Resources Research Center of Guam Univ. Agana. (Available from National Technical Information Service, Springfield VA 22161 as PB80-194228).

Nelson, T.A., J. Olson, L. Imhoff, and A.V. Nelson. 2010. Aerial exposure and desiccation tolerances are correlated to species composition in "green tides" of the Salish Sea (northeastern Pacific). *Bot. Mar.* 53: 103-111.

Neori, A., I. Cohen, and H. Grodin. 1991. *Ulva lactuca* biofilter for marine fishpond effluents II. Growth rate, yield and C:N ratio. *Bot Mar*. 34: 483-489.

Neori, A., S.P. Ellner, C.E. Boyd, and M.D. Krom. 1993. The integration of seaweed biofilters with intensive fish ponds to improve water quality and recapture nutrients. In: Moshiri, G.A. (Ed.), Constructed Wetlands for Water Quality Improvement. Lewis Publishers, Boca Raton, FL, pp. 603–607.

Neori, A., M.D. Krom, S.P. Ellner, C.E. Boyd, D. Popper, R. Rabinovitch, P.J. Davison, O. Dvir, D. Zuber, M. Ucko, D. Angel, and H. Gordin. 1996. Seaweed biofilters as regulators of water quality in integrated fishseaweed culture units. *Aquaculture* 141: 183-199.

Neori, A., N.L.C. Ragg, and M. Shpigel. 1998. The integrated culture of seaweed, abalone, fish and clams in modular intensive land-based systems. II. Performance and nitrogen partitioning within an abalone (Haliotis tuberculata) and macroalgae culture system. *Aquacult. Eng.* 17 (4): 215-239.

Neori, A., M. Shpigel, and D.M. Ben-Ezra. 2000. A sustainable integrated system for culture of fish, seaweed and abalone. *Aquaculture* 186: 279–291.

Neori, A., F.E. Msuya, L. Shauli, A. Schuenhoff, F. Kopel, and M. Shpigel. 2003. A novel three stage seaweed (*Ulva lactuca*) biofilter design for integrated mariculature. *J. Appl. Phycol*. 15: 543–553.

Neori, A., T. Chopin, M. Troell, A.H. Buschmann, G.P. Kraemer, C. Halling, M. Shpigel, and C. Yarish. 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. *Aquaculture* 231: 361–391.

Neori, A., M.D. Krom, and J. Van-Rijn. 2007. Biogeochemical processes in intensive zero-effluent marine fish culture with recirculating aerobic and anaerobic biofilters. *J. Exp. Mar. Biol. Ecol*. 349: 235-247.

New, M.B., and U.N. Wijkström. 2002. Use of fishmeal and fish oil in aquafeeds. Further thoughts on the fishmeal trap. FAO fisheries circular (FAO Fish. Circ.). No. 975, 61 pp.

Newell, R.I.E. 2004. Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. *J. Shellfish Res*. 23: 51-62.

Ng, W.J., T.S. Sim, and K.K. Ong. 1990. The effect of Elodea densa on aquaculture water quality. *Aquaculture* 87: 267-276.

Nguyen, C., W. Yan, F. Le Tacon, and F. Lapayrie. 1992. Genetic variability of phosphate solubilising activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus *Laccaria bicolor* (Maire) P.D. Orton. *Plant Soil* 143: 193-199.

Nhapi, I. 2004. Potential for the use of duckweed-based pond systems in Zimbabwe. *Water SA* 30 (1).

Nichols, J.W., G.A. Wedemeyer, F.L. Mayer, W.W. Dickhoff, S.V. Gregorgy, W.T. Yasutake, and S.D. Smith. 1984. Effects of fresh water exposure to arsenic trioxide on the par-smolt transformation of coho salmon, *Oncorhynchus kisutch*. *J. Environ. Toxicol. Chem.* 3: 143.

Nicula, M. 2004. Fiziologia Organismelor Acvatice. I. Fiziologia peştilor, first ed. Mirton, Timişoara, Romania.

Nikinma, M., and W.H. Huestis. 1984. Adrenergic swelling of nucleated erythrocytes: cellular mechanisms in a bird, domestic goose, and two teleosts, striped bass and rainbow trout. *J. Exp. Biol*. 113, 215-224.

Noble, T.H., C. Smith-Keune, and D.R. Jerry. 2014. Genetic investigation of the large-scale escape of a tropical fish, barramundi, *Lates calcarifer*, from a sea-cage facility in northern Australia. *Aquacult. Environ. Interact*. 5: 173-183.

Nobre, A.M., D. Robertson-Andersson, A. Neori, and K. Sankar. 2010. Ecological economic assessment of aquaculture options: comparison between abalone monoculture and integrated multi-trophic aquaculture of abalone and seaweeds. *Aquaculture* 306: 116-126.

Noctor, G., L. Gomez, H. Vanacker, and C.H. Foyer. 2002. Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *J. Exp. Bot*. 53: 1283-1304.

North, B.P., J.F. Turnbull, T. Ellis, M.J. Porter, H. Migaud, J. Bron, and N.R. Bromage. 2006. The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 255: 466-479.

Nouanthavong, T., and T.R. Preston. 2011. Comparison of natural pond system with an intensive (indoor) system for raising Cachama (*Colossoma macropomu*) and Tilapia (*Oreochromis niliticus*). http://www.lrrd.org/lrrd23/4/tick23100.htm.

Nout, M.J.R. 2009. Rich nutrition for the poorest-cereal fermentations in Africa and Asia. *Food Microbiol.* 26: 682-685.

NRC-NAS. 1977. Nutrient requirements of warm water fishes, National Research Council—National Academy of Sciences, Washington, DC, p. 78.

Nussey, G., J.H.J. Van Vuren, and H.H. Du-Preez. 1995. Effect of copper on the haematology and osmoregulation of the Mozambique tilápia Oreochromis mossambicus (Cichlidade). *Comp. Biochem. Physiol*. 111: 369-380.

Okoye, P.C., F. Daddy, and B.D. Jiesanmi. 2002. The nutritive value of water hyacinth (*Eichhornia crassipes*) and its utilisation in fish feed. National Institute for Freshwater Fisheries Research, P. M. B. 6606, New Bussa, Niger State.

Olguín, E.J., F.J. Melo-Jiménez, V.J. Hernández-Landa, R.E. González-Portela, G. Sánchez-Galván. 2015. Evaluation of a phytoremediation system operated in continuous mode using *Pistia stratiotes*. IV SOLABIAA Latin American Conggress. Brazil.

Oliveira, C.A., V.M.C. Alves, I.E. Marriel, E.A. Gomes, M.R. Scotti, N.P. Carneiro, C.T. Guimara˜es, R.E. Schaffert, and N.M.H. Sa. 2009. Phosphate solubilising microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. *Soil Biol. Biochem.* 41: 1782-1787.

Ormandy, E.G., J. Dale, and G. Griffin. 2011. Genetic engineering of animals: Ethical issues, including welfare concerns. *Can Vet J.* 52: 544-550.

Oron, G., L.R. Wildschut, and D. Porath. 1984 Wastewater recycling by duckweed for protein production and effluent renovation. *Wat. Sci. Tech.* 17:803-817.

Oron, G., D. Porath, and H. Jansen. 1987. Performance of duckweed species *Lemna gibba* on municipal water for effluent renovation and protein production. *Biotechnol. Bioengineer* 29: 258-267.

Oron, G., A. De-Vegt, and D. Porath. 1988. Nitrogen removal and conversion by duckweed grown on wastewater. *Water Res*. 22 (2): 179-184.

Oron, G. 1994. Duckweed culture for wastewater renovation and biomass production. *Agric. Water Manage.* 26, 27-40.

ORR. 1999. Final Report: Office of Regulatory Reform Task Force Review of Regulatory Arrangements in the Victorian Aquaculture Industry. Office of Regulatory Reform, Melbourne.

Osofero, S.A., S.O. Otubusin, J.A. Daramola. 2009. Effect of stocking density on tilapia (*Oreochromis niloticus* Linnaeus 1757) growth and survival in bamboo-net cages trial. *Afr. J. Biotechnol.* 8: 1322–1325.

O'Toole, G., H.B. Kaplan, and R. Kolter. 2000. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* 54: 49-79.

Owen, L., and S.I. Sunram-Lea. 2011. Metabolic agents that enhance ATP can improve cognitive functioning: a review of the evidence for glucose, oxygen, pyruvate, creatine, and L-carnitine. *Nutrients* 3: 735-755.

Ozengin, N., and A. Elmaci. 2007. Performance of duckweed (*Lemna minor* L.) on different types of wastewater treatment. *J Environ. Biol.* 28: 307-314.

Paez-Osuna, F. 2001. The environmental impact of shrimp aquaculture: causes, effects, and mitigating alternatives. *Environ. Manage.* 28 (1): 131 – 140.

Paez-Osuna, F., A. Gracia, F. Flores-Verdugo, L.P. Lyle-Fritch, R. Alonso-Rodriguez, A. Roque, and A.C. Ruiz Fernandes. 2003. Shrimp aquaculture development and the environment in the Gulf of California ecoregion. *Marine Pollut. Bull.* 46: 806-815.

Pankhurst, N.W., and M. Dedual. 1994. Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. *J. Fish Biol.* 45:1013-1025.

Pankhurst, N.W. 2011. Neuroendocrine system controlling the stress response. *Gen. Comp. Endocrinol.* 170: 265-275.

Papoutsoglou, S., N. Karakatsouli, G. Pizzonia, A. Christina, A. Polissidis, and Z. Papoutsoglou. 2006. Effects of rearing density on growth, brain neurotransmitters and liver fatty acid composition of white sea-bream *Diplodus sargus* L. *Aquacult. Res*. 37: 87-95.

Park, J., N. Renukdas, T. Luna, and L.A. Roy. 2015. The effects of biomass density on size variability and growth performance of juvenile largemouth bass, *Micropterus salmoides*, in a semi-closed recirculating system. *J. World. Aquacult. Soc.* 46: 283-291.

Patra, A.K. 2015. Evaluation of the duckweed (*Lemna minor*) meal as partial replacement for fish meal on the growth performance of *Labeo rohita* (Ham.) fry. *European J. Experiment. Biol.* 5: 18-23.

Pauly, D., J. Alders, E. Bennett, V. Chrisensen, P. Tyedemers, and R. Watson. 2003. The Future of Fisheries. *Science* 302: 1359-1361.

Peng, S.H., W.M. Wan-Azha, W.Z. Wong, W.Z. Go, E.W. Chai, K.L. Chin, and P.S. Hung 2013. Effect of using agro-fertilizers and N-fixing *Azotobacter* enhanced biofertilizers on the growth and yield of corn. *J. Appl Sci.* 13: 508-512.

Percival, P.A. 1999. Physiological stress response of barramundi (*Lates calcarifer*). PhD Thesis. James Cook University, Queensland.

Perna, A., and E.P. Demilio. 1986. Influence of submerged macrophytes on the oxygen content of a river used for trout farming (R. Ticino, Central Italy). *Riv. Ital. Piscic. Ittiopatol*. 21: 138-40.

Person-Le Ruyet, J., L. Labbé, N. Le Bayon, A. Sévère, A. Le Roux, H. Le Delliou, and L. Quéméner. 2008. Combined effects of water quality and stocking density on welfare and growth of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Living Resour.* 21, 185-195.

Petersen, T.D.P., P.W. Hochavhka, and R.K. Saurez. 1987. Hormonal control of gluconeogenesis in rainboe trout hepatocytes: regulatory role of pyruvate kinase. *J. Exp. Zool*. 243: 173-180.

Petrucio, M.M., and F.A. Esteves. 2000. Uptake rates of nitrogen and phosphorus in the water by *Eichhornia crassipes* and *Salvinia auriculata*. *Rev. Brasil. Biol.* 60: 229-236.

Pfeiffer, T., and R. Malone. 2006. Nitrification performance of a propeller-washed bead clarifier supporting a fluidized sand biofilter in a recirculating warmwater fish system. *Aquacult. Eng*. 34: 311-321.

Phillips, J.C., and C.L. Hurd. 2004. Kinetics of nitrate, ammonium, and urea uptake by four intertidal seaweeds from New Zealand. *J. Phycology* 40: 534-545.

Phillips, M.J., and D.J. Macintosh. 1997. Aquaculture and the environment: Challenges and opportunities, p.159-170. In K.P.P. Nambiar and T. Singh (eds.) Sustainable Aquaculture. Proceedings of INFOFISH-AQUATECH '96 International Conference on Aquaculture. Kuala Lumpur, Malaysia, 25-27 September 1996. Kuala Lumpur, INFOFISH. 248 p.

Pickering, A.D., and A. Stewart. 1984. Acclimation of the internal tissue of the brown trout, Salmo trutta L., to chronic crowding stress. *J. Fish Biol*. 24: 731-740.

Pickering, A.D., and T.R. Pottinger. 1987. Crowding causes prolonged leucopenia in salmonid fish, despite interrenal acclimation. *J. Fish Biol*. 30: 701-712.

Pickering, A.D., and T.G. Pottinger. 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of cortisol. *Fish Physiol Biochem* 7:253-258.

Pickering, A.D. 1990. Stress and the suppression of somatic growth in teleosts fish. In: Prasad MRN (ed) Progress in comparative endocrinology. Wiley, New York, pp 112-134.

Pickering, A.D. 1992. Rainbow trout husbandary: management of the stress response. *Aquaculture* 100: 125-139.

Pickering, A.D. 1993. Growth and stress in fish production. *Aquaculture* 111: 51-63.

Piedrahita, R.H. 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. *Aquaculture* 226: 35-44.

Petersen, E.H., B.D. Glencross, N.V. Tien, L.A. Tuan, V.A. Tuan, and T.H. Phuong. 1987. Recent changes in the bio-economic of finfish mariculture in Vietnam. *J. Aquac. Res. Development* 6: 3.
Pinhassi, J., F. Azam, J. Hemphala, R.A. Long, J. Martinez, U.L. 1999. Coupling between bacterioplankton species composition, population dynamics, and organic matter degradation. *Aquat. Microb Ecol.* 17: 13–26.
Placinta, S., C. Victor, C.M. Tiberiu, and P.S. Mihai. 2014. Hematological profile of juvenile European catfish (*Silurus glanis*) reared under different stocking densities in recirculating system conditions. *Zooteh. Bioteh.* 47: 246-251.
Policar, T., V. Stejskal, J. Kristan, P. Podhorec, V. Svinger, and M. Blaha. 2013. The effect of fish size and stocking density on the weaning success of pond-cultured pikeperch (*Sander lucioperca*) L. juveniles. *Aquacult Int.* 21: 869–882.
Ponmurugan, P., and C. Gopi. 2006. In vitro production of growth regulators and phosphate activity by phosphate solubilising bacteria. *Afr J Biotech* 5: 348-350.
Popovic, N.T., R. Coz-Rakovac, and I. Strunjak-Perovic. 2007. Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria: a review. *Vet Med 52: 49-53*.
Porath, D., and J. Pollock. 1982. Ammonia stripping by duckweed and its feasibility in circulating aquaculture. *Aquat. Bot.* 13: 125-131.
Porath, D., and M. Agami. 1977. Enhancement of protein production in fish ponds with duckweed (*Lemnaceae*). *Israel J. Bot.* 26: 51-51.
Pottinger, T.G., M. Rand-Weaver, and J.P. Stumper. 2003. Over- winter fasting and refeeding in rainbow trout: plasma growth hormones and cortisol levels in relation to energy mobilization. *Comp. Biochem. Physiol* 95: 313- 317.
Pouey, J.L.O.F., S.R.N. Piedras, C.B. Rocha, R.A. Tavares, and J.D.M. Santos. 2011. Productive performance of silver catfish, *Rhamdia quelen*, juveniles stocked at different densities. *Ars Veterinaria* 27: 241-245.
Power, D.M., L. Llewellyn, M. Faustino, M.A. Nowell, B.T. Björnsson, I.E. Einarsdottir, A.V.M. Canario, and G.E. Sweeney. 2001. Thyroid hormones in growth and development of fish. Comp. Biochem. Physiol., Part C: *Toxicol. Pharmacol.* 130: 447-459.
Pradhan, N., and L.B. Sukla. 2005. Solubilization of inorganic phosphate by fungi isolated from agriculture soil. *Afr J Biotechnol* 5: 850-854.
Prak Kea, T.R. Preston, and J. Ly. 2003. Effect of level of fish meal on growth and feed conversion of pigs fed a basal diet of water spinach supplemented with palm oil and broken rice. MSc thesis in Sustainable Livestock Production, Swedish University of Agricultural Science, Uppsala, pp. 41-51.
Procarione, L.S., T.B. Barry, and J.A. Malison. 1999. Effects of high rearing densities and loading rates on the growth and stress response of juvenile rainbow trout. *N Am J Aquacult* 61:91-96.
Pullin, R.S.V. 1989. Third world aquaculture and the environment. NAGA; The ICLARM Quarterly. January 1989.
Rakocy, J.E., and M.P. Masser. 2006. Recirculating Aquaculture Tank Production Systems: Aquaponics—Integrating Fish and Plant Culture. SRAC Publication.
Ramírez-Godínez, J., R.I. Beltrán-Hernández, C. Coronel-Olivares, E. Contreras-López, M. Quezada-Cruz, and G. Vázquez-Rodríguez. 2013. Recirculating Systems for Pollution Prevention in Aquaculture Facilities. *J Water Res Protect* 5: 5-9.
Ramsay, J., G.Z. Varga, M.K. Westerfield, and M.C. Schreck. 2006. Whole-body cortisol as indicator or crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* 258: 565-574.

Ran, N., M. Agami, and G. Oron. 2004. A pilot study of constructed wetlands using duckweed (*Lemna gibba* L.) for treatment of domestic primary effluent. *Water Res*. 38: 2241–2248.
Randall, D.J., and T.K.N. Tsui. 2002. Ammonia toxicity in fish. *Mar. Poll. Bull.* 45: 17-23.
Randall, D. J., and S.F. Perry. 1992. Catecholamine. In: Hoar, W. S., Randall, D. J., Farrell, T. P. (eds) Fish Physiology, Vol. XII, Academic Press, New York.
Rao, J.V. 2006. Biochemical alterations in euryhaline fish, *Oreochromis mossambicus* exposed to sub-lethal concentrations of an organophosphorus insecticide, monocrotophos. *Chemosphere* 65 (10): 1814-20.
Rashid, M., S. Khalil, N. Ayub, S. Alam, and F. Latif. 2004. Organic acids production and phosphate solubilization by phosphate solubilising microorganisms (PSM) under in vitro conditions. *Pak. J. Biol. Sci.* 7: 187-196.
Ray, A.K., and I. Das. 1992. Utilization of diets containing composted aquatic weed (Salvinia cuculata) by the Indian major carp, rohu, (*Labeo rohita* Ham.) fingerlings. *Biores. Technol*. 40: 67–72.
Razzolini, M.T., M. Di Bari, P.S. Sanchez, and M.I. Sato. 2008. Aeromonas detection and their toxins from drinking water from reservoirs and drinking fountains. *J Water Health* 1: 117-123.
Redding, J.M., C.B. Schreck, E.K. Birks, and R.D. Ewing. 1991. Cortisol and its effects on plasma thyroid hormone and electrolyte concentrations in fresh water and during seawater acclimation in yearling coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol*. 81 (3): 373-382.
Redding, T., S. Todd, and A. Midlen. 1997. The treatment of aquaculture wastewater - A botanical approach. *J. Environ. Manage.* 50: 283-299.
Reddy, K.R., K.L. Campbell, D.A. Graetz, and K.M. Portier. 1982. Use of biological filters for treating agricultural drainage effluents. *J. Environ. Qual*. 11: 591-595.
Reddy, K.P., and W.F. DeBusk. 1985. Growth characteristics of aquatic macrophytes cultured in nutrient enriched water: II. Azolla, duckweed, and salvinia. *Econ. Bot*. 39, 200-208.
Reddy, K.R., and D.A. Graetz. 1987. Carbon and nitrogen dynamics in wetland soils. In: Hook, D.D. et al., Eds., The ecology and management of wetlands, Springer, Berlin, 307-318.
Reddy, K.R., and W.H. Smith. 1987. Aquatic plants for wastewater treatment and resource recovery. Magnolia Publishing Inc., Orlando, FL, USA.
Reddy, P.M., and M. Bashamohideen. 1989. Fenvalerate and cypermethrin induced changes in the haematological parameters of *Cyprinus carpio*. *Acta Hydrochim. Hydrobiol.* 17: 101-107.
Refstie, T., and A. Kittelsen. 1976. Effect of density on growth and survival of artificially reared Atlantic salmon. *Aquaculture* 8: 319-326.
Refstie, T. 1977. Effect of density on growth and survival of rainbow trout. *Aquaculture* 1: 329-334.
Reid, S.G., M.M. Vijayan, and S.F. Perry. 1996. Modulation of catecholamine storage and release by pituitary- interregnal axis in the rainbow trout, *Oncorhynchus mykiss*. *J. Comp. Physiol*. B 165: 665-676.
Ren, M.C., Q.H. Ai, K.S. Mai, H.M. Ma, and X.J. Wang. 2011. Effect of dietary carbohydrate level on growth performance, body composition, apparent digestibility coefficient and digestive activities of juvenile cobia, Rachycentron canadum L. *Aquac Res* 42: 1467-1475.
Ren, Y.X., X.L. Zhu, D.D. Fan, P. Ma, and H. Liang. 2013. Inoculation of phosphate solubilising bacteria for the improvement of lead accumulation by *Brassica juncea*. *Environ. Technol.* 34: 463-469.

Reynolds, C.S., and P.S. Davies. 2001. Sources and bioavailability of phosphorus fractions in freshwaters: a British perspective. *Biol. Rev.* 76 (1): 27-64.

Riche, M., C.R. Weirich, and P.S. Wills. 2013. Stocking density effects on production characteristics and body composition of market size cobia, *Rachycentron canadum*, reared in recirculating aquaculture systems. *J. World Aquacult. Soc.* 44: 259-266.

Ridha, M.T. 2005. Comparative study of growth performance of three strains of Nile tilapia, *Oreochromis niloticus*, L. at two stocking densities. *Aquacult. Res*. 37: 172-179.

Riise, J.C., and N. Roose. 1997. Benthic metabolism and the effects of bioturbation in a fertilised polyculture fish pond in northeast Thailand. *Aquaculture* 150: 45-62.

Rimmer, M.A., A.W. Reed, M.S. Levitt, and A.T. Lisle. 1994. Effects of nutritional enhancement of live food organisms on growth and survival of barramundi, Lates calcarifer (Bloch), larvae. *Aquacult. Res*. 25(2): 143-156.

Rimmer, M.A., and D.J. Russell. 1998. Aspects of the biology and culture of *Lates calcarifer*. In: de Silva, S.S. (Eds.), Tropical Mariculture, Academic Press, U.S.A., pp. 449-476.

Robinson, E.H., R.P. Wilson, and W.E. Poe. 1981. Arginine requirement and apparent absence of a lysine–arginine antagonist in fingerling channel catfish. *J. Nutr*. 11: 46–52.

Robledo, D., L. Navarro-Angulo, D.V. Lozano, and Y. Freile-Pelegrin. 2014. Nutrient removal efficiency of Hydropuntia cornea in an integrated closed recirculation system with pink shrimp *Farfantepenaeus brasiliensis*. Aquacult. Res. 45: 1648-1658.

Rodriguez, H., and R. Fraga. 1999. Phosphate solubilising bacteria and their role in plant growth promotion. *Biotechnol Adv* 17: 319-339.

Romarheim, O., H.A. Skrede, Y. Gao, A. Krogdahl, V. Denstadli, E. Lilleend, and T. Storebakken. 2006. Comparison of white flakes and toasted soybean meal partly replacing fish meal as protein source in extruded feed for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 256: 354-364.

Roncarati A., P. Melotti, A. Dees, O. Mordenti, and L. Angellotti. 2006. Welfare status of cultured seabass (*Dicentrarchus labrax* L.) and seabream (*Sparus aurata* L.) assessed by blood parameters and tissues characteristics. *J. Appl. Ichthyol.* 22: 225–234.

Roque D'Orbcastel, E., G. Lemarié, B. Gilles, P. Tommaso, M. Giovanna, T. Sébastien, D. Gilbert, F. Sveinung, C. Jean-Luc, and J.P. Blancheton. 2010. Effects of rearing density on sea bass (*Dicentrarchus labrax*) biological performance, blood parameters and disease resistance in a flow through system. *Aquat Liv. Res*. 23(1): 109-117.

Roques, J.A.C., E. Schram, T. Spanings, T. Van Schaik, W. Abbink, J. Boerrigter, P. De Vries, H. Van de Vis, and G. Flik. 2015. The impact of elevated water nitrite concentration on physiology, growth and feed intake of African catfish *Clarias gariepinus* (Burchell 1822). *Aquacult. Res.* 46: 1384-1395.

Rotlant, J., M. Pavlidis, M. Kentouri, M.E. Abad, and L. Tort.1997. Non-specific immune responses in the red porgy Pagrus pagrus crowding stress. *Aquaculture* 156:279-290.

Rottman, R.W., R. Fransis- Floyd, and R. Durborow. 1992. The role of stress in fish disease. SRAC Publication no. 474, 4p.

Rowland, S.J., C. Mifsud, M. Nixon, and P. Boyd. 2006. Effects of stocking density on the performance of the Australian freshwater silver perch (*Bidyanus bidyanus*) in cages. *Aquaculture* 253: 301-308.

Rubim, M.A.L., P.L. Isolino Sampaio, and P. Parolin. 2015. Biofilter efficiency of Eichhornia crassipes in wastewater treatment of fish farming in Amazonia. YTON 84: 244-251.

Ruane, N.M., E.C. Carballo, and J. Komen. 2002. Increased stocking density influences the acute physiological stress response of common carp *Cyprinus carpio* (L.). *Aquacult. Res.* 33: 777-784.

Russell, J.B., and G.M. Cook. 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. *Microbiol. Rev.* 59: 48–62.
Russell, A.J.M., P.A. Grotz, S.K. Kriesemer, and D.E. Pemsl. 2008. Country case study: development and status of freshwater aquaculture in Malawi. WorldFish Center Studies and Reviews: viii + 52 pp.
Ryder, E., S.G. Nelson, C. Mckeon, E.P. Glenn, K. Fitzsimmons, and S. Napolean. 2004. Effect of water motion on the cultivation of the economic seaweed *Gracilaria parvispora* (Rhodophyta) on Molokai, Hawaii. *Aquaculture* 238: 207–219.
Ryther, J.H., J.C. Goldman, C.E. Gifford, J.E. Huguemin, A.S. Wing, J.P. Clarner, L.D. Williams, and B.E. Lapointe. 1975. Physical models of integrated waste recycling marine polyculture systems. *Aquaculture* 5: 163-177.
Sabra, W.A., A.P. Zeng, H. Lunsdorf, and W.D. Deckwer. 2000. Effect of oxygen on formation and structure of *Azotobacter vinelandii*: alginate and its role in protecting nitrogenase. *Appl. Environ. Microbiol*. 66: 4037-4044.
Sadler, J., N.W. Pankhurst, P.M. Pankhurst, and H. King. 2000. Physiological stress responses to confinement in diploid and triploid Atlantic salmon. *J. Fish Biol*. 56: 506-518.
Said, M., M. Saleem, and U. Noreen. 2005. Study on the growth performance and feed conversion ratio of *Labeo rohita* fed on soybean meal, blood meal and corn gluten 60%. *Pak. Vet. J.* 25(3): 121-126.
Saillant, E., A. Fostier, P. Haffray, B. Menu, S. Laureau, J. Thimonier, and B. Chatain. 2003. Effects of rearing density, size grading and parental factors on sex ratios of the sea bass (*Dicentrarchus labrax* L.) in intensive aquaculture. *Aquaculture* 221: 183-206.
Samad, M.A., M.A. Islam, and M.A. Khaleque. 2005. Effect of stocking density on the growth and survival rate of magur (*Clarias batrachus*) fry in laboratory and nursery ponds. *Pak. J. Biol. Sci.* 8: 338-344.
Sammouth, S., E.R. D’Orbcastel, G. Gasset, G. Lemarié, G. Breuil, J.L. Marino, S. Coeurdacier, A.D. Fivelst, and J.P. Blancheton. 2009. The effect of density on sea bass (*Dicentrarchus labrax*) performance in a tank-based recirculating system. *Aquacult. Engineer.* 40: 72-78.
Sandifer, P.A., and J.S. Hopkins. 1996. Conceptual design of a sustainable pond-based shrimp culture system.*Aquacult. Eng.* 15:41-52.
Sandu, S.I., G.D. Boardman, B.J. Watten, and B.L. Brazil. 2002. Factors influencing the nitrification efficiency of fluidized bed filter with a plastic bead medium. *Aquacult. Eng*. 26: 41-59.
Sanni, S., and O.I. Forsberg. 1996. Modeling pH and carbon dioxide in single-pass seawater aquaculture systems. *Aquacult. Eng*. 15: 91-110.
Satheeshkumar, P., G. Ananthan, D. Senthilkumar, A.B. Khan, and K. Jeevanantham. 2012. Comparative investigation on haematological and biochemical studies on wild marine teleost fish from Vellar estuary, southeast coast of India. *Comparative Clinical Pathology* 21: 275-281. http://dx.doi.org/10.1007/s00580-010-1091-5.
Saunders, R.L., S.D. Mccormick, E.B. Henderson, J.G. Eales, and C.E. Jhonson. 1985. The effect of orally administered 3, 5, 3’-triiodo-thyronine on growth and salinity tolerance of the Atlantic salmon, *Salmo solar*. *Aquaculture* 45: 143-156.
Schanderi, S.H. 1970. Method in Food Analysis. Academic Press, New York.
Schipp, G., J. Bosmans, and J. Humphrey. 2007. NT Barramundi faming handbook. Darwin Aquaculture Centre, Department of Primary Industry, Fisheries and Mines.
Schneider, O., V. Sereti, E.H. Eding, and J.A.J. Verreth. 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. *Aquacult. Eng.* 32 (3-4): 379-401.

Schneider, O., J.P. Blancheton, L. Varadi, E.H. Eding, and J.A.J. Verreth. 2006. Cost price and production strategies in European recirculation systems. Linking Tradition & Technology Highest Quality for the Consumer, Firenze, Italy, WAS.

Scholten, M.C.T., E.M. Foekema, H.P. Van Dokkum, N.H.B.M. Kaag, and R.G. Jak. 2005. Eutrophication Management and Ecotoxicology. Springer. The Netherlands.

Schram, E., J.W. Van der Heul, A. Kamstra, and M.C.J. Verdegem. 2006. Stocking density dependent growth of dover (*Solea solea*). *Aquaculture* 252: 239-247.

Schreck, C.B. 1982. Stress and rearing of salmonids. *Aquaculture* 28: 241-249.

Schreck, C.B., R. Pating, C.K. Pring, J.R. Winto, and J.E. Holway. 1985. Effect of rearing density on indices of smoltification and performance of coho salmon (*Oncorhynchus kisutch*). *Aquaculture* 45: 345-358.

Schreck, C.B., B.L. Olla, and M.W. Davis. 1997. Behavioral responses to stress. In: Iwana, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish stress and health in aquaculture. Society for Experimental Biology, Seminar Series 62, Cambridge University Press, Cambridge, pp. 145-170.

Schreck, C.B. 2000. Accumulation and long-term effects of stress in fish. pp. 147-158. In: Moberg, G. P. & J. A. Mench (eds.). The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare, New York, Cabi Publishing, 485p.

Schreier, H.J., N. Mirzoyan, and K. Saito. 2010. Microbial diversity of biological filters in recirculating aquaculture systems. *Curr Opin Biotechnol* 21: 318–325.

Schuenhoff, A., M. Shpigel, L. Lupatsh, A. Ashkenazi, F.E. Msuya, and A. Neori. 2003. A semi recirculating inof fish and seaweed. *Aquaculture* 2001: 167-181.

Schuenhoff, A., L. Mata, and R. Santos. 2006. The tetrasporophyte of Asparagopsis armata as a novel seaweed biofilter. *Aquaculture* 252: 3–11

Schulz, C., J. Gelbrecht, and B. Rennert. 2004. Constructed wetlands with free water surface for treatment of aquaculture effluents. *J. Appl. Ichthyol*. 20: 64-70.

Schwartz, M.F., and C.E. Boyd. 1995. Constructed wetlands for treatment of channel catfish pond effluents. *Prog. Fish-Cult.* 57: 255-266.

Segner, H., R. Oesch, H. Schmidt, K. von Poeppinghausen. 1989. Digestive enzymes in larval *Coregonus larvaretus* L. *J. Fish Biol*. 35: 249-263.

Selvarani, A.J., P. Padmavathy, A. Srinivasan, P. Jawahar. 2015. Performance of Duckweed (*Lemna minor*) on different types of wastewater treatment. *Int J. Fish. Aquat. Stud.* 2 (4): 208-212.

Shah, K.A., S. Sumbul, and S.A. Andrabi. 2010. A Study on Nutritional Potential of Aquatic Plants. *VetScan* 5(1).

Shah, M., H.N. Hashmi, A. Ali, and A.R. Ghumman. 2014. Performance assessment of aquatic macrophytes for treatment of municipal wastewater. *J. Environ. Health Sci Eng.* 12:106.

Shahab, S., N. Ahmed, and N.S. Khan. 2009. Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs. *Afr J Agric Res* 4: 1312-1316.

Sharma, J., and R. Chakrabarti. 2003. Role of stocking density on growth and survival of Catla, *Catla catla*, and Rohu, *Labeo rohita*, larvae and water quality in a recirculating system. *J. App. Aquaculture* 14: 171-178.

Sharma, K.P., R.K. Chaturvedi, S. Sharma, and K. Sharma. 2000. Scavenging potential of hydrophytes for copper removal from textile dye wastewaters. *Bull. Environ. Contam. Toxicol*. 65: 330-336.

Sharma, S., V. Kumar, and R.B. Tripathi. 2011. Isolation of phosphate solubilising microorganism (PSMs) From Soil. *J. Microbiol. Biotech. Res*. 1: 90-95.

Sharma, R., K. Sharma, N. Singh, and A. Kumar. 2013. Rhiszophere biology of aquatic microbes in order to access their bioremediation potential along with different aquatic macrophytes. *Rec. Res Sci. Technol*. 5: 29-32.

Sharrer, M.J., S.T. Summerfelt, G.L. Bullock, L.E. Gleason, and J. Taeuber. 2005. Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. *Aquacult. Eng*. 33: 135-149.
Shereif, M.M., M.E. Easa, M.I. El-Samra, K.H. Mancy. 1995. A demonstration ofwastewat er treatment for reuse applications in fish production and irrigation in Suez, Egypt. *Water Sci. Technol.* 32: 137-144.
Shireman, J.V., and C.R. Smith. 1983. Synopsis of biological data on grasscarp, Ctenopharyngodon idella (Cuvier and Valenciennes, 1844). FAO Fish Synop. (1350: pp. 47–50).
Shpigel, M., A. Neori, D.M. Popper, and H. Gordin. 1993. A proposed model for ‘‘environmentally clean’’ landbased culture of fish, bivalves and seaweeds. *Aquaculture* 117: 115-128.
Shpigel, M., and Neori, A. 1996. Abalone and seaweeds intensive cultivation in integrated land-based mariculture system: I. Proposed design and cost analyses. *Aquacult. Eng.* 15: 313-326.
Silberman, D.M., M. Wald, and A.M. Genaro. 2002. Effects of chronic mild stress on lymphocyte proliferative response. Participation of serum thyroid hormones and corticosterone. *J. Int. Immunopharmacol*. 2: 487-497.
Silva, F.C.P., J.R. Nicoli, J.L. Zambonino-Infante, M.M. Le Gall, S. Kaushik, and F.J. Gatesoupe. 2010. Influence of partial substitution of dietary fish meal on the activity of digestive enzymes in the intestinal brush border membrane of gilthead sea bream, Sparus aurata and Goldfish, *Carassius auratus*. *Aquaculture* 306: 233–237.
Singh, M., and N.T. Prakash. 2012. Characterisation of phosphate solubilising bacteria in sandy loam soil under chickpea cropping system. *Indian J. Microbiol.* 52, 167-173.
Sipauba-Tavares, L., E. Favero, and F. Braga. 2002. Utilization of macrophyte biofilter in effluent from aquaculture. I. Floating plant. *Braz. J. Biol.* 62: 713-723.
Sirakov, I., and K. Velichkova. 2013. The usage of aquatic floating macrophytes (Lemna and Wolffi a) as biofilter in recirculation aquaculture system (RAS). *Turkish J. Fish Aquat. Sci.* 13: 101-110.
Skillicorn, P., W. Spira, and W. Journey. 1993. Duckweed aquaculture: A new aquatic forming system for developing countries. The World Bank: Washington DC, USA.
Skujins, J.J., L. Braal, and A.D. Mc Laren. 1962. Characterisation of phosphatase in a terrestrial soil sterilized with an electron beam. *Enzymologia* 25: 125-133.

Smith, V.H. 2003. Eutrophication of freshwater and coastal marine ecosystems. A global problem. *Environ Sci Pollut Res* 10: 126-139.
Smith, M.D., and I. Moelyowati. 2001. Duckweed based wastewater treatment (DWWT): design guidelines for hot climates. *Water Sci. Technol.* 43 (11): 291-299.
Soengas, J.L., P. Rey, G. Rozas, M.D. Andres, and M. Aldegunde. 1992. Effect of cortisol and thyroid hormone treatment on the glycogen metabolism of selected tissues of domesticated rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 101: 317-328.
Sorphea, S., T. Lundh, T.R. Preston, and K. Borin. 2010. Effect of stocking densities and feed supplements on the growth performance of tilapia (*Oreochromis* spp.) raised in ponds and in the paddy field. *Livest. Res. Rural Dev*. 22, 227.
Southworth, B.E., C.R. Engle, and K. Ruebush. 2009. The effect of understocking density of channel catfish stockers in multiple-batch production. *J. Appl. Aquacult.* 21: 21–30.
Spyridakis, P., R. Metailler, J. Gabaudan, and A. Riaza. 1989. Studies on nutrient digestibility in European sea bass (*Dicendrarchus labrax*). 1. Methodological aspects concerning faeces collection. *Aquaculture* 77: 61-70.

Staley, J., D. Brenner, and N. Krieg. 2005. The Proteobacter, Part B: The Gammaproteobacteria. In: Garrity, G.M., editor. Bergey's Manual of Systematic Bacteriology 2. Springer, Michigan, pp. 384-402.

Steffens, W. 1989. Principles of Fish Nutrition. Ellis Horwood, Chichester.

Stickney, R.R. 1994. Principles of Aquaculture. Wiley, New York.

Subandar, A., R.J. Petrell, and P.J. Harrison. 1993. Laminaria culture for reduction of dissolved nitrogen in salmon farm affluent. *J. Appl. Phycol.* 5: 455-463.

Subbarao, N.S. 1998. Phosphate solubilising microorganisms. In: Biofertilizers in agriculture and forestry. Regional Biofert Dev Centre Hissar India. p. 133-42.

Sugita, H., S. Yamamoto, C. Asakura, and T. Morita. 2005. Occurrence of *Listonella anguillarum* in seed production environments of Japanese flounder *Paralichthys olivaceus* (Temminck et Schlegel). *Aquacult. Res.* 36: 920-926.

Summerfelt, S.T., J. Bebak, and S. Tsukuda. 2001. Fish Hatchery Management. Second edition, PA Fish and Boat Commission.

Sumpter, J.P. 1997. The endocrinology of stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish stress and health in aquaculture. Cambridge University Press, Cambridge, pp. 95-118.

Sun, L., and H. Chen. 2014. Effects of water temperature and fish size on growth and bioenergetics of cobia (*Rachycentron canadum*). *Aquaculture* 426–427:172–180.

Sushanta, B., P. Sunita, M.J.B.D. Gonsalves, A. Das, M.B.L. Mascharena-Pereira, and P.A. Loka Barathi. 2012. Phosphate solubilising bacteria: comparison between coastal and deep sea sediments. *J Coast Environ* 3, 153-164.

Sutton, D.L., and W. H. Ornes. 1975. Phosphorus removal from Static Sewage Effluent using Duckweed. *J. Environ. Qual.* 4: 367-370.

Tabatabai, M.A., and J.M. Bremner. 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biol Biochem* 1: 301-307.

Tacon, A.G.J., and M. Metian. 2008. Global overview on the use of fishmeal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* 285: 146-158.

Tal, Y., J.E.M.Watts, S.B. Schreier, K.R. Sowers, and H.J. Schreier. 2003. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. *Aquaculture* 215:187-202.

Tantikitti, C., W. Sangpong, S. Chiavareesajja. 2005. Effects of defatted soybean protein levels on growth performance and nitrogen and phosphorus excretion in Asian seabass (*Lates calcarifer*). *Aquaculture* 248 (1-4): 41-50.

Tavares-Dias, M., E.F. da Silva-Sandrim, F.R. de Moraes, P.C.F. Carneiro. 2001. Physiological responses of "Tambaqui" *Colossoma Macropomum* (Characidae) to acute stress. Boletim do Instituto de Pesca, São Paulo, 27 (1): 43-48.

Tavares, F.D.A., F.R. Lapolli, R. Roubach, M.K. Jungles, D.M. Fracalossi, A.M.D. Moraes. 2010. Use of domestic effluent through duckweeds and red tilapia farming in integrated system. PANAMJAS 5 (1): 1-10.

Tejera, J.C., C. Lluch, M. Martinez, and J. Gonzales. 2005. Isolation and characterization of *Azotobacter* and *Azospirillum* strains from the sugarcane rhizosphere. *Plant Soil* **7**: 223-232.

Teles, M., M. Pacheco, and M.A. Santos. 2007. Endocrine and metabolic responses of *Anguilla anguilla* L. caged in a freshwater–wetland (Pateira de Fermentelos—Portugal). *Sci. Total Environ.* 372: 562-570.

Terova, G., R. Gornati, S. Rimoldi, G. Bernardini, and M. Saroglia. 2005. Quantification of a glucocorticoid receptor in sea bass (*Dicentrarchus labrax* L.) reared at high stocking density. *Gene* 357: 144-151.

The World Bank. 2013. Fish to 2030. Prospects for Fisheries and Aquaculture. World Bank Report, Number 83177-GLB.

Thompson, F.L., P.C. Abreu, and W. Wasielesky. 2002. Importance of biofilm for water quality and nourishment in intensive shrimp culture. *Aquaculture* 203: 263-278.

Thorne, T. 2002. The translocation of barramundi (*Lates calcarifer*) for aquaculture and recreational fishery enhancement in Western Australia. Fisheries Management Paper No. 159. Department of Fisheries of WA.

Tian, X., D. Li, S. Dong, X. Yan, Z. Qi, G. Liu, and J. Lu. 2001. An experimental study on closed-polyculture of penaeid shrimp with tilapia and constricted tagelus. *Aquaculture* 202: 57–71.

Timmons, M.B., J.M. Ebeling, F.W. Wheaton, S.T. Summerfelt, and B.J. Vinci. 2002. Recirculating Aquaculture Systems, second ed. Cayuga Aqua Ventures, Ithaca. NRAC Publication, New York.

Tintos, A., J.M. Miguez, J.M. Mancera, and J.L. Soengas. 2006. Development of a microtitre plate indirect ELISA for measuring cortisol in teleost fish, and evaluation of stress responses in rainbow trout and gilthead sea bream. *J. Fish Biol*. 68: 251-263.

Tolbert, N.E., C. Benker, and E. Beck. 1995. The oxygen and carbon dioxide compensation points of C3 plants: possible role in regulating atmospheric oxygen. Proc. Natl Acad. Sci. USA 92, 11230-11233.

Tolussi, C.E., A.W.S. Hilsdorf, D. Caneppele, and R.G. Moreira. 2010. The effects of stocking density in physiological parameters and growth of the endangered teleost species piabanha, *Brycon insignis* (Steindachner, 1877). *Aquaculture* 310: 221-228.

Tran-Duy, A., J.W. Schrama, A.A. van Dam, and J.A.J. Verreth. 2008. Effects of oxygen concentration and body weight on maximum feed intake, growth and hematological parameters of Nile tilapia, *Oreochromis niloticus*. *Aquaculture* 275:152-162.

Tripathi, B.D., J. Srivastava, and K. Misra. 1991. Nitrogen and phosphorus removal-capacity of 4 chosen aquatic macrophytes in tropical fresh-water ponds. *Environ Conserv.* 18: 143-147.

Tripathi, N.K., K.S. Latimer, C.R. Gregory, B.W. Ritchie, R.E. Wooley, and R.L. Walker. 2005. Development and evaluation of an experimental model of cutaneous columnaris disease in Koi *Cyprinus carpio*. *J Vet Diagn Investig.* 17 (1): 45-54.

Tripathy, P.P., and S. Ayyappan. 2005. Evaluation of Azotobacter and Azospirillum as biofertilizers in aquaculture. *World J. Microbiol Biotechnol.* 21: 1339-1343.

Trivedi, P., and T.M. Sa. 2008. *Pseudomonas corrugata* (NRRL B-30409) mutants increased phosphate solubilization, organic acid production and plant growth at lower temperatures. *Curr. Microbiol.* 56: 140-144.

Troell, M., C. Halling, A. Nilsson, A.H. Buschmann, N. Kautsky, and L. Kautsky. 1997. Integrated marine cultivation of Gracilaria chilensis (Gracilariales, Rhodophyta) and salmon cages for reduced environmental impact and increased economic output. *Aquaculture* 156: 45-61.

Troell, M., P. Ronnback, C. Halling, N. Kautsky, and A. Buschmann. 1999a. Ecological engineering in aquaculture: use of seaweed for removing nutrients from intensive mariculture. *J. App Phycol.* 11: 89-97.

Troell, M., N. Kautsky, and C. Folke. 1999b. Applicability of integrated coastal aquaculture systems. *Ocean Coast. Manage*. 42: 63-69.

Troell, M., C. Halling, A. Neori, T. Chopin, A.H. Buschmann, N. Kautsky, and C. Yarish. 2003. Integrated mariculture: asking the right questions. *Aquaculture* 226: 69-90.

Troell, M., D. Robertson-Andersson, R. Anderson, J. Bolton, G. Maneveldt, C. Halling, and T. Probyn. 2006. Abalone farming in South Africa: an overview with perspectives on kelp

resources, abalone feed, potential for on-farm seaweed production and socio-economic importance. *Aquaculture* 257: 266-281.

Troell, M., 2009. Integrated marine and brackish water aquaculture in tropical regions: research, implementation and prospects. In: Integrated Mariculture: a Global Review, pp: 47-131. Soto, D. (ed.). FAO Fisheries and Aquaculture Technical Paper. No. 529. FAO, Rome, Italy.

Trzebiatowski, R., J. Filipiak, and R. Jakubowski. 1981. Effect of stock density on growth and survival of rainbow trout (*Salmo gairdneri*, Richardson). *Aquaculture* 22: 289-295.

Tsavkelova, E.A., T.A. Cherdyntseva, S.G. Botina, and A.I. Netrusov. 2007. Bacteria associated with orchid roots and microbial production of auxin. *Microbiol. Res.* 162: 69-76.

Tubongbanua, E.S. 1987. Development of artificial feeds for seabass (*Lates calcarifer*). In Coplan, J.W., and D.L. Grey (eds). Management of wild and cultured seabass/barramundi (*Lates calcarifer*). ACIAR Proceedings No. 20, Canberra, Australia, pp. 186-188.

Tucker, C.S., and J.A. Hargreaves. 2008. Environmental best management practices for aquaculture. Blackwell Publishing Professional 2121 State Avenue, Ames, Iowa 50014, USA.

Turcois, A.E., and J. Papenberock. 2014. Sustainable Treatment of Aquaculture Effluents-What Can We Learn from the Past for the Future?. *Sustainability* 6: 836-856.

Turnbull, J., A. Bell, C. Adams, J. Bron, J., and F. Huntingford. 2005. Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. *Aquaculture* 243: 121-132.

Umar, K.J., L.G. Hassan, D.M. Dangoggo, and M.J. Ladan. 2007. Nutritional composition of water spinach (*Ipomoea aquatic* Forsk.) leaves. *J Appl. Sci.* 7: 803-809.

Urbinati, E.C., and P.C.F. Carneiro. 2004. Práticas de manejo e estressedos peixes em piscicultura intensiva. In: Cyrino, J.E.P.; Urbinati,E.C.; Fracalossi, D.M., Castagnolli, N. (Eds). Tópicos especiaisem piscicultura de água doce tropicas intensiva. TecArt, São Paulo, pp.171-194.

Vaintraub, I.A., and N.A. Lapteva. 1988. Colorimetric determination of phytate in unpurified extracts of seed and the products of their processing. *Anal. Biochem.* 17: 227-230.

Valeta, J.S., and M.C.M. Verdegem. 2009. Phosphate removal from aquaculture effluent by algal turf scrubber technology. *Malawi J. Aquacult. Fish* 1: 19-22.

Van Anholt, R.D., F.A. Spanings, W.M. Koven, and S.E. Bonga. 2003. Effects of acetylsalicylic acid treatment on thyroid hormones, prolactins, and the stress response of tilapia (*Oreochromis mossambicus*). *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 285: 1098-1106.

Van der Geyten, S., K.A. Mol, W. Pluymers, E.R. Kühn, V.M. Darras. 1998. Changes in plasma T_3 during fasting/refeeding in tilapia (*Oreochromis niloticus*) are mainly regulated through changes in hepatic type II iodothyronine deiodinase. *Fish. Physiol. Biochem*. 19: 135-143.

Van der Steen, P., A. Brenner, and G. Oron. 1998. An integrated duckweed and algae pond system for nitrogen removal and renovation. *Wat. Sci. Technol.* 38: 335-343.

Van Rijn, J. 1996. The potential for integrated biological treatment systems in recirculating fish culture-A review. *Aquaculture* 139: 181-201.

Vazzana, M., M. Cammarata, L. Cooper, and N. Parrinello. 2002. Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leucocyte cytotoxicity. *Aquaculture* 210: 231-243.

Vessey, J. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* 255: 571-586.

Vermaat, J. E., and M. K. Haniff. 1998. Performance of common duckweed species (Lemnacae) and the water ferm Azolla filiculoides on different types of waste water. *Wat. Res*. 32, 2569-2576.
Vijila, K. 2000. Estimation of IAA Production in Nitrogen Fixing Microorganisms. Practical-manual Microbial Interaction in Soil. Tamil Nadu Agricultural University. Coimbatore, pp. 38-39.
Vijayan, M.M., and J.F. Leatherland. 1988. Effect of stocking density on the growth and stress-response in brook charr, *Salvelinus fontinalis*. *Aquaculture* 75: 159-170.
Vohra, P., F.H. Kratzer, and M.A. Joslyn. 1996. The growth depressing and toxic effects of tannins to chicks. *Poultry Sci.* 46: 135–142.
Wagner, E.J., T. Jensen, R. Arndt, M.D. Routledge, and Q. Brddwisch. 1997. Effects of rearing density upon cutthroat hematology, hatchery performance, fin erosion and general health and condition. *The Prog Fish Cult* 59: 173-187.
Wagner, T., and J.L. Congleton. 2004. Blood chemistry correlates of nutritional condition, tissue damage and stress in migrating juvenile Chinook salmon (*Oncorhynchus tshawaytscha*). *Can. J. Fish. Aquat. Sci*. 61: 1066- 1074.
Waite, R., M. Beveridge, R. Brummett, S. Castine, N. Chaiyawannakarn, S. Kaushik, R. Mungkung, S. Nawapakpilai, and M. Phillips. 2014. Installment 5 of "Creating a Sustainable Food Future". Improving Productivity and Environmental Performance of Aquaculture. Working Paper. World Resources Institute.
Walpita, C.N., S.V.H. Grommen, V.M. Darras, and S. Van der Geyten. 2007. The influence of stress on thyroid hormone production and peripheral deiodination in the Nile tilapia (*Oreochromis niloticus*). *Gen.Comp. Endocrinol.* 150: 18-25.
Walton, M.J., and C.B. Cowey. 1992. Aspects of intermediary metabolism in salmonid fish. *Comp. Biochem. Physiol*. 73B: 59-79.
Wandelaar-Bonga, S.E. 1997. The stress response in fish. *Physiol. Rev*. 77: 591-625.
Wang, W. 1987. Root elongation method for toxicity testing of organic and inorganic pollutants. *Environ. Toxicol. Chem.* 6: 409-414.
Wang, J.Q., D. Li, S. Dong, K. Wang, and X. Tian. 1998. Experimental studies on polyculture in closed shrimp ponds: I. Intensive polyculture of Chinese shrimp (*Penaeus chinensis*) with tilapia hybrids. *Aquaculture* 163 (1-2):11-27.
Wang, J.K. 2003. Conceptual design of a microalgae-based recirculating oyster and shrimp system. *Aquacult. Eng*. 28: 37-46.
Waring, C.P., R.M. Stagg, and M.G. Poxton. 1996. Physiological responses to handling in the turbot. *J. Fish Biol*. 48: 161-173.
Wedemeyer, G.A. 1997. Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. Soc. Exp. Biol. Semin. Ser. 62. Cambridge University Press, UK, pp. 35-71.
Wedemeyer, G.A., and W.T. Yasutake. 1997. Clinical methods for the assessment of the effect of environmental stress on fish health. Tech. Pap. USFWS. No. 89.
Wee, K.L. 1991. Use of non-conventional feedstuff of plant origin as fish feeds is it practical and economically feasible. In: DeSilva, S.S. (Ed.), Fish Nutrition Research in Asia Proceedings of the Fourth Asian Fish Nutrition Workshop. Asian Fisheries Society Special Publication, vol. 5. Asian Fisheries Society, Manila, Philippines, p. 205.
Weon, H.Y., B.Y. Kim, S.H. Yoo, S.W. Kwon, E. Stackebrandt, and S.J. Go. 2008. *Chryseobacterium soli sp*. nov. and *Chryseobacterium jejuense sp*. nov., isolated from soil samples from Jeju, Korea. *Int. J. Syst Evol Microbiol.* 58: 470–473.
Wheaton, F.W., J.N. Hochheimer, G.E. Kaiser, M.J. Krones, G.S. Libey, C. Easter. 1994. Nitrification filter principles. In: Timmons, M.B., Losordo, T.S. (Eds.), aquaculture

water reuse systems: engineering design and management. Amsterdam: Elsevier, Chap. 4.
Whitehead, C., and J.A. Brown. 1989. Endocrine responses of brown trout, *Salmo trutta* L., to acid, aluminium and lime dosing in Welsh hill stream. *J. Fish Biol*. 35: 59-71.
Whitmarsh, D.J., E.J. Cook, and K.D. Black. 2006. Searching for sustainability in aquaculture: an investigation into the economic prospects for an integrated salmon–mussel production system. *Mar. Policy* 30 (3): 293-298.
Wietz, M., M.R. Hall, and L. Hoj. 2009. Effects of seawater ozonation on biofilm development in aquaculture tanks. *Syst. Appl. Microbiol.* 32: 266-277.
Wik, T.E.I., B.T. Lindén, and P.I. Wramner. 2009. Integrated dynamic aquaculture and wastewater treatment modelling for recirculating aquaculture systems. *Aquaculture* 287: 361-370.
Wilkinson, R.J., N. Paton, and M.J.R. Porter. 2008. The effects of pre-harvest stress and harvest method on the stress response, rigor onset, muscle pH and drip loss in barramundi (*Lates calcarifer*). *Aquaculture* 282: 26-32.
Willett, D., B. Rutherford, C. Morrison, and W. Knibb. 2003. Tertiary treatment of Ayr municipal wastewater using bioremediation: a pilot study. Report to the Burdekin Shire Council and the Burdekin Rangelands Reef Initiative. Queensland Department of Primary Industries. 14 pp.
Wills, P.S., R. Baptiste, C. Robinson, T. Pfeiffer, and M. Davis. 2009. Maximizing production densities on marine recirculating aquaculture to rear advanced juvenile red drum for stock enhancement. Harbor Branch Oceanographic Institute at Florida Atlantic University.
Wolters, W., A. Masters, B. Vinci, and S. Summerfelt. 2009. Design, loading, and water quality in recirculating systems for Atlantic salmon (*Salmo salar*) at the USDA ARS National Cold Water Marine Aquaculture Center (Franklin, Maine). *Aquacult. Eng*. 41: 60-70.
Wolverton, B.C. 1979. Engineering design data for small vascular aquatic plants wastewater treatment systems. In: Proc. EPA Seminar on Aquaculture Systems for wastewater treatment, EPA 430/9-80-006.
Woo, S.M., M.K. Lee, I.S. Hong, S. Poonguzhali, and T.M. Sa. 2010. Isolation and characterization of phosphate solubilising bacteria from Chinese cabbage. 19th World Congress of Soil Science, Soil Solutions for a Changing World 1-6 August 2010, Brisbane, Australia.
Woo, N.Y.S., A.S.B. Chung, and T.B. Ng. 1991. Influence of oral administration of 3, 5, 3'-triiodothyronine on growth, digestion, food conversion and metabolism in the underlying red sea bream, *Chrysophryus major* (Temmink and Schlegel). *J. Fish Biol*. 39: 450-468.
Wood, C.M., A.P. Farrell, and C.J. Brauner. 2012. Homeostasis and toxicology of essential metals (1st ed.). Amsterdam; New York: Academic Press.
Wright, R.T., and R.B. Coffin. 1984. Measuring microheterotrophic grazing of marine bacterioplankton with differential filtration and incubation. *Microb. Ecol. 10*: 137-149.
Wu, M., S. Ka Kan Mak, X. Zhang, and P. Quian. 2003. The effect of co-cultivation on the pearl yield of *Pinctada martensi* (Dumker). *Aquaculture* 221: 347-356.
Xu, J., and G. Shen. 2011. Growing duckweed in swine wastewater for nutrient recovery and biomass production. *Bioresource Technol*. 102: 848-853.
Yadav, J., S. Yadav, and S.G. Singh. 2011. Plant growth promotion in wheat crop under environmental condition by PSB as bio-fertilizer. *Res. J. Agril. Sci*. 2(1): 76-78.

Yang, H., Y. Zhou, Y. Mao, X. Li, Y. Liu, and F. Zhang. 2005. Growth characters and photosynthetic capacity of *Gracilaria lemaneiformis* as a biofilter in a shellfish farming area in Sanggou Bay, China. *J. Appl. Phycol*. 17: 199-206.
Yao, H., C.D. Campbell, S.J. Chapman, T.E. Freitag, G.W. Nicol, and B.K. Singh. 2013. Multi-factorial drivers of ammonia oxidizer communities: evidence from a national soil survey. *Environ Microbiol* 15: 2545–2556.
Yarahmadi, P., H.K. Miandare, S.H. Hoseinifar, N. Gheysvandi, and A. Akbarzadeh. 2014. The effects of stocking density on hemato-immunological and serum biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Int.* 23: 55-63.
Yildiz, H.Y., and S. Altunay. 2011. Physiological stress and innate immune response in gilthead sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax) exposed to combination of trimethoprim and sulfamethoxazole (TMP-SMX). *Fish Physiol Biochem.* 37 (3): 401-409.
Yılmaz, S., and S. Ergün. 2012. Effects of garlic and ginger oils on hematological and biochemical parameters of seabass, *Dicentrarchus labrax*. *J. Aquat Anim. Health* 24:219 -224.
Yilmaz, E., I. Akyurt, and G. Gu¨nal. 2004. Use of duckweed, *Lemna minor*, as a protein feedstuff in practical diets for common carp, *Cyprinus carpio*, fry. *Turk J. Fish Aquat. Sci.* 4: 105-109.
Ying-Ning, H., M. Dony-Chacko, H. Shu-Chuan, S. Chun-Hao, C. Mei-Fang, C. Hsing-Mei, and H. Chieh-Chen. 2012. Selection and application of endophytic bacterium *Achromobacter xylosoxidans* strain F3B for improving phytoremediation of phenolic pollutants. *J. Hazard Mater.* 219: 43-49.
Yu, X., X. Liu, T.H. Zhu, G.H. Liu, and C. Mao. 2011. Isolation and characterization of phosphate-solubilising bacteria from walnut and their effect on growth and phosphorus mobilization. *Biol. Fertil. Soils* 47:437-446.
Yue, S., I.J.H. Duncan, and R.D. Moccia. 2006. Do differences in conspecific body size induce social stress in domestic rainbow trout. *Env Biol Fish* 76: 425–431.
Zern, T.L., K.L. West, and M.L. Fernandez. 2003. Grape polyphenols decrease plasma triglycerides and cholesterol accumulation in the aorta of ovariectomized guinea pigs. *J Nutr*. 133: 2268-2272.
Zhao, B., Y.L. He, J. Hughes, and X.F. Zhang. 2010. Heterotrophic nitrogen removal by a newly isolated *Acinetobacter calcoaceticus* HNR. *Bioresource Technol.* 101: 5194-5200.
Zhang, S.Y., G. Li, H.B. Wu, X.G. Liu, Y.H. Yao, L. Tao, and H. Liu. 2011. An integrated recirculating aquaculture system (RAS) for land-based fish farming: The effects on water quality and fish production. *Aquacult Eng* 49: 93-102.
Zhang, T.C., Y.C. Fu, and P.L. Bishop. 1995. Competition for substrate and space in biofilms. *Water Environ. Res*. 67: 992-1003.
Zhang, Y., W. Ji, Y. Wu, H. Han, J. Qin, and Y. Wang. 2014. Replacement of dietary fish meal by soybean meal supplemented with crystalline methionine for J apanese seabass (*Lateolabrax japonicas*). *Aquacult. Res*. 47: 243-252.
Zhao, X., G.K. Moates, N. Wellner, S.R. Collins, M.J. Coleman, and K.W. Waldron. 2014. Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (*Lemna minor*). *Carbohydr Polym.* 13: 410-418.
Zhou, Q.C., K.S. Mai, B.P. Tan, and Y.J. Liu. 2005. Partial replacement of fishmeal by soybean meal in diets for juvenile cobia (*Rachycentron canadum*). *Aquacult. Nutr.* 11: 175–182.
Zhou, Y., H. Yang, H. Hu, Y. Liu, Y. Mao, H. Zhou, X. Xu, and F. Zhang. 2006. Bioremediation potential of the macroalga *Gracilaria lemaneiformis* (Rhodophyta) integrated into fed fish culture in coastal waters of North China. *Aquaculture* 252: 264-276.

Zhou, Y.R., X.Y. Yuan, J.R. Wang, T.F. Bao, and W.M. Shi. 2010. Screening of duckweed (Lemnaceae) species for efficient removal of water body's nitrogen in the Tai Lake region and preliminary study on nitrogen removal mechanism. *Soils* 42: 390-397.
Zhou, Y.R., Y.F. Lu, H.L. Zhang, and W.M. Shi. 2013. Aerobic denitrifying characteristics of duckweed rhizosphere bacterium RWX31. *Afr. J Microbiol Res* 7: 211-219.
Zhu, Y.J., D.G. Yang, J.W. Chen, J.F. Yi, and W.C. Liu. 2011. An evaluation of stocking density in the cage culture efficiency of Amur sturgeon *Acipenser schrenckii*. *J. Appl. Ichthyol*. 27: 545-549.
Zimmo, O. 2003. Nitrogen transformations and removal mechanisms in algal and duckweed waste stabilization ponds. Ph.D Thesis, International Institute for Infrastructural, Hydraulic and Environmental Engineering, Delft, the Netherlands.
Zucker, D.A. 1999. A Dynamic, Stochastic Model of a Land-Based Summer Flounder Paralichthys dentatus Aquaculture Firm. *J. World Aquacult. Soc*. 30, 219.

"Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged."

APPENDIX A

The current Publications from the thesis

Thesis Title : Efficacy of duckweed (*Lemna minor* Linnaeus) integrated in barramundi recirculating aquaculture system (RAS)

Thesis Chapter	Publication Status
I. The abundance and diversity of heterotrophic bacteria as a function of harvesting frequency of duckweed (*Lemna minor* L.) in recirculating aquaculture systems. Letters in Applied Microbiology 63, 53-59.	This chapter has been published in Letters in Applied Microbiology 63, 53-59.
II. Water quality, growth and stress responses of juvenile barramundi (*Lates calcarifer* Bloch), reared at four different densities in integrated recirculating aquaculture systems. Aquaculture 458, 113-120.	This chapter has been published in Aquaculture 458, 113-120.
III. Can duckweed (*Lemna minor* Linnaeus) harvested from IRAS be recycled into barramundi (*Lates calcarifer* Bloch) diets as a partial replacement of protein from the fishmeal?	This chapter is submitted to Aquaculture