Norwegian University of Life Sciences
Faculty of Veterinary Medicine and Biosciences
Department of Animal and Aquacultural Sciences

Master Thesis 2016
30 credits

Development of Research Tool to Evaluate the Potential of Using Chlorella sorokiniana as Bio-Filter In Recycled Tilapia Production

Muhammad Saqib Latif

Development of Research Tool to Evaluate the Potential of Using *Chlorella sorokiniana* as Bio-Filter In Recycled Tilapia Production

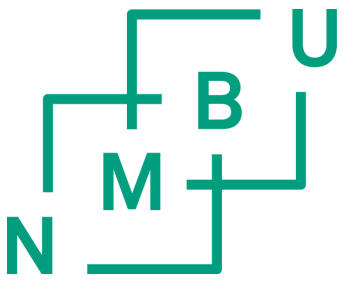
Master of Science Thesis (30 credits)

Muhammad Saqib Latif

Department of Animal and Aquacultural Sciences

Norwegian University of Life Sciences

Ås (2016)



Acknowledgements

Before all else, I thank Almighty Allah for His infinite mercy to render me alive to see this day. I thank to Allah for all the strengths and abilities He has given to me to come with this write up.

This period of master thesis has been a real adventure for me. Startup of something innovative is always a challenge, but if you are determined, dedicated and devoted, surely you can do it.

I feel pleasure to express my heartiest gratitude to my supervisor Dr. Trond Storebakken for his scholastic and constructive support throughout the accomplishment of this manuscript. Thanks for your scientific knowledge and skills in enabling me to become a junior scientist.

I am deeply thankful to Mr Thorsten Heidorn for providing algae culture as well as valuable comments and guidance, Dejan Knezevic and Sodbilig Wuriyanghai for their engineering participation in the construction of photo-bioreactor and Keke Zheng for water chemistry measurements during the experiment.

I am more earnestly obliged to my adorable father Muhammad Latif whose valuable guidance financial assistance and little pushes enable me to join the higher ideas of life, my angelic mother who raised me and always prayed for my success.

At last but not least of course, I am heartily thankful to my lovely and sweet wife Sadia, for her patience and support and my son Shayaan. Whatever I am is purely because of my family.

Muhammad Saqib Latif

Ås, March 2016

Abstract

The current study was attempted to develop the research tools in order to evaluate if *Chlorella sorokiniana* has a potential to perform as a bio-filter in recycle water tilapia production. The overall objective was to test the hypothesis that *C. sorokiniana* will effectively remove nitrogenous catabolites from the water and benefit the tilapia with oxygen and nutrients by photosynthesis. Removal of ammonia and nitrite from the water is improved by fertilization with phosphate, the 1st limiting factor for primary production in freshwater. A total of 9 tanks were used for the experiment, and were divided into three treatment groups CON, HPG and FPG. One group was kept as control group (CON), while other two groups were fertilized with phosphate, either a full (FPG) or half (HPG) phosphate concentration. Phosphate fertilization levels were determined by considering the adjusting the N:P ratio to 16:1, which is considered optimal for growth of green algae found in the water (FPG), providing half of this phosphate dose (HPG), and only giving the algae access to the phosphate excreted by the fish (CON). Ammonium and nitrite values from each tank were calculated based on their molecular weight. This obtained value was then calculated based on the molecular weight of Na_2HPO_4 and the water volume in the tanks. The resulted value (3.73 mg/l) was a full phosphate concentration i.e. 16:1 ratio, and was simply divided by two to get the half phosphate concentration (1.86 mg/l). The nitrogen excretion level used for the calculation was estimated by monitoring excretion of NH_4^+ into the water by Nile tilapia fed a fully plant based diet in a system that was not inoculated with algae.

A wooden table and board (placed on ground) was used for placing tanks and plastic tubes respectively. Each tank was filled with 12 L of water and was equipped with a heater, air stone and a pump. In addition, each pump was connected with two ends of plastic tube (L = 10m, D = 1cm) to make a closed system. Tubes were placed on a wooden board in such a way that approximately 8.5 meters of each tube was exposed to the light. Dissolved oxygen, temperature and pH were monitored regularly. Light was provided to the algae for photosynthesis purpose. The whole experiment lasted for 9 days.

From day 1 to 4, a general pattern of ammonium, nitrite and phosphate concentrations in the water after introduction of feed was followed. On day 5 and 6 same concentrations were followed, after inoculating the system with algae and fertilization with different levels of phosphate. In these two days no feed was offered to the fish. On day 7 and 8, as the system was re-inoculated with algae and fertilized with phosphate at the three defined levels, and the

concentrations of ammonium, nitrite and phosphate were monitored. A control measurement was repeated on day 9 without feeding and phosphate fertilization.

Ammonium, nitrite and phosphate concentrations (n = 9) before and after algae inoculation were 1.35, 0.02, 0.09 mg/l and 0.13, 0.01, 0.1 mg/l respectively. Similarly, concentrations before and after fertilization in CON (0.26, 0.04, 0.02 mg/l and 1.13, 0.13, 0.09 mg/l), HPG (0.15, 0.05, 0.04 mg/l and 0.42, 0.06, 0.11 mg/l) and FPG (0.11, 0.03, 0.07 mg/l and 0.34, 0.03, 0.08 mg/l).

A strong linear decrease in ammonium and nitrite concentration with the increase of phosphate dose was found i.e. FPG performed the best followed by HPG and CON. The decrease was stronger for ammonium than nitrite, which suggest ammonium as the preferred nitrogen source for assimilation in *Chlorella*.

Based on the data, it is proposed that *Chlorella sorokiniana* has a potential to be used as a bio-filter in recycled tilapia production. Further studies needed to be undertaken for appropriate understanding of the system before practical application.

Key words. *Chlorella*, bio-filter, phosphate-fertilization, tilapia, recirculation, ammonium, nitrite

Table of contents

List of figures and tables

List of abbreviations

C. sorokiniana	*Chlorella sorokiniana*
CON	Control group
DO	Dissolved oxygen
FPG	Full phosphate group
HPG	Half phosphate group
mg/l	Milligram/liter
NH_4^+	Ammonium
NO_2^-	Nitrite
OD	Optical density
PO_4^{3-}	Phosphate
WCMs	Water chemistry measurements
$\mu mol/m^2/s$	Micromole/square meter/second
mL	Milliliter
NH_3-N	Ammonia-nitrogen
NH_4-N	Ammonium-nitrogen
μM	Micromole
Na_2HPO_4	Disodium phosphate
ml/min	Milliliter/minute
LED	Light-emitting diode

1 Introduction

1.1 Environmental biology and nutritional requirements of Nile Tilapia

Tilapia refers to a broad group of cichlid fishes which are primarily native to Africa. There are three economically important genera of tilapia named as *Tilapia*, *Oreochromis* and *Sarotherodon*. All are commonly called "tilapia" (Mjoun et al., 2010a). Tilapias are known as "aquatic chickens" due to their high growth rate and adaptability to a wide range of environmental conditions (El-Sayed, 2006). Some of the key water quality parameters for optimal growth of tilapia includes water temperature, salinity, dissolved oxygen, pH and ammonia level (Mjoun et al., 2010a).

In general, tilapia is highly tolerant to these conditions up to a specific upper and lower limit but in order to gain optimum growth, optimal conditions are necessary. Temperature is one of the these limiting factor that shift metabolic changes quite effectively, e.g. growth is reduced below 20°C, stops at temperature below 16°C, and tilapias are unable to survive below 10°C (Pullin and Lowe-McConnell; 1982, Mjoun et al., 2010a). Tilapia are therefore very tolerant to high water temperature in contrast to their low water temperature tolerance and can survive at a temperature as high as 42°C. Though, the typical water temperature considered optimal for tilapia growth is in the range of 22°C to 29°C.

Salinity is also a factor for the tilapia that affects growth and reproduction. Tilapia culture in saline water is biologically sound up to 36 parts per thousand, but optimal growth ranges between 10 to 20 parts per thousand (Suresh and Lin, 1992). Dissolved oxygen is another water parameter, and it is documented that tilapia is highly tolerant to low dissolved oxygen concentration, and can survive with as little O_2 in the water as 0.1mg/l (Magid and Babiker, 1975). Although, growth can be attained at oxygen concentration higher than 3mg/l but the most favorable oxygen concentration for optimum growth exists between 6 to 6.5 mg/l (Abdel-Tawwab et al., 2015).

Ammonia and pH affect tilapia growth. Tilapia can tolerate pH level between 3.7 and 11, while best growth is attained at pH from 7 to 8/9 (Ross, 2000 (cited by Mjoun et al., 2010a)); El-Sherif and El-Feky, 2009). Nitrogenous wastes of fish are excreted mostly through gills including ammonia that is highly toxic to fish. Two forms of ammonia occurred in water in a balanced form as unionized NH_3 and ammonium ions NH_4^+ and the ratio is dependent on water temperature and pH. The latter one is not toxic to fish (Pullin and Lowe-McConnell, 1982). The

toxic range of ammonia exists between 2.5 to 7.1mg/l whereas optimum concentration should be below 0.05mg/l (El-Sherif and El-Feky, 2008).

Tilapia are herbivores/omnivorous and feed on phytoplankton, zooplankton, larval fish, periphyton, detritus and higher plants. Beside this range, they readily accept the pelleted feed containing proteins and lipids of animal or plant origin (Mjoun et al., 2010b). A well balanced diet (protein, lipids, carbohydrates, vitamins, minerals) is necessary for rapid growth of the fish. They demand a balanced inclusion of essential amino acids, and protein to energy ratio by keeping in mind the fish age and size (Santiago and Lovell, 1988; El-Sayed and Teshima, 1992; Mjoun et al., 2010b).

Similarly, fish do not have a specific requirement of carbohydrates but addition provides an inexpensive source of energy and secures good physical quality of the pelleted or extruded feeds. Vitamins and minerals on other hand are considered essential for normal fish metabolism and therefore the dietary supply is necessary, especially in intensive systems where production of algae is not sufficient to satisfy these demands (Mjoun et al., 2010b).

1.2 Biology and limiting factors in production of *Chlorella*

Chlorella is unicellular green algae which contains two green photosynthetic pigments in its chloroplast i.e. chlorophylls a and b. *Chlorella* has many structural resemblances with plants such as cell wall, cytoplasm, mitochondria and chloroplast (Safi et al., 2014). Morphologically it is spherical in shape with a diameter of 2-10 μm. The mode of reproduction in *Chlorella* is asexual and natural or artificial production is attained by different means, for instance autotrophically (with light) common in open pond systems, closed photo-bioreactor and heterotrophically (without light) (Safi et al., 2014).

Generally, microalgae have a capacity to grow in both fresh and marine water, at nearly all kind of environmental conditions. Maximum growth can be achieved by optimizing different factors that affect *Chlorella* growth, such as light intensity, regimes and wavelength, pH, CO_2 concentration, nitrogen and phosphorous concentrations and temperature (Lustigman et al., 1995; Tam and Wong, 1996; Widjaja et al., 2009; Chinnasamy et al., 2009; Seyfabadi et al., 2011; Liang et al., 2013; Atta et al., 2013; Blair et al., 2014).

Considering above mentioned conditions it is possible to achieve nutrient-targeted growth. For instance, increased production of carbohydrate and lipid contents by optimized light wavelength and intensity (Atta et al., 2013), phosphorous concentration (Liang et al., 2013), temperature, CO_2 concentration and nitrogen depletion (Widjaja et al., 2009;

Chinnasamy et al., 2009). Similarly, high chlorophyll and increased protein contents are feasible with increased substrate nitrogen concentration, light regime and intensity (Tam and Wong, 1996; Seyfabadi et al., 2011; Guerrero-Cabrera et al., 2014; Safi et al., 2014).

Light is an essential energy source for photosynthesis and is required for autotrophic growth. Growth evaluation of *Chlorella* under different light wavelength was conducted by Blair et al. (2014). They reported that white light had highest cell density, growth rate and volumetric biomass compared to blue and red lights. Light intensity and regime also affect growth rate and nutrient contents. Atta et al. (2013) studied growth of *Chlorella* under different light wavelength, light intensities and photoperiod regimes. They reported that *C. vulgaris* gained maximum growth rate, cell dry weight and lipid content at blue LED light intensity of 200 μmol/m^2/s and a light regime of 12/12 hour light and dark.

Temperature and CO_2 are also important elements for growth of *Chlorella* and therefore optimum levels are necessary. Increase in biomass and chlorophyll contents by elevated CO_2 to 6% compared to ambient (0.036%) and temperature at 30°C were reported by Chinnasamy et al. (2009). Enhanced growth is viable by increasing CO_2 flow rate until 50 ml/min (Widjaja et al., 2009).

Nitrogen and phosphorous also play a vital role in the growth of microalgae. *Chlorella* can effectively grow in nitrogen concentration ranging from 20mg/l to 250mg/l either in the form of NH_3-N or NH_4-N without any significant differences in specific growth (Tam and Wong, 1996; Guerrero-Cabrera et al., 2014). It is reported that lipid content and lipid productivity is increased at low phosphorous concentrations 16 to 32 μM, while protein remained unaffected with phosphorous concentration. Carbohydrate contents are directly correlated to phosphorous concentration (Liang et al., 2013). Growth curve of *C. vulgaris* at different pH (2, 4, 6, 8, 10) shows that it is unable to grow at pH 2 or below. Most rapid growth of *Chlorella* takes place at pH 6 followed by pH 8.

1.3 Significance of algae in aquaculture

Generally, algae have broad range of applications in many areas, such as in biofuels, human nutrition, agrochemical etc. Beside these applications, algae have a great potential to be used in aquaculture and there are two perspectives in this regard either as feed additive or as bio-filter. Algae, being photosynthetic organisms, maintain the water quality parameters by removing ammonia, nitrite, CO_2 into their biomass and increase water oxygen, provide essential nutrients, as well as minimize the unwanted microbial population (Priyadarshani et al., 2012).

Algae are being tested/used as an alternative protein source in feed additive for animal/aquaculture species, enrichment of zooplankton, and for the purification of water (Brown, 2002; Gál et al., 2007; Bertoldi et al., 2008; Velichkova, 2014; Guerrero-Cabrera et al., 2014; Sirakov et al., 2015).

1.3.1 Potential of *Chlorella* as protein source

The use of algae as an alternative protein source is not infancy as the efforts has been made since the early fifties. Application of algae in aquaculture industry is mainly for feed purposes as a protein source. According to current calculations about 30% of world algal production is used for animal feed (Becker, 2007) and *Chlorella* is one of the most commonly used genus of algae in aquaculture in this sense (Sirakov et al., 2015). One of the particular reason for this may be the more balanced chemical composition compared to other algal species. *Chlorella* generally contains 51-58% protein, 12-17% carbohydrate and 14-22% lipids in its dry matter (Becker, 2007). In addition, to be used in aquaculture, algae have to present certain characteristics that make them attractive for usage. For instance, easy culturing, non-toxic, right size and shape, good nutritional properties especially protein contents, and digestible cell wall (Priyadarshani et al., 2012). It is now well documented through investigations that algae present promising features as novel source of protein and their quality is equal or even higher than conventional plant protein feed ingredients (Becker, 2007).

1.3.2 Potential of *Chlorella* as bio-filter

Aquaculture is the most rapidly growing animal production sector all over the world. Because of this increase, it is important to consider the negative environmental impacts of aquaculture. Since the target is to achieve maximum growth so protein rich diets are formulated. In this regard, fish metabolic wastes together with uneaten feed are rich in CO_2 and nitrogen compounds. Which in turn contribute to deteriorate the water chemistry and are unsuitable for cultured organisms (Yusoff et al., 2011). The process of nitrogen transformation in an earthen fish pond can be understand by a simple schematic diagram (fig.1).

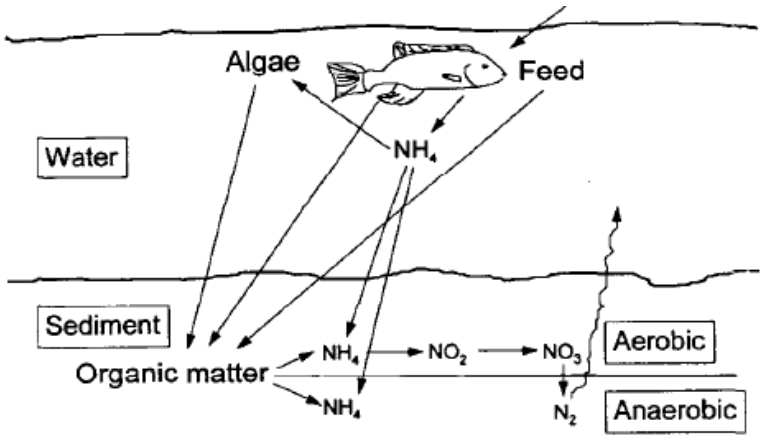


Figure 1. Schematic illustration of nitrogen transformation in an earthen fish pond (Adapted from Rijn, 1996).

In aquatic system algae can be a potential candidate for purification of these wastes. Algae needs nitrogen, phosphorous, CO_2 and light for the photosynthesis. As algae has the capability to efficiently remove inorganic nutrients from the water into their biomass and produce oxygen that is useful for the culturing organism (Gál et al., 2007). Several studies have reported the potential of *Chlorella* to remove CO_2, nitrogen and phosphorous from aquaculture and wastewater effluents (Tam and Wong, 1996; Widjaja et al., 2009; Kim et al., 2010; Liang et al., 2013; Velichkova, 2014; Guerrero-Cabrera et al., 2014). The general mechanism involved converting CO_2 to biomass by algae is well explained by Hailing-Sørensen et al. (1996) and is presented in fig. 2.

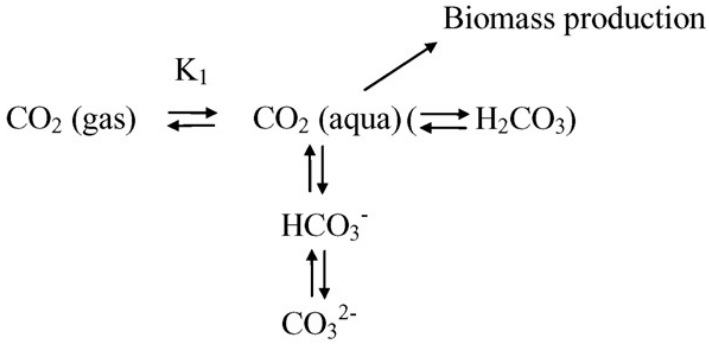


Figure 2. Mechanism involved in conversion of CO2 to the algal biomass.

If the CO_2 concentration in water is high pH will be decreased as excess will be converted to H_2CO_3, but in case of low concentration pH will be increased as utilization will be from HCO_3^-. Widjaja et al. (2009) further clarified the relationship between pH and CO_2 concentration. They observed different range of pH at different flow rate of CO_2 gas under an air flow rate of 6l/min, e.g. 6.86-8.33, 6.74-7.15, 6.16-7.01, 5.44-6.44 at a flow rate of 0ml/min, 20ml/min, 50ml/min and 200ml/min respectively. Out of these, highest growth curve was reported at pH 6.2-7.0, at an air flow rate at 50ml/min.

Gál et al. (2007) developed a combined aquaculture-algae system consisted of intensive fish tank, algal pond and fish pond. They reported that nutrient transformation efficiency by algae using nutrients (organic carbon, nitrogen and phosphorous) from wastes of intensive fish tanks and converting them to their biomass as a food source for fish pond is likely possible. In this way algae functioned both as bio-filter and in providing essential nutrients.

According to Velichkova (2014), *Chlorella* has a great potential to use nitrogen into the biomass from the aquaculture wastewater present either in the form of urea or ammonium nitrate. Guerrero-Cabrera et al. (2014) reported high reduction in ammonium and phosphorus contents in tilapia effluent medium compared to basal bold medium during algae growth suggesting its high survival and nutrient removal capacity. *Chlorella* can remove or utilize more than 95% of nitrogen if the culture media contains 40 to 80mg/N. Also, it has the great ability to assimilate nitrogen in the form of ammonia (Tam and Wong, 1996).

Kim et al. (2010) shown high efficiency of *Chlorella* to accumulate nitrogen (NH_3, NO_3^-, NO_2^- and NH_4^+) and inorganic carbon from wastewater effluent. *Chlorella* successfully removed the concentrations of inorganic carbon (58.6±0.28 mg/l) and nitrogen (7.7±0.19 mg/l) at pH 7. Their gained biomass was comparable to these concentrations. In another study, they tested the potential of *Chlorella* to remove nitrogen and phosphorous from the secondary wastewater effluent collected from municipal wastewater treatment plant and revealed that nitrogen and phosphorous can be removed within 48h if the algal cell density is approximately 350 mg/l and CO_2 is supplied in addition. (Kim et al., 2013).

1.4 Effect of phosphate fertilization on fish and algal growth

The basic dogma to use fertilizers to aquaculture ponds is to enhance photosynthetic activity of phytoplankton which in turn will benefit the cultured species. There are two management considerations in this sense, nutrient input requirement and frequency of fertilizer application (Knud-Hansen and Batterson, 1994). Fertilization would give rise to primary production, dissolved oxygen, pH and total phosphorous (Qin et al., 1995 (cited by Elnady et al., 2010)). A simple pathway from nutrients input (organic or inorganic fertilizers) to fish harvest is presented in fig. (3).

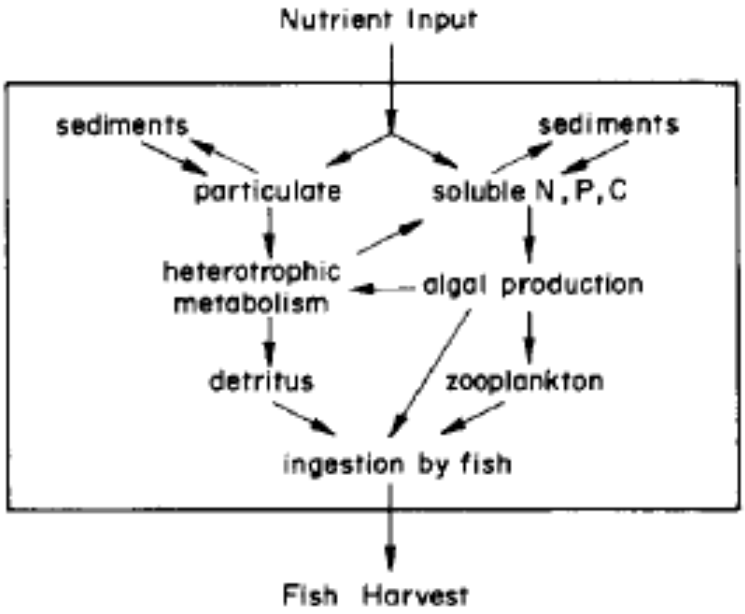


Figure 3. Schematic illustration of nutrients input (organic or inorganic fertilizers) to fish harvest (Adapted from Knud-Hansen et al., 1993)

It is extrapolated that phosphorous has significant effect on algal growth (Liang et al., 2013). To achieve the development of food organisms in pond, a weekly ratio of N:P (20:1) is recommended (Qin et al., 1995). Growth of fish in addition is effected by use of organic or inorganic fertilizers as well as by fertilization frequency (Knud-Hansen and Batterson, 1994; Elnady et al., 2010).

1.5 Symbiotic production of algae and tilapia

The term symbiotic production literally means production by mutual benefit. Symbiotic culturing of algae and fish may give an economic and sustainable aquaculture. In this closed system, fish provides inorganic nitrogen, CO_2, and turbulence to the algae, while algae on the other hand use these nutrients into their biomass which are toxic to fish and supply back dissolved oxygen and nutrients beneficial to fish (Pack, 1991).

This system somehow resembles to aquaponics, which is a bio-integrated system that connect conventional recirculating aquaculture to hydroponics production of plants in a symbiotic way (Diver, 2000). So, the basic theme or principle is the same in both systems i.e. recirculation of water by the removal and utilization of toxic nutrients and converting them to the valuable nutrients for each other. The only difference is that in former system, both plants and fishes are grown in the same place, while in the latter system, they are grown in different compartments or places.

Compulsory element that needs to be undertaken in an aquaponics system is the choice of fish and plant species. In this regard, physiological behavior and limiting factors involved for their production must be contemplated. These elements can be for instance, nutrients present in aquaculture effluents and the capability of algae or plants to remove these effluents effectively to their biomass.

1.6 Objectives of the research

The current study was attempted to develop the research tools in order to evaluate if *Chlorella sorokiniana (C. sorokiniana)* has a potential to perform as a bio-filter in recycled tilapia production. The main objectives of the research work were following.

- To evaluate, if *C. sorokiniana* can replace mixed microbial populations in a bio-filter
- To find out, if phosphate supplementation to water together with excreta from fish will facilitate efficient removal of nitrogen-catabolites.

The overall objective was to test the hypothesis that *C. sorokiniana* will effectively remove nitrogen-catabolites and phosphorous and benefit the tilapia with oxygen and nutrients by photosynthesis.

2 Materials and methods

2.1 Fish rearing and acclimatization

The experiment was conducted at the Department of Animal and Aquacultural Sciences, of the Norwegian University of Life Sciences (NMBU). Nile tilapias were reared in glass aquariums with following dimensions (70x50x50cm). The fish were anesthetized by tricanine methanesulfonate (MS-222, 0.1 g/L water) and were transferred to the small experimental tanks for acclimatization. A total of twenty fishes with a weight variation of 41 to 106 g were weighed individually. Two tilapias were then distributed into each of 10 experimental tanks The resulted average weight variation in each tank after this attention was between 119-147 g. Number of fishes per tank was reduced to one after day 1 of the experiment and the resulted weight varied from 59 to 98 g.

The experimental tanks were 10 rectangular plastic storage boxes with the following dimensions (39x29x24.5 cm) and with a capacity of 20 L were used as experimental tanks. These boxes were purchased from a local hardware store (Clas Ohlson, Sweden). Each tank was filled to 12 liters of water and equipped with a heater (EHIEM 3611, thermostatic 25watt, 20 to 25 L capacity, temperature range 18-34°C, Germany) to stabilize the temperature (27±2°C), and a filter (Marina JF50, 3.3 watt, 50L/h, Hagen Inc. China) of which filter part was manually removed and made it functional to be used as a pump.

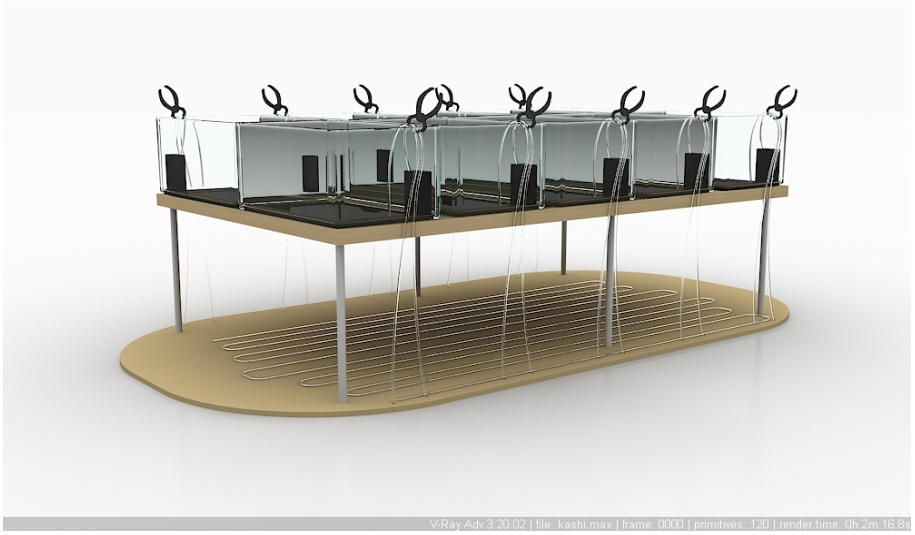
2.2 Algae inoculation

Pure culture of *C.sorokiniana* (strain NIVA, CHL176) were obtained from NIBIO (Norwegian Institute of Bioeconomy Research). The optical density was measured at 750nm for the cultured algae and the sample was diluted 20 times to get the results at this wavelength. The value of algae after OD_{750} calculation was 7.1. Initially, a 100ml of this algae biomass (OD_{750}, 7.1) was inoculated in each tank. Due to rapid drop in oxygen concentration and filtering of algae by fish, another 100ml of same algae biomass was re-inoculated after four hours.

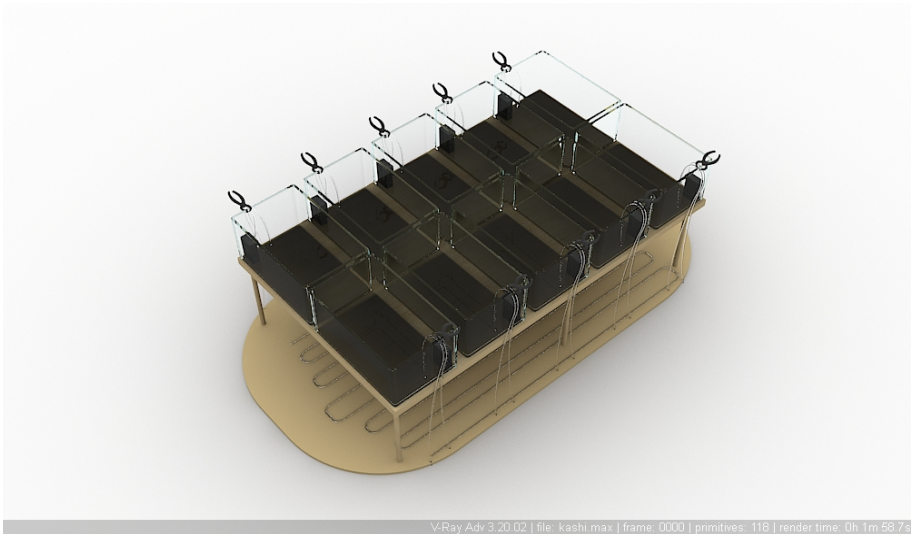
2.3 Construction of photo-bioreactor

A pilot-scale photo-bioreactor was constructed on a wooden board for the photosynthesis of algae (fig. 4). For this purpose, a board with following dimension (150x90cm) was used. The whole board was covered with thin reflective material (Mylar blanket or safety blanket) in order

to get back the light reflection. A total of 10 tubes (1cm diameter, pruechased from local hardware store, Biltema, Norway), each with 10-meter length, were fixed on this board with the help of cable clips, in such a way that approximately 8.5-meter of each hose was mounted on board. The two ends of each hose were ended on top of the table for making an inlet and outlet of the tank. The tubes were dispersed in a fashion that they occupied an area of 1.5 m^2 on wooden board. Tubes board was laid straight on ground surface and a table was then laid on top of this where fish tanks were going to be placed. In addition to this, 10cm thick Styrofoam plates laminated with aluminum were placed on the four edges between tubes board and table, for maximum capturing of light within the designated area.



a



b

Figure 4. A simple view of 3D model (without reflective material and lights) of photo bioreactor with rearing tanks and dispersion of tubes on a wooden board (without Styrofoam plates), (a) side view and (b) top view.

Four identical LED light plates (13.2 cm^2) provided by EVOLYS ^{TM} (Norway), each equipped with 16 diodes were used. These four LED light plates were connected in a series with a distance of 28 cm from each other. The flow of current was regulated by LED Driver (INVENTRONICS-, Model EUD200S070BT) having an output voltage of 143-286 VDC and input voltage of 127-250 VDC. Both light plates and LED driver were tightened together on an aluminum rod. This rod was then hanged under the table in order to illuminate the tubes board. Light intensity measured at a distance of 60 cm between table and tubes board was 165 $\mu molm^{-2}s^{-1}$ in the middle and 95 $\mu molm^{-2}s^{-1}$ over the edges.

2.4 Experimental design

A total of 9 tanks were used for the experiment, and were divided into three treatment groups CON, HPG and FPG. One group was kept as control group (CON), while other two groups were fertilized with phosphate, either a full (FPG) or half (HPG) phosphate concentration. The last tank was an extra tank and was given the same conditions as CON (tank 1). The purpose was to have a backup just in case of fish mortality in any of the tank. The experiment was run for a total of 9 days. The first three days for fish acclimatization, day 4 for feeding establishment, day 5 and 6 for establishment of algae and phosphate fertilization, day 7 and 8 for combined establishment of feeding, algae and phosphate fertilization, while day 9 was only for a control check. These establishment procedures are described below in detail.

2.4.1 Establishment of system with feeding and without algae and phosphate

At the start of day 1 fishes were starved for the previous two days. Following steps were followed on day 1.

1. Due to high turbidity in water, about half (6/6) of the water was changed twice.
2. Samples were then taken for water chemistry measurements (WCMs).
3. After that feed was fed for half an hour but was rejected by the fish.
4. Experiment was postponed for further two days so that the fish properly get acclimatized to this environment.
5. Also the number of fish was reduced to one fish per tank after observing the high NH4+ concentration in the water.
6. Water was regularly changed with two intervals (morning and evening) between these two days (day 2 and 3).

After a break interval of two days, on day 4 the following procedure was pursued.

1. At the start, water was changed by three quarter 8/12 liters and WCMs were taken at 11 a.m. before feeding.
2. Fishes were then fed a weighed feed for half an hour (11 a.m. to 11:30 a.m.) and uneaten feed pellets were collected by siphon method and weighed (method described later).
3. Half an hour after feeding, WCMs were chased for consecutive five hours starting from 12 p.m. to 04 p.m.
4. At the end of the day 4, water was changed again the same way (8/12 liters) and the whole setup was left as it over night.

2.4.2 Establishment of system with algae inoculation and phosphate fertilization

After establishment of the system with feeding, system was established with algae inoculation and phosphate fertilization on day 5 and day 6. For this purpose, following steps were pursued on day 5.

1. Fishes were kept starved for whole day and WCMs were recorded before changing water at 10 a.m.
2. After the first WCMs, water was changed by three quarter and samples were taken again for WCMs at 11 a.m.
3. Next step was to inoculate the algae, so before doing this, one end of each hose was connected to the filter to made a close circulation.
4. Pure culture of *C. sorokiniana* was then inoculated (100 ml) in each tank at 02 p.m. and oxygen concentration was measured every 15 minutes in the first hour and thereafter two-hours, four-hours and five-hours respectively.
5. After two hours of algae inoculation, both FPG and HPG tanks (number mentioned above) were fertilized (at 04 p.m.) with a full disodium phosphate and half disodium phosphate concentration by considering the N:P ratio (method described later).
6. WCMs were done at 04 p.m. before phosphate fertilization, and 06 p.m. after phosphate fertilization.
7. When the tanks were inoculated with algae, all the samples were spun before calculating the WCMs values.
8. Later at 6:30 p.m. another 100 ml of *C. sorokiniana* culture was inoculated to make the concentration higher in the tanks.
9. Oxygen was regulated by introducing the air stones in each tank at 7 p.m. and the whole setup was left as it till next day.

On the next day i.e. day 6 following steps were done.

1. WCMs values were taken without any water changing, feeding and fertilization at 10 a.m.
2. Selected tanks were then fertilized at 12 p.m. and both WCMs and OD samples were measured after every second hour (at 2 and 4 p.m.).
3. Some feces samples were collected from each tank.
4. No further WCMs were done on this day and setup was left overnight.

2.4.3 Establishment of system with feeding, algae and phosphate fertilization

After the system was established individually with feeding, algae inoculation and phosphate fertilization, it was evaluated with all these parameters together. This process was done for two days in a row i.e. day 7 and day 8. Following procedure was followed on day 7.

1. WCMs samples together with OD samples were taken without feeding and fertilization at 9 a.m.
2. Selected tanks were fertilized at 10 a.m. on day 7 and fishes were fed for half an hour (10 a.m. to 10:30 a.m.).
3. Uneaten feed pellets were collected by siphon and were counted and weighed.
5. After half an hour of feeding, WCMs and OD samples were observed for consecutive six hours starting from 11 a.m. to 04 p.m.
6. After the final reading on day 7 whole setup was left as it till next day.

On day 8, same procedure was followed as of day 7 with one exception i.e. timing of phosphate fertilization, which was done at 12 p.m. on day 8. On the last day i.e. day 9 a control check was made. Samples were measured for WCMs once at 10 a.m. without feeding and fertilization.

2.5 Feed formulation and preparation

For the whole experiment only one plant based feed was fed to the fish. The following feed ingredients were used for feed formulation i.e. soybean meal, corn gluten, potato starch, rapeseed oil, mono calcium phosphate, premix, vitamin C, choline chloride, calcium chloride, essential amino acids and sodium alginate. The diet was formulated with 30% crude protein, 6% fat and 30% starch. Table 1 summarizes the formulation of the experimental diet.

Preparation of the feed was done in the feed laboratory at NMBU. Four kg of feed was prepared for the whole experiment. All ingredients were accurately weighed. Soybean meal was pre-treated with phytase (2500 phytase units/kg dry matter) by adding a preheated water

Table 1. Formulation of the experimental diet.

Experimental diet ingredients	Dry matter basis, g/kg
Soybean meal [a]	224
Corn gluten [b]	293
Potato starch [c]	337
Rapeseed oil [d]	43
MCP [e]	10
Premix [f]	10
Vitamin C [g]	10
Lysine [h]	17.82
Threonine [i]	6.8
Tryptophan [j]	2.14
Arginine [k]	10.8
Valine [l]	7.04
Choline chloride [m]	10
Calcium chloride [n]	0.71
Sodium alginate [o]	20

[a]Soybean meal, Denosoy, Denofa, Fredristad, Norway. [b]Corn gluten, Cargill 13864. [c]Potato starch, Sweden. [d]Food grade Eldorado, Oslo, Norway. [e]MCP Bolifor, Yara, Norway. [f] Contents per Kg: Vitamin A 2500.0 IU; Vitamin D3 2400.0 IU; Vitamin E 0.2 IU; Vitamin K3 40.0 mg; Thiamine 15.0 mg; Riboflavin 25.0 mg; d-Ca-Pantothenate 40.0 mg; Niacin 150.0 mg; Biotin 3.0 mg; Cyanocobalamine 20.0 g; Folic acid 5.0 mg; Pyridoxine 15.0 mg; Vitamin C: 0.098 g (Stay-C 35, ascorbic acid phosphate, DSM Nutritional Products, Basel, Switzerland); Cu: 12.0 mg; Zn: 90.0 mg; Mn: 35.0 mg; I: 2.0 mg; Se: 0.2 mg; Cd = 3.0 g; Pb = 28.0 g; total Ca: 0.915 g; total K 1.38 g; total Na 0.001 g; total Cl 1.252 g; Trouw Nutrition, LA Putten, The Netherlands. [g]Stay-C 35, ascorbic acid phosphate DSM Nutritional Products, Basel, Switzerland. [h]L-lysine, Ajinomoto, Japan. [i]Threonine, Ajinomoto, Japan. [j]Tryptophan, Adisseo Brasil Nutricao Animal Ltd., Sao Paulo, Brazil. [k]Arginine, Ajinomoto, Japan. [l]Valine, Sigma Aldrich Co, St. Louis, USA. [mn]Choline and calcium chloride, Qianjiang Yongan Pharmaceutical Co., Ltd., Hubei, China. [o]Sodium alginate, Pronova Biopolymer, Drammen, Norway.

(50°C) up to 40% of moisture in the feed mix and incubating for half an hour. Mixing of incubated soybean meal and major ingredients was done with a kitchen mixer for 5 five minutes. After this, alginate and micronutrients were included and mixed for another 5 minutes. The feed mix was then transferred to a pasta dough mixer (Moretti Forni Grain, Italy) and mixed for another 20 min. During this process, cold water was added to achieve 40% of total feed weight, and pre-weighed rapeseed oil was also added gently. The whole material was then fed through a pasta extruder (P55DV Italgy, Carasco, Italy) for proper conditioning and shaping of pellets.

Feed was cut into 3mm size with the help of pellet cutter at the outer end of the pasta extruder machine. The process was repeated thrice and the temperature of the die and pellet (about 50°C) was monitored. Afterwards, produced feed was dried in a hot air drying cabinet, set at 75°C for five hours. Moisture level for the feed was approximately 7% when stored in a cooling room at 4°C.

2.6 Feed intake evaluation

Fishes were fed a plant based diet on day 4, 7 and 8. Before feeding, feed was individually weighed for each tank. Feed was then provided to fish in each tank for about half an hour and uneaten feed was collected by siphoning afterwards. In order to know the feed intake by individual fish, total feed was subtracted with the feed left after feeding to get the value for given feed. Weight of the uneaten feed, after counting number of pellets was subtracted from the given feed to get the value for eaten feed. The weight of the uneaten feed was standardized by taking 10 dry pellets and weighed them five times on a scale. The average value was 0.15 g from the 5 samples.

2.7 Calculation of N:P ratio

Selected tanks were fertilized with disodium phosphate (Na_2HPO_4 Molecular weight = 141.96, Merck, Dramstadt Germany) in powder form. Tentative optimal N:P ratio present in water was set to 16:1 and to get the nitrogen and phosphorous ratio based on this standardization, following calculations were done. According to the atomic table, molecular weight of NH_4^+ and NO_2^- becomes 18.04 and 46.01 respectively. Since these values were in mg/l and in order to get them into µmol/l, following formula was used.

Micromoles = Concentrations (mg/l) /1000 / molecular weight (g/g-mol) $*10^{-6}$

After having NH_4^+ and NO_2^- values in µmol/l, they were summed up and an average value (35.0 µmol/l) of the nine tanks was obtained. This resulted value was then divided by 16

to obtained the value for phosphorous (2.1903 µmol/l). The final N:P ratio became 35.0 µmol/l :2.19 µmol/l. As the molecular weight of Na_2HPO_4 was 141.96 and the total volume of water in each combined tank and tube was 12 liters so following calculation was made to get the weight of disodium phosphate in milligram (mg),

2.1903 µmol/l *141.96 (g/g-mol) *12 liters/1000 = 3.731 mg

This resulted value was a full phosphate concentration i.e. 16:1 ratio. A half phosphate concentration was then simply made by dividing the full phosphate concentration value (3.73 mg/l) by two.

2.8 Assessment of water chemistry parameters

2.8.1 Dissolved oxygen

For the measurement of dissolved oxygen, an oxygen probe (OxyGurad, Handy MK III, Denmark) was used. Every time before measurements, oxygen probe was calibrated by turning on the device and let the reading in the display to be 100% atmospheric oxygen. In case of low or high percentage, device was manually fixed with a side screw to 100% atmospheric oxygen. After calibration, device was set to desired measurement unit i.e. mg/l for taking dissolved oxygen readings. Average dissolved oxygen concentration was around 6.33 mg/l during the experiment.

2.8.2 Temperature and pH

Temperature of the water was manually checked by using a simple laboratory thermometer. All the pH measurements were done by using pH probe VWR pH 100 (VWR International, USA.). The pH probe was calibrated every time before usage, first with pH 4 and then with pH 7. Average value for temperature and pH during the whole experiment was 27.8°C and 7.68 respectively.

2.8.3 Ammonium (NH_4^+), Nitrite (NO_2^-) and phosphate (PO_4^{3-})

For measuring the ammonium, nitrite and phosphate concentration in the water, Spectroquant® NOVA 60 photometer accompanied with testing kits ((NH_4^+, NO_2^-, and PO_4^{3-}) was used. Measurements of ammonium (NH_4^+), nitrite (NO_2^-) and phosphate (PO_4^{3-}) were performed according to the manufacturer's instructions. Water sample were taken with the help of a pipette, each time exactly 5ml for ammonium and phosphate and 10 ml for nitrite. No spinning was done before algae inoculation. But when the system was inoculated with

C.sorokiniana, samples were spun down at 3000 rpm for 5 min by using a centrifuge (Wifug Lab Centrifuge, Wifug Ltd., England) to avoid the possible error in measuring the samples.

2.8.4 Optical density (OD)

Optical density for algae concentration was done at OD_{750} by using UV-VIS Spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto Japan). All the samples were directly taken from each tank and were checked for optical density.

2.9 Statistical analysis

The results were subject to two-way analysis of variance (ANOVA) and linear regression analysis by the SAS computer software (SAS Institute Inc. Cary, NC, USA). Factors in the ANOVA were phosphate fertilization level and time (hours) post feeding. Statistically significant ($P<0.05$) were ranked by least-square means in the P-diff procedure.

3 Results

For the ease of understanding the whole experiment, results are categorized and described on daily basis. From day 1 to 4, a general pattern of ammonium, nitrite and phosphate concentrations in the water after introduction of feed was followed. While on day 5 and 6 same concentrations were followed, after inoculating the system with *Chlorella* and fertilization with different levels of phosphate. In these two days no feed was offered to the fish. On day 7 and 8, as the system was inoculated with *Chlorella* and fertilized with phosphate, the feed was offered to the fish, and again the same concentrations were followed. A control measurement was performed on day 9 without feeding and phosphate fertilization.

3.1 Day 1, 2 and 3 observations

On day 1, the feed was offered but was poorly eaten or rejected by the fish, possibly because of the stress element. The stress factor was due to high level of ammonium concentration observed. The distribution of ammonium, nitrite and phosphate levels in all the ten tanks is presented in the fig. 5. As it can be seen from the fig. 5, the ammonium concentration was higher than 1mg/l in each tank. Initially, two fishes per tank were introduced but after the observed high ammonium concentration, fish number was reduced to one fish per tank. Moreover, the experiment was postponed for two days so that the fish could fully adapt to the environment. Because of that reason no measurements were done on day 2 and day 3, and the water was partly replaced twice/day.

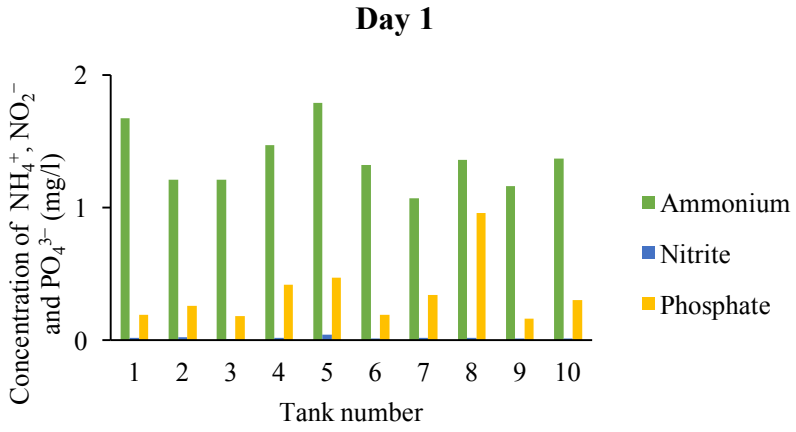


Figure 5. Concentrations of ammonium, nitrite and phosphate in all ten tanks on day 1.

3.2 Day 4 observations

The experiment was resumed on day 4 with the introduction of feed to the fish. A general pattern of different concentrations of ammonium, nitrite and phosphate in ten tanks at different

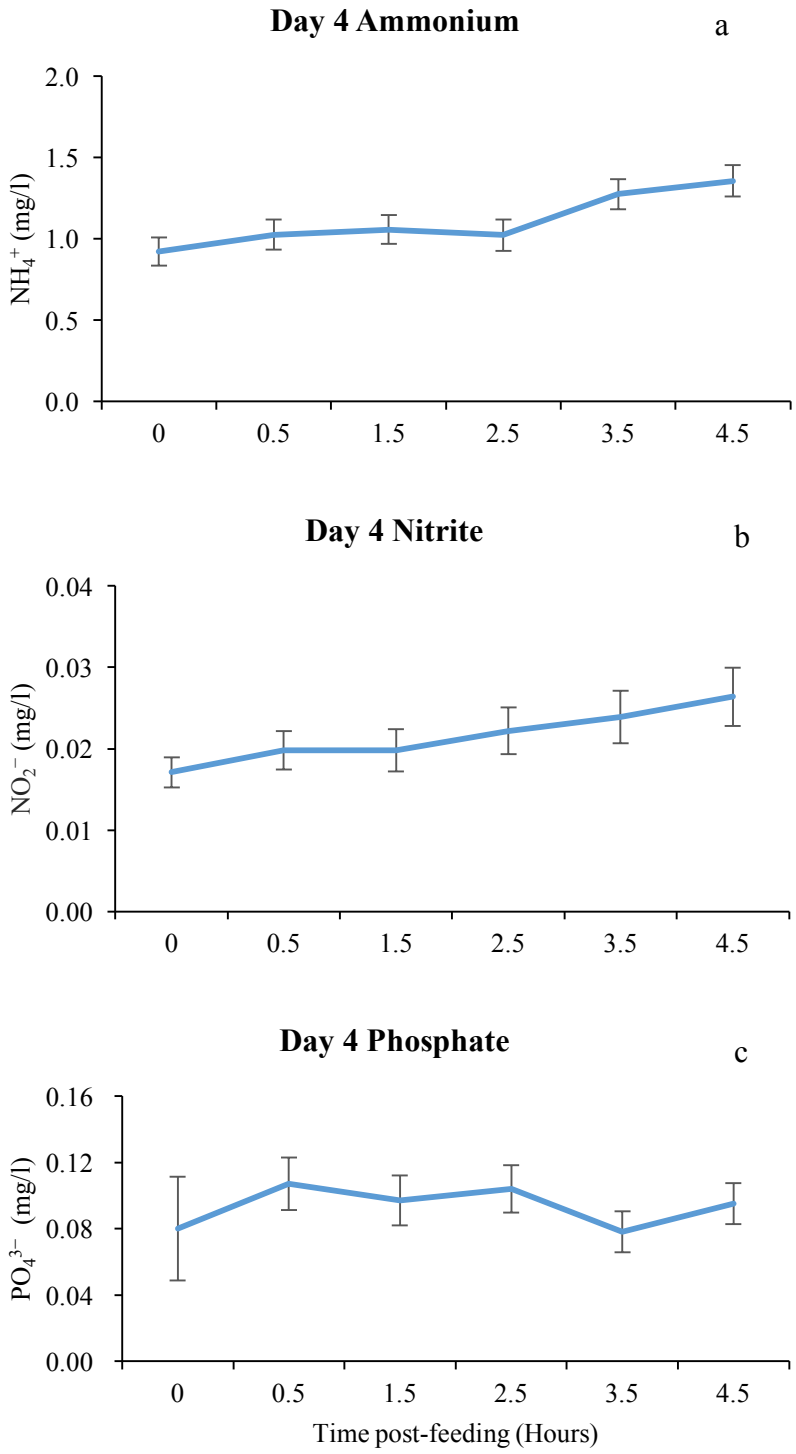


Figure 6. Development of the general pattern of (a) ammonium, (b) nitrite and (c) phosphate concentrations (mean ± SE) in ten tanks at different time points of post-feeding on day 4.

time points post-feeding is presented in the fig. (6). The values indicate response to feeding with increasing and decreasing values of ammonium, nitrite and phosphate at different time point. The starting values for ammonium were quite high due to the presence of the fish in the tanks (fig. 6a). In case of ammonium, there was a linear increase in the concentration. After post-feeding, values ascended slightly from half an hour to 2.5 hours, and increased rapidly between 2.5 hours to 4.5 hours. A maximum concentration of ammonium was observed after 4.5 hours (1.35 mg/l ± 0.09) post-feeding. On the next morning, a measurement was carried out after about 22.5 hours (1.22 mg/l ± 0.08), which showed a decline in the concentration, but the value was still higher than the initial value (0.92 mg/l ± 0.08).

Nitrite concentration was ascended in a smooth linear proportion from 0 hour (0.017 mg/l ± 0.002) post-feeding to 4.5 hours (0.026 mg/l ± 0.004) post-feeding (fig. 6b). The concentration boosted to a level of 0.057 mg/l ± 0.009 when measured after 22.5 hours post-feeding. In case of phosphate, rather an irregular pattern in concentration was observed (fig. 6c). The concentration increased at 0.5 hours, 2.5 hours and 4.5 hours, whereas decrease was recorded at 1.5 hours and 3.5 hours. Analysis after 22.5 hours post-feeding (0.02 mg/l ± 0.006), revealed a decline in the concentration compared to the starting value (0.08 mg/l ± 0.03).

3.3 Day 5 and Day 6 observations

On day 5, *Chlorella* was inoculated to the system with an initial concentration of 1dl followed by another deciliter after four hours. The system was also fertilized with pre-calculated amount of phosphate after dividing the tanks into three categories i.e. control group (CON), half phosphate group (HPG) and full phosphate group (FPG).

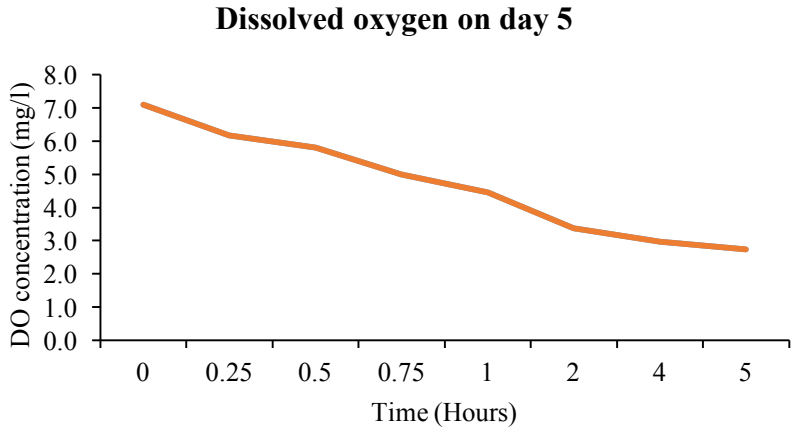


Figure 7. A summary of the drop of oxygen concentration in nine tanks (mean values) after removal of aeration on day 5.

A vigorous drop in oxygen concentration was observed in the system after being inoculated with the algae (fig. 7). The drop in concentration was due to the removal of aeration which was being provided to the system before algae inoculation. Concentration was measured for every 15 minutes in the first hour and thereafter two-hours, four-hours and five-hours respectively. On day 6, green feces were observed in the tanks, indicating that fish was filtering *Chlorella* through the gills and ingesting it.

Progress of ammonium, nitrite and phosphate concentrations on day 5 and 6 in three groups (CON, HPG, FPG) is shown in fig. (8). The mean initial and final values obtained for ammonium, nitrite and phosphate for the three groups, ranged from 0.65 to 0.13 mg/l, 0.04 to 0.01 mg/l and 0.05 to 0.17 mg/l respectively. The mean values present a decrease in ammonium and nitrite concentrations (fig. 8a, b) while a mix of increase and decrease in phosphate concentration (fig. 8c) to a dose response to algae inoculation and phosphate supplementation. For individual time point, significant differences ($p < 0.05$, $p < 0.01$, $p < 0.001$) or tendencies ($p < 0.10$) post-algae inoculation and phosphate supplementation is illustrated. This is shown at time points 2 ($p = 0.007$), 4 ($p = 0.024$), 20 ($p = 0.052$) and 24 ($p = 0.097$) hours in case of phosphate (fig. 8c).

No significant differences in ammonium and nitrite concentrations were observed between the three groups. The starting concentrations of ammonium and nitrite at time point 0-hour in all the three groups were slightly different but at a time point of 43 hours they were nearly similar fig. (8a, b). On the other hand, phosphate concentration was similar at the time point 0-hour, where as at time point 43 hours, there was high concentration in FPG followed by CON and HPG. From the fig. (8a and b) it is clear that system was got established at time point of 20 hours. As the concentrations were nearly similar both in ammonium and nitrite case.

3.4 Day 7 observations

On day 7, when the system was stabilized with algae, a follow up of ammonium, nitrite and phosphate concentrations was accomplished. The fish was fed and the tanks were supplemented with predefined phosphate concentrations.

Development in ammonium, nitrite and phosphate concentrations on day 7 in all the three groups is presented in fig. (9). The mean initial and final values obtained for ammonium, nitrite and phosphate for the three groups, ranged from 0.13 to 0.54 mg/l, 0.01 to 0.05 mg/l and 0.11 to 0.13 mg/l respectively. The mean values indicate a response to feeding and phosphate

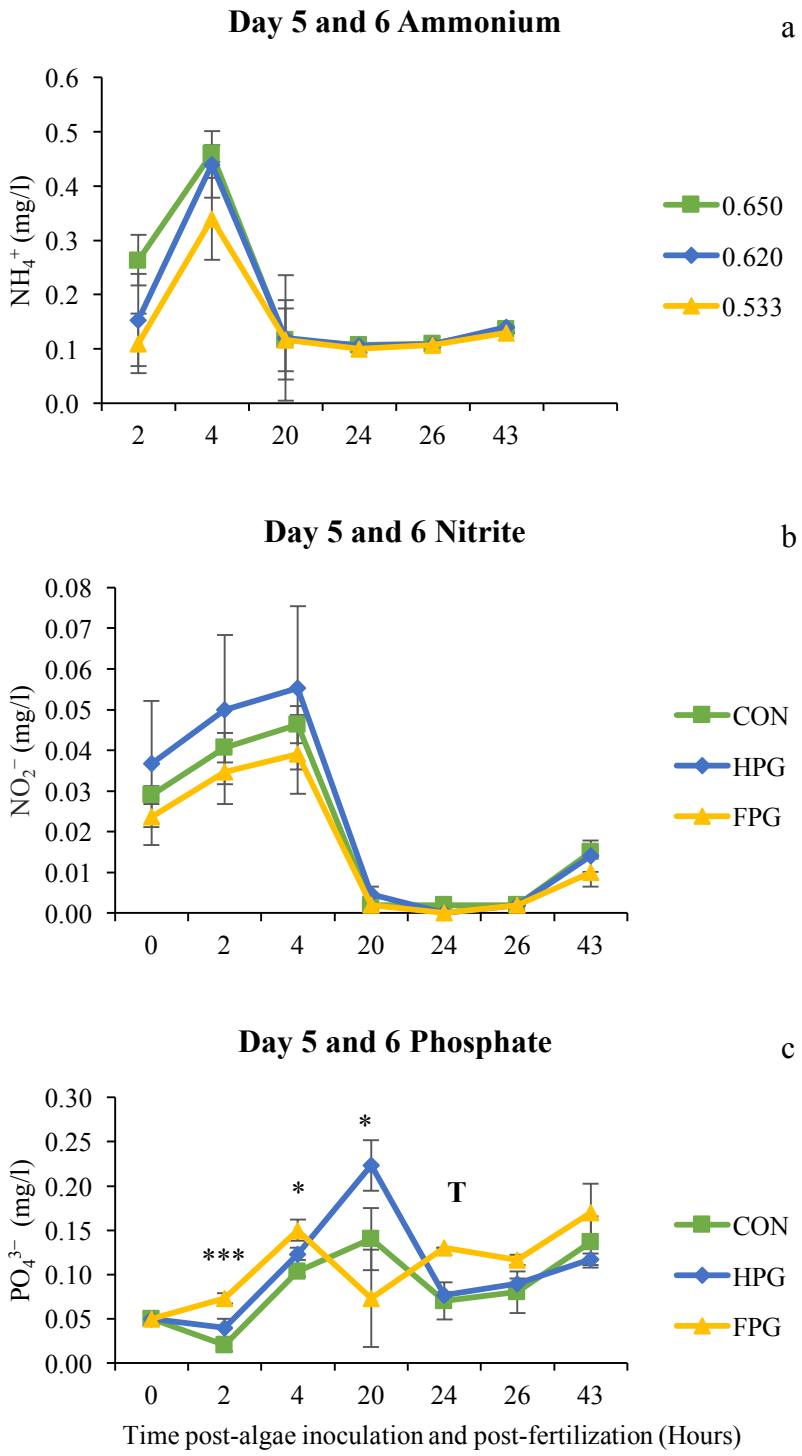


Figure 8. Development of (a) ammonium, (b) nitrite and (c) phosphate concentrations (mean ± SE) on day 5 and 6 in three groups (CON, HPG, FPG) after being inoculated with C.sorokiniana and Na_2HPO_4 fertilization. Tendencies (T) and significant differences (T = $p<0.1$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

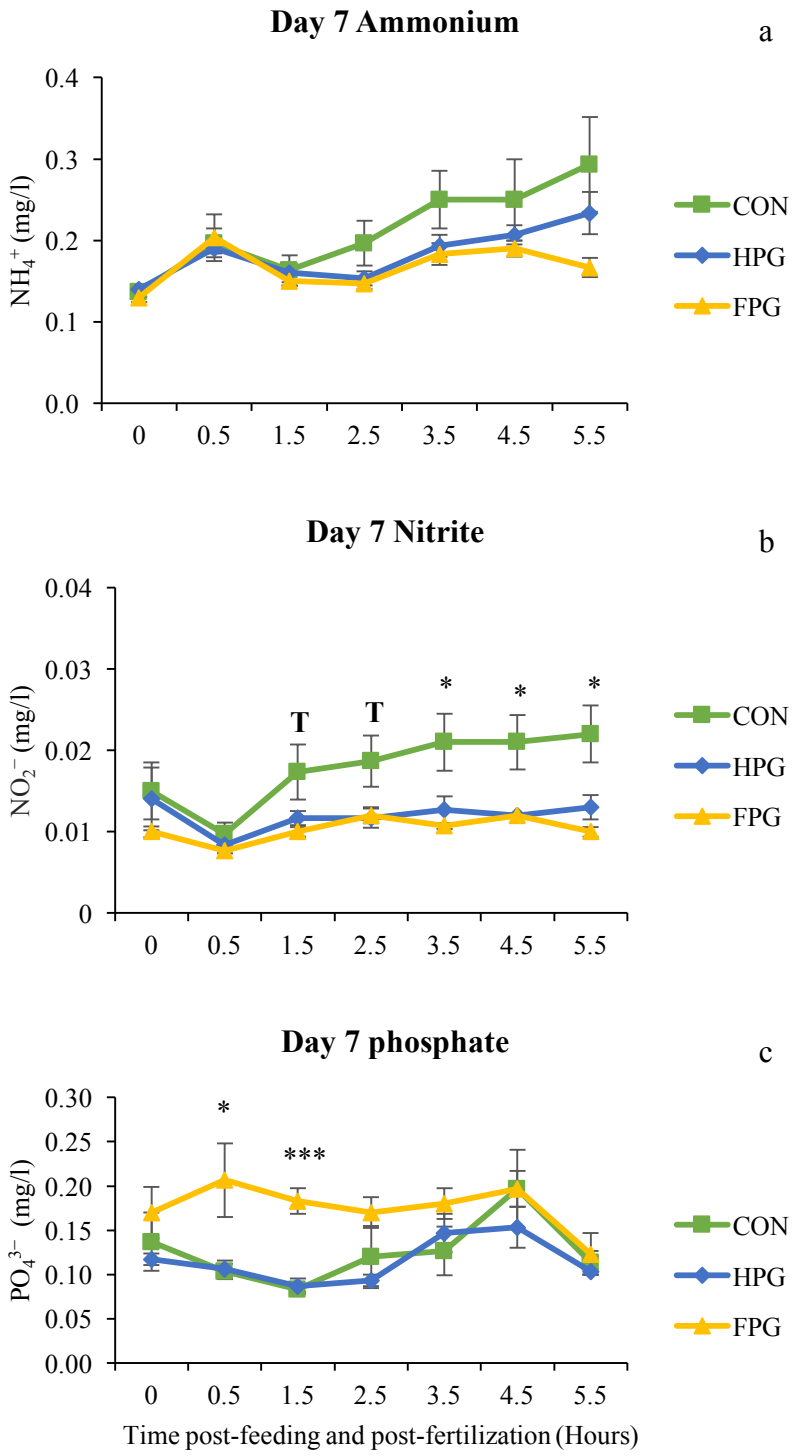


Figure 9. Development of (a) ammonium, (b) nitrite and (c) phosphate concentrations (mean ± SE) on day 5 and 6 in three groups (CON, HPG, FPG) after being inoculated with C.sorokiniana and Na_2HPO_4 fertilization. Tendencies (T) and significant differences (*, **, ***) & sign illustrations (T = $p<0.1$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

supplementation, with decreasing values with increasing phosphate dose. The general trend was similar in ammonium and nitrite concentrations i.e. FPG has lowest concentration after time-point 5.5 hours followed by HPG and CON. Whereas HPG has the lowest phosphate concentration followed by CON and FPG. It can be seen from the fig. (9a and b), a linear pattern of either increase and or decrease in concentration for the three groups, both in ammonium and nitrite.

In case of ammonium, at time point 0 the concentration was nearly similar in all the groups. No significant difference was observed at any time point; however numeric differences were observed from time point 2.5 to 5.5 hours. A tendency in difference of nitrite concentration was commenced at time point 1.5 hours ($p = 0.09$) followed by time point 2.5 hours ($p = 0.08$), thereafter significant differences were observed at time point 3.5 ($p = 0.03$), 4.5 ($p = 0.03$) and 5.5 ($p = 0.02$) hours (fig. 9b). Phosphate concentration was significantly different at time point 0.5 ($p = 0.04$) and 1.5 ($p = 0.0006$) hours post feeding and phosphate supplementation (fig. 9c).

3.5 Day 8 and 9 observations

Day 8 was a repetition of the day 7 in a sense that same procedure was followed. But we have observed that system was more established on day 8. For instance, FPG in comparison to HPG and CON had quite low concentrations in both ammonium and nitrite than day 7 in terms of numerical values. The trend in concentrations observed was also more linear than day 7.

Development of ammonium, nitrite and phosphate concentrations on day 8 in all three groups is presented in fig. (10). The mean initial and final values obtained for ammonium, nitrite and phosphate for the three groups, ranged from 0.24 to 1.13 mg/l, 0.01 to 0.134 mg/l and 0.10 to 0.11 mg/l respectively. As it is obvious from the figs. (10a and b) both ammonium and nitrite concentrations showed a smooth trend from 0 hours to 5.5 hours post-feeding and phosphate supplementation, indicating the establishment of the system. At time point 5.5 hours, FPG kept the lowest concentration in both ammonium and nitrite followed by HPG and CON. Phosphate concentration was nearly similar in all three groups after 4.5 hours (fig. 10c). A rapid rise in concentration in FPG and HPG at time point 5.5 hours was due to the phosphate supplementation.

In case of nitrite, tendencies were observed at time points 0 ($T = 0.06$), 0.5 ($T = 0.07$) and 1.5 ($T = 0.07$) hours post-feeding and phosphate supplementation. In case of phosphate, significant difference was observed only at time point 0.5 hour post-feeding and phosphate on day 9 after time point of 22.5 hours, one control measurement was done for ammonium, nitrite

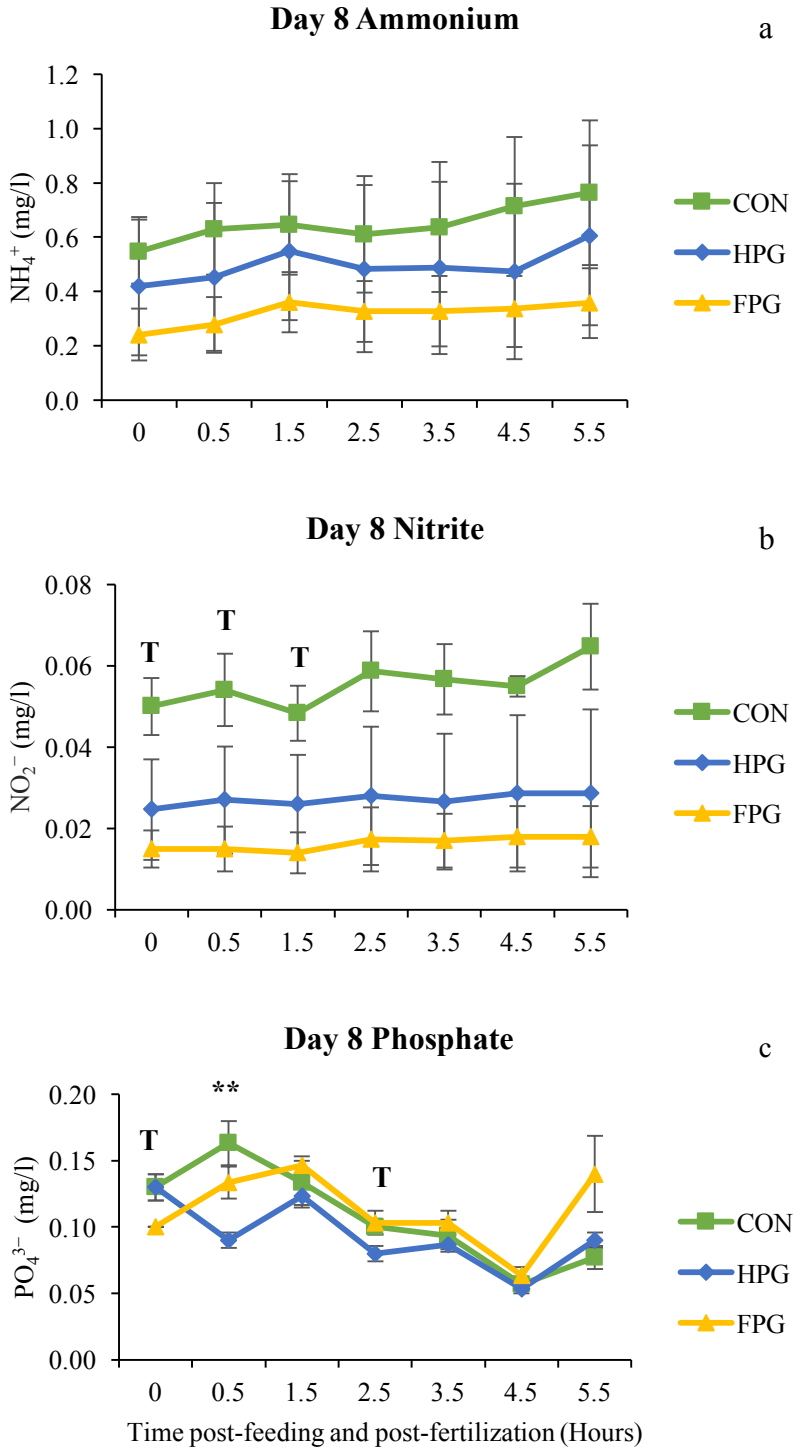


Figure 10. Development of (a) ammonium, (b) nitrite and (c) phosphate concentrations (mean ± SE) on day 8 in three groups (CON, HPG, FPG) after feeding and Na2HPO4 fertilization. Tendencies (T) and significant differences (*, **, ***) & sign illustrations (T = p<0.1, * = p<0.05, ** = p<0.01, *** = p<0.001).

and phosphate before post-feeding and phosphate supplementation. The measurement showed no significant differences, but rather showed high numerical differences both for ammonium and nitrite concentrations between the three groups. For instance, ammonium concentrations for FPG, HPG and CON were 0.34 mg/l ± 0.18, 0.42 mg/l ± 0.26, 1.13 mg/l ± 0.44 respectively. Similarly, nitrite concentrations for FPG, HPG and CON were 0.03mg/l ± 0.02, 0.06 mg/l ± 0.05, 0.13 mg/l ± 0.03.

3.6 System response to phosphate fertilization on day 7 and day 8

Statistically, data from day 7 and day 8 showed tendencies and significant differences in nitrite and phosphate concentrations at certain time-points of post-feeding and post-fertilization. The data did not reveal any significant difference in ammonium concentration in term of time-points for these two days. Nevertheless, a strong linear response was found when mean values of ammonium and nitrite concentrations at different time-points were plotted in terms of phosphate supplementation. In this FPG performed the best followed by HPG and CON. The response was stronger for ammonium concentration than nitrite (fig. 11a, b, c, d). The system response increased with the increasing dose of phosphate, or in other way, concentrations decreased with the increasing dose of phosphate.

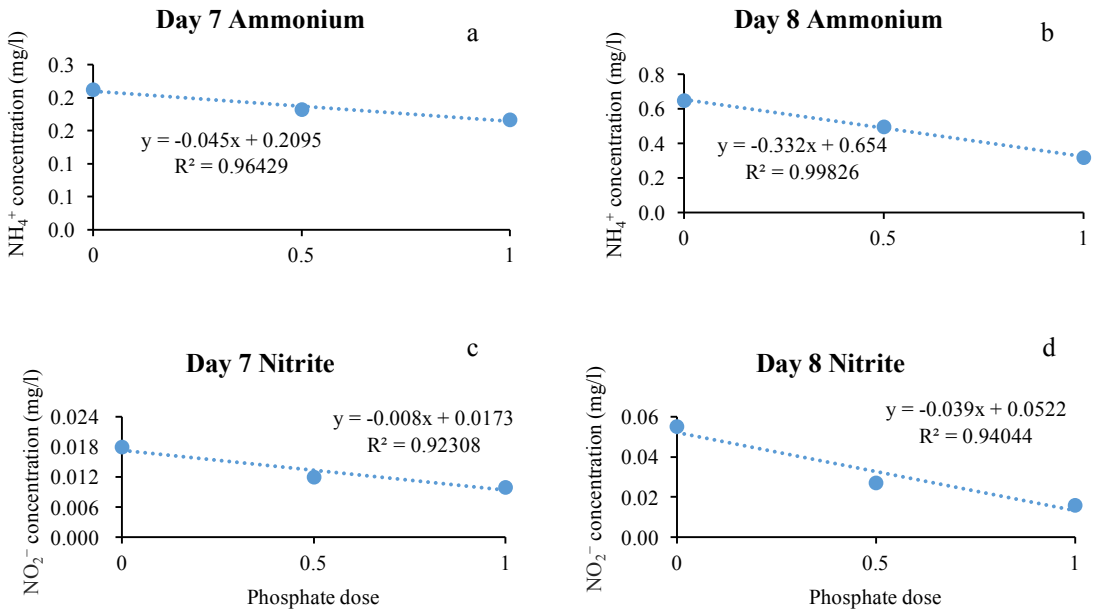


Figure 11. Mean values for concentration of (a, b) ammonium and (c, d) nitrite in response to phosphate fertilization.

3.7 Algae concentration from day 6 to 8

Chlorella concentration was measured for 51 hours from day 6 to 8 by using UV-VIS Spectrophotometer at OD_{750}. The graph showed an irregular pattern in concentration of *Chlorella* in all the three groups (CON, HPG, FPG). As the initial concentration was high and it dropped down after 20 hours. The rapid concentration was achieved between 20 to 22 hours, and was maintained until 25 hours, but thereafter began to fall again and showed an irregularity once again.

Two-way ANOVA analysis revealed that HPG has significantly higher ($p = 0.003$) algae concentration from CON and has the tendency ($p = 0.05$) to be higher from FPG. Which indicates that cell concentration was higher in HPG (when measured at OD_{750}). Visual observance, however revealed that the light exposed plastic tubes at FPG contained more green colonizing material than HPG and CON. This suggests that nutrients were removed by the algae that were colonized in the tubes in the illuminated area rather than freely moving.

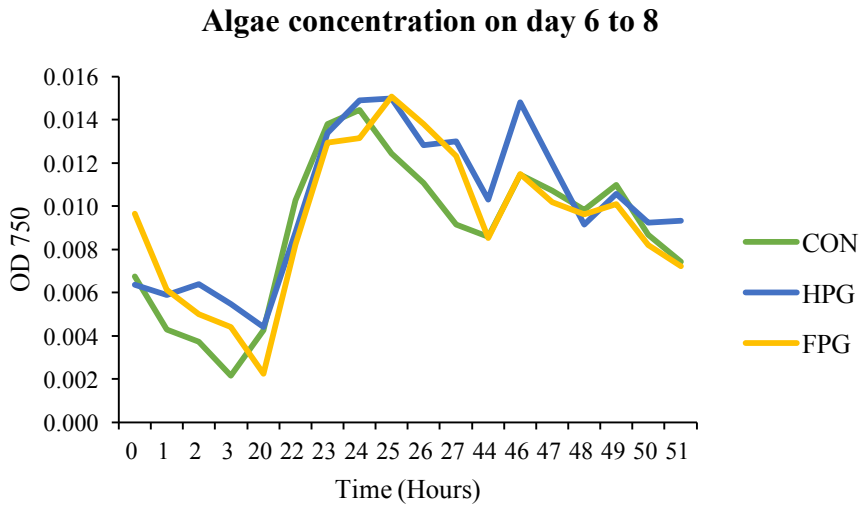


Figure 12. Measurement of algae concentration from day 6 to day 8 in three different groups at optical density of 750nm.

4 Discussion

On day 1, the feed was offered but was poorly eaten or rejected by the fish. This poor feed intake or rejection was may be due to the stress element such as handling and or pollutants such as ammonia. Ammonium concentration was observed higher than 1mg/l in each tank on day 1. This poor feed intake due to stress and high ammonia is in agreement with the previous findings (Bonga, 1997; Israeli-Weinstein and Kimmel, 1998; Leal et al., 2011).

Average value for temperature during the whole experiment was 27.8°C. Optimal temperature range for tilapia growth ranges between 22°C to 29°C and the observed value is in the range as previously described (Mjoun et al., 2010a). The average pH observed during the experiment was 7.7 which is considered as crucial for optimum growth performance and survival rate of tilapia. This pH is within the recommendation range depicted by El-Sherif and El-Feky (2009).

Dissolved oxygen is another important factor for the normal feed intake and of tilapia. The value of average oxygen concentration in all three groups remained 6.3 mg/l during the experiment except for the day 5 when the aeration was stopped before algae inoculation. The oxygen concentration dropped down from 7.1mg/l to 2.7 mg/l from 0 to 5 hours respectively (Fig. 7). The observed oxygen concentration is within the optimum range i.e. 2.5 to 7.1mg/l (El-Sherif and El-Feky, 2008). Survival of tilapia at low oxygen concentration and having no mortality is in accordance with the earlier findings (Magid and Babiker, 1975).

Optimal temperature and pH are equally important for growth of *Chlorella*. Temperature reported for optimum growth of *Chlorella* is between 25 to 35°C with 30°C being the best (Chinnasamy et al., 2009; Cassidy, 2011). Temperature (27.8°C) observed during this study is within this temperature range. *Chlorella* has the capacity to grow over a wide range of pH and grows best between 6 to 8, but is unable to grow at pH 2 or below (Lustigman et al., 1995). The pH (7.7) value monitored in this study is therefore within the reported range for *Chlorella* growth. Algae utilize CO_2 into biomass and this utilization affect pH of water (Hailing-Sørensen et al., 1996; Kim et al., 2010 and 2013). There are two phenomena in this regard. If CO_2 concentration in water is high, pH will decrease as excess will be converted to H_2CO_3. In case of low concentration, pH will be increased as utilization will be from HCO_3^-. No significant change in pH was monitored in the system even after algae inoculation on day 5 and onwards. This non-significant change in pH hence reveals that there was optimal concentration of CO_2 in the system. CO_2 was not measured in this study, however.

On day 1, high concentration of ammonium was present although no mortality was observed. The value of that total ammonia can be similar at different temperature and pH while the unionized ammonia varies with the change in temperature and pH (Wurts, 2003). According to a standard pH- temperature table this suggest that concentration of un-ionized ammonia in this study ranged between 0.015 to 0.042 mg/l (Francis-Floyd et al., 2009). This calculated concentration is below the toxic range of ammonia for tilapia i.e. 0.05 mg/l (El-Sherif and El-Feky, 2008).

The increase in excretion rate after feeding is reported by many previous studies (Handy and Poxton, 1993; Ballestrazzi et al., 1994; Engin and Carter, 2001). On day 4, ammonium concentration increased linearly after feeding the fish and the peak value was obtained after 4.5 hours, while a decline was noticed afterwards. This process is supported by earlier investigations (Dosdat et al., 1996; Peres and Oliva-Teles, 2006). Ammonium concentration after 22.5 hours (1.22 mg/l ± 0.08) was higher than the initial concentration (0.92 mg/l ± 0.08). This observation is in accordance with the findings of Engin and Carter (2001), who observed a higher concentration of ammonium after 24 hours compared to the initial value. Nitrite increase was also linear, and a measurement after 22.5 hours (0.05 ± 0.009) disclosed a higher concentration compared to 4.5 hours (0.01 ± 0.002). This increase in nitrite concentration may suggest the bacterial activity in the water i.e. oxidation of ammonia to nitrite. It is well known that nitrogen is a mandatory substrate for ammonia-oxidizing bacteria to maintain their growth (Kowalchuk and Stephen, 2001). In case of phosphate, concentration increased at 0.5 hours, 2.5 hours and 4.5 hours of post-feeding and declined after 22.5 hours. A similar pattern in dietary phosphorous excretion, after feeding sea bass with different protein sources has been documented (Ballestrazzi et al., 1994).

The day after (day 6), when algae was inoculated to the system, green feces were observed in the tanks. This indicates that fish was filtering algae through the gills and ingesting it. Many studies have shown that tilapia can filter and ingest the microalgae, but variations (ingestion, filtration and assimilation efficiency) occur in terms of fish size, type and concentration of algae (Northcott et al., 1991; Turker et al., 2003; Lu et al., 2004). This study was not designed to quantify filtration and or ingestion efficiency. But it should be a positive aspect for future study when the fish growth is a concern. However, existence of green feces confirmed that tilapia can use algae directly from the water either by filtration or direct ingestion by drinking algae-rich water.

On day 5, when algae were inoculated to the system, a vigorous drop in ammonium and nitrite concentrations was observed in all the three groups (CON, HPG, FPG). The mean initial

and final values obtained for ammonium and nitrite for the three groups were from 0.65 to 0.13 mg/l and 0.04 to 0.01 mg/l, respectively. Removal of ammonium and nitrite by algae has been reported in many studies (Widjaja et al., 2009; Kim et al., 2010; Liang et al., 2013; Velichkova, 2014; Guerrero-Cabrera et al., 2014). It is documented that nitrogen is required as the major nutrient, and is the only plant nutrient which is available in both cationic (NH_4^+) and anionic (NO_3^-) form (Forde and Clarkson, 1999). The absorption of ammonium is a passive while of nitrate is an active process and priority of uptake is somewhat unclear and have many doctrines. For instance, above a threshold level of ammonium, nitrate is inhibited and vice versa (Haynes and Goh, 1978; Solsac et al., 1987; Dortch, 1990). Cells who are well-stocked with nitrogen from ammonium cannot instantly use nitrate (Syrett, 1981, (cited by Flynn et al., 1997)). Further, ammonium-inhibition of nitrate uptake is not because of ammonium, but rather a product of its assimilation (Cresswell and Syrett 1979). A modelling study, however assumed a simultaneous change in the nutrient status (Flynn et al., 1997). Based on the first three concepts, ammonium is supposed to be the primary source of nitrogen for *Chlorella*, while according to the model study it can be both. The immediate reduction of ammonium in the first 2 hours of post-algae inoculation and increase in nitrite until 4 hours on day 5 (Fig. 8a, b) in all three groups is supported by these studies (Haynes and Goh, 1978; Cresswell and Syrett 1979; Solsac et al., 1987; Dortch, 1990). Both nitrite and ammonium concentrations were decreased on day 6, and after 20 hours of post-algae inoculation these remained at the same level until 26 hours. The persistence of both at the same level from 20 to 26 hours corresponds with the modelling study done by Flynn et al. (1997). The nitrite concentration after 26 hours (0.002 ± 0.00) was exactly similar in CON (no phosphate-fertilization), HPG (1.86 mg/l) and FPG (3.73 mg/l). At 43-hours an increase was observed in all the groups.

Observations from day 7, 8 and 9 showed that nitrite was largely increased in CON (0.01 ± 0.004 to 1.13 ± 0.44) and HPG (0.01 ± 0.004 to 0.06 ± 0.05), while it only slightly increased in FPG (0.01 ± 0.004 to 0.03 ± 0.02). The nitrite difference in three groups was probably due to the ammonia-competition between algae and ammonia-oxidizing bacteria. As a direct competition between algae and ammonia-oxidizing bacteria occurs and algae are superior competitors in this regard, in terms of growth and nitrogen uptake (Risgaard-Petersen et al., 2004). This suggest higher abundance of ammonia-oxidizing bacteria in CON followed by HPG and FPG.

On day 7, 8 and 9 a linear interaction between the removal of both ammonium and nitrite was observed for all the three groups (CON, HPG, FPG). A plot of mean values of ammonium

and nitrite concentration at different time-points showed a better fit ammonium than nitrite, which supports ammonium as primary preference.

The system was evaluated on the basis of effect of phosphate fertilization in the three groups (CON, HPG, FPG) from day 5 to 9. Two measures are discussed in this aspect, one is the algal growth rate and second is the removal capacity of ammonium, nitrite and phosphate. It was noticed that removal capacity of ammonium, nitrite and phosphate between three groups was not varying on day 5 and 6, and all the groups responded in a similar fashion even when they were fertilized with phosphate, meaning growth was similar at this level. Significant differences were observed on days 7, 8 and 9. These results indicate higher growth of algae in the fertilized groups with the following order FPG>HPG than CON. The enhancement of algal growth with the application of phosphate fertilization is previously reported (Bisoyi and Singh, 1988; Knud-Hansen and Batterson, 1993 and 1994; Elnady et al., 2010). On day 7, ammonium and nitrite concentrations in HPG and FPG were significantly lower than that of CON. Similarly, on day 8 and 9, lower concentration of ammonium and nitrite was found in FPG and HPG than CON. This difference in CON, HPG and FPG concentrations suggest a difference in growth rate of the algae.

There is a direct relation between the algal growth and decrease in water nitrogen content (Tam and Wong, 1996; Kim et al., 2010). Decrease in concentrations (ammonium, nitrite, phosphate) was observed in the following order, FPG<HPG<CON. In other words, full phosphate fertilization resulted in lower concentrations of ammonium, nitrite and phosphate followed by HPG and CON. On day 7 and 8, low concentrations of ammonium and nitrite were observed in FPG followed by HPG and CON. Phosphate concentration was observed similar in all the groups at the end of day 7, 8 and 9. This difference in the removal capacity for ammonium and nitrite and indifferent phosphate was might be due to the N/P ratio. As the removal capacity of nitrogen is influenced by N/P ratio, while phosphorous removal is independent (Xin et al., 2010). The results from day 8 and 9 showed that ammonium and nitrite were not completely removed in FPG, which was assumed to have higher algal growth and N/P ratio. One possible reason for that might be that the concentration of CO_2 may have been insufficient for maximum photosynthesis, since the CO_2 also has a stimulatory effect on nutrient removal (Kim et al., 2013). The correlation between phosphate fertilization level and removal capacity for ammonium and nitrite showed that concentrations decreased with the increasing level of phosphate fertilization. This was because of higher algal growth in FPG followed by HPG and CON as phosphate is the limiting nutrient for algal growth (Bisoyi and Singh, 1988; Xin et al., 2010). These results are in correspondence with the work of Kim et al. (2013).

Algae concentration in the tank with fish was measured for 51 hours from day 6 to 8 at OD_{750}. Visual observance revealed that the light exposed plastic tubes at FPG contained more green colonizing material than HPG and CON. These dense colonies of algae grew and fastened more intensely in the illuminated part of tubes than the part without light (part that was going in and out of the tanks). Algae like all other plants need light to assimilate inorganic carbon for conversion into organic matter by the process of photosynthesis (Lavens and Sorgeloos, 1996). Increased growth and colonization of algae in the tubes part, exposed to the light, is well supported by many studies (Lustigman et al., 1995; Tam and Wong, 1996; Atta et al., 2013; Blair et al., 2014). The two-way ANOVA analysis revealed that HPG had significantly higher algae concentration from CON and tended to be higher from FPG. This analysis showed conflicting results from nitrogen removal, where FPG proved most efficient in removing ammonium and nitrite. This suggests that nutrients were removed by the algae that were colonized in the tubes in the illuminated area. Lower concentration in FPG than HPG is may be because of this factor that most of algae in FPG was fastened to the tubes. This also provides the reason that FPG tubes were more green than HPG as were seen by naked eyes.

5 Conclusions

Stocking of starved fish for two days in experimental tanks without algae caused ammonium concentration higher than 1 mg/l. A linear response in the increase of ammonium and nitrite over time was observed after feeding. Tilapia was found to filter algae through the gills and ingesting it as indicated by the presence of green feces in the tanks. Further, tilapia survived at dissolved oxygen level of around 2 mg/l.

Chlorella sorokiniana in this study demonstrated to have a profound effect on removal of tilapia wastes (ammonium and nitrite) and phosphate. A strong linear decrease in ammonium and nitrite concentration is found with the increase of phosphate dose i.e. FPG performed the best followed by HPG and CON. The decrease was stronger for ammonium concentration than nitrite, confirming that ammonium is the preferred nitrogen source for assimilation in *Chlorella*. Algae grew and fastened maximum to the illuminated area (tubes) than the tanks as seen by the naked eyes. A higher concentration of algae in HPG than FPG and CON at OD_{750} was found. This suggest that although HPG possessed higher concentration but nutrient removal is more dependent on algae colonizing the tubes exposed to light than algae freely moving in the water. Based on the data, it is proposed that

Chlorella sorokiniana has a great potential to be used as a bio-filter in recycled tilapia production. The system provides basic understandings to remove fish wastes and phosphate, and ratifies the combine culturing of algae and fish. This could be an economical but challenging way which open new doors for recirculation of wastewater from fish. Further studies needed to be undertaken for appropriate understanding of the system before any practical applications.

6 References

Abdel Magid, A., & Babiker, M. (1975). Oxygen consumption and respiratory behaviour of three Nile fishes. *Hydrobiologia, 46*(4), 359-367.

Abdel-Tawwab, M., Hagras, A. E., Elbaghdady, H. A. M., & Monier, M. N. (2015) Effects of dissolved oxygen and fish size on Nile tilapia, *Oreochromis niloticus* (L.): growth performance, whole-body composition, and innate immunity. *Aquaculture International*, 1-14.

Atta, M., Idris, A., Bukhari, A., & Wahidin, S. (2013). Intensity of blue LED light: a potential stimulus for biomass and lipid content in fresh water microalgae *Chlorella vulgaris*. *Bioresource Technology, 148*, 373-378.

Ballestrazzi, R., Lanari, D., D'agaro, E., & Mion, A. (1994). The effect of dietary protein level and source on growth, body composition, total ammonia and reactive phosphate excretion of growing sea bass (*Dicentrarchus labrax*). *Aquaculture, 127*(2-3), 197-206.

Becker, E. W. (2007). Micro-algae as a source of protein. *Biotechnology Advances, 25*(2), 207-210. doi:10.1016/j.biotechadv.2006.11.002

Bertoldi, F., Sant'Anna, E., & Barcelos-Oliveira, J. (2008). *Chlorella vulgaris cultivated in hydroponic wastewater.* Paper presented at the International Symposium on Soilless Culture and Hydroponics 843.

Bisoyi, R., & Singh, P. (1988). Effect of phosphorus fertilization on blue-green algal inoculum production and nitrogen yield under field conditions. *Biology And Fertility Of Soils, 5*(4), 338-343.

Blair, M. F., Kokabian, B., & Gude, V. G. (2014). Light and growth medium effect on *Chlorella vulgaris* biomass production. *Journal of Environmental Chemical Engineering, 2*(1), 665-674.

Bonga, S. W. (1997). The stress response in fish. *Physiological Reviews, 77*(3), 591-625.

Brown, M. R. (2002). Nutritional value and use of microalgae in aquaculture. *Avances en Nutrición Acuícola VI. Memorias del VI Simposium Internacional de Nutrición Acuícola, 3*, 281-292.

Cassidy, K. O. (2011). Evaluating algal growth at different temperatures. 2011. *Thesis and Dissertations-Biosystems and Agricultural Engineering. Cited 17-11-2015,* Available from :http://uknowledge.uky.edu/bae_etds/3.

Chinnasamy, S., Ramakrishnan, B., Bhatnagar, A., & Das, K. C. (2009). Biomass production potential of a wastewater alga Chlorella vulgaris ARC 1 under elevated levels of CO2 and temperature. *International Journal of Molecular Sciences, 10*(2), 518-532.

Cresswell, R., & Syrett, P. (1979). Ammonium inhibition of nitrate uptake by the diatom, *Phaeodactylum tricornutum*. *Plant Science Letters, 14*(4), 321-325.

Diver, S. (2000). Aquaponics-Integration of Hydroponics and Aqua- culture; ATTRA-National Sustainable Agriculture Information Service: Fayetteville, AR.

Dortch, Q. (1990). The interaction between ammonium and nitrate uptake in phytoplankton. *Marine ecology progress series. Oldendorf, 61*(1), 183-201.

Dosdat, A., Servais, F., Metailler, R., Huelvan, C., & Desbruyeres, E. (1996). Comparison of nitrogenous losses in five teleost fish species. *Aquaculture, 141*(1), 107-127.

El-Sayed, A.-F. M. (2006). *Tilapia culture*: CABI.

El-Sayed, A.-F. M., & Teshima, S.-i. (1992). Protein and energy requirements of Nile tilapia, *Oreochromis niloticus*, fry. *Aquaculture, 103*(1), 55-63.

El-Sherif, M. S., & El-Feky, A. M. (2008, October). Effect of ammonia on Nile tilapia (*O. niloticus*) performance and some hematological and histological measures. In *INTERNATIONAL SYMPOSIUM ON TILAPIA IN AQUACULTURE* (Vol. 8, pp. 513-530).

El-Sherif, M. S., & El-Feky, A. M. I. (2009). Performance of Nile tilapia (*Oreochromis niloticus*) fingerlings. I. Effect of pH. *International Journal of Agriculture and Biology, 11*(3), 297-300.

Elnady, M., Alkobaby, A., Salem, M., Abdel-Salam, M., & Asran, B. (2010). Effect of fertilization and low quality feed on water quality dynamics and growth performance of Nile tilapia (*Oreochromis niloticus*). *Journal of American Science, 6*(10), 1044-1054.

Engin, K., & Carter, C. (2001). Ammonia and urea excretion rates of juvenile Australian short-finned eel (*Anguilla australis australis*) as influenced by dietary protein level. *Aquaculture, 194*(1), 123-136.

Flynn, K. J., Fasham, M. J., & Hipkin, C. R. (1997). Modelling the interactions between ammonium and nitrate uptake in marine phytoplankton. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 352*(1361), 1625-1645.

Forde, B. G., & Clarkson, D. T. (1999). Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. *Advances in botanical research, 30*, 1-90.

Francis-Floyd R, Watson C, Petty D, Pouder DB (2009) Ammonia in aquatic systems. UF/IFAS University of Florida (UF)/Institute of Food and Agricultural Sciences (IFAS), FA 16, p 5.

Gál, D., Pekár, F., Kerepeczki, E., & Váradi, L. (2007). Experiments on the operation of a combined aquaculture-algae system. *Aquaculture International, 15*(3-4), 173-180.

Guerrero-Cabrera, L., Rueda, J. A., García-Lozano, H., & Navarro, A. K. (2014). Cultivation of Monoraphidium sp., Chlorella sp. and Scenedesmus sp. algae in Batch culture using Nile tilapia effluent. *Bioresource Technology, 161*, 455-460.

Hailing-Sørensen, B., Nyhohn, N., & Baun, A. (1996). Algal toxicity tests with volatile and hazardous compounds in air-tight test flasks with CO 2 enriched headspace. *Chemosphere, 32*(8), 1513-1526.

Handy, R., & Poxton, M. (1993). Nitrogen pollution in mariculture: toxicity and excretion of nitrogenous compounds by marine fish. *Reviews in Fish Biology and Fisheries, 3*(3), 205-241.

Haynes, R., & Goh, K. M. (1978). Ammonium and nitrate nutrition of plants. *Biological Reviews, 53*(4), 465-510.

Israeli-Weinstein, D., & Kimmel, E. (1998). Behavioral response of carp (*Cyprinus carpio*) to ammonia stress. *Aquaculture, 165*(1), 81-93.

Kim, J., Lingaraju, B. P., Rheaume, R., Lee, J.-Y., & Siddiqui, K. F. (2010). Removal of ammonia from wastewater effluent by *Chlorella vulgaris*. *Tsinghua Science & Technology, 15*(4), 391-396.

Kim, J., Liu, Z., Lee, J.-Y., & Lu, T. (2013). Removal of nitrogen and phosphorus from municipal wastewater effluent using *Chlorella vulgaris* and its growth kinetics. *Desalination and Water Treatment, 51*(40-42), 7800-7806.

Knud-Hansen, C. F., & Batterson, T. R. (1994). Effect of fertilization frequency on the production of Nile tilapia (*Oreochromis niloticus*). *Aquaculture, 123*(3), 271-280.

Knud-Hansen, C., Batterson, T., & McNabb, C. (1993). The role of chicken manure in the production of Nile tilapia, *Oreochromis niloticus* (L.). *Aquaculture Research, 24*(4), 483-493.

Kowalchuk, G. A., & Stephen, J. R. (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annual Reviews in Microbiology, 55*(1), 485-529.

Lavens, P., & Sorgeloos, P. (1996). *Manual on the production and use of live food for aquaculture*: Food and Agriculture Organization (FAO).

Leal, E., Fernández-Durán, B., Guillot, R., Ríos, D., & Cerdá-Reverter, J. M. (2011). Stress-induced effects on feeding behavior and growth performance of the sea bass

(Dicentrarchus labrax): a self-feeding approach. *Journal of Comparative Physiology B, 181*(8), 1035-1044.
Liang, K., Zhang, Q., Gu, M., & Cong, W. (2013). Effect of phosphorus on lipid accumulation in freshwater microalga *Chlorella* sp. *Journal of Applied Phycology, 25*(1), 311-318.
Lu, J., Takeuchi, T., & Satoh, H. (2004). Ingestion and assimilation of three species of freshwater algae by larval tilapia *Oreochromis niloticus*. *Aquaculture, 238*(1), 437-449.
Lustigman, B., Lee, L., & Khalil, A. (1995). Effects of nickel and pH on the growth of Chlorella vulgaris. *Bulletin Of Environmental Contamination And Toxicology, 55*(1), 73-80.
Mjoun, K., Rosentrater, K. A., & Brown, M. L. (2010a). Tilapia: profile and economic importance.
Mjoun, K., Rosentrater, K. A., & Brown, M. L. (2010b). Tilapia: environmental biology and nutritional requirements.
Northcott, M. E., Beveridge, M. C., & Ross, L. G. (1991). A laboratory investigation of the filtration and ingestion rates of the tilapia, Oreochromis niloticus, feeding on two species of blue-green algae. *Environmental Biology of Fishes, 31*(1), 75-85.
Pack, M. Y. (1991). Symbiotic production method for microalgae and fishes: Google Patents.
Peres, H., & Oliva-Teles, A. (2006). Effect of the dietary essential to non-essential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*). *Aquaculture, 256*(1), 395-402.
Priyadarshani, I., Sahu, D., & Rath, B. (2012). Algae in Aquaculture. *International Journal of Health Sciences and Research, 2*(1), 108-114.
Pullin, R. S., & Lowe-McConnell, R. H. (1982). *The Biology and Culture of Tilapias: Proceedings of the International Conference on the Biology and Culture of Tilapias, 2-5 September 1980 at the Study and Conference Center of the Rockefeller Foundation, Bellagio, Italy*: WorldFish.
Qin, J., Culver, D., & Yu, N. (1995). Effect of organic fertilizer on heterotrophs and autotrophs: implications for water quality management. *Aquaculture Research, 26*(12), 911-920.
Risgaard-Petersen, N., Nicolaisen, M. H., Revsbech, N. P., & Lomstein, B. A. (2004). Competition between ammonia-oxidizing bacteria and benthic microalgae. *Applied And Environmental Microbiology*, *70*(9), 5528-5537.
Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., & Vaca-Garcia, C. (2014). Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renewable and Sustainable Energy Reviews, 35*, 265-278.
Salsac, L., Chaillou, S., Morot-Gaudry, J.-F., Lesaint, C., & Jolivet, E. (1987). Nitrate and ammonium nutrition in plants. *Plant physiology and biochemistry, 25*(6), 805-812.
Santiago, C. B., & Lovell, R. T. (1988). Amino acid requirements for growth of Nile tilapia. *The Journal Of Nutrition, 118*(12), 1540-1546.
Seyfabadi, J., Ramezanpour, Z., & Khoeyi, Z. A. (2011). Protein, fatty acid, and pigment content of *Chlorella vulgaris* under different light regimes. *Journal of Applied Phycology, 23*(4), 721-726.
Sirakov, I., Velichkova, K., Stoyanova, S., & Staykov, Y. (2015). The importance of microalgae for aquaculture industry. Review. *International Journal of Fisheries and Aquatic Studies* 2(4), 81-84
Suresh, A. V., & Lin, C. K. (1992). Tilapia culture in saline waters: a review. *Aquaculture, 106*(3), 201-226.
Syrett, P. J. (1981). Nitrogen-metabolism of microalgae. *Canadian Bulletin of Fisheries and Aquatic Sciences*(210), 182-210.
Tam, N., & Wong, Y. (1996). Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. *Bioresource Technology, 57*(1), 45-50.

Turker, H., Eversole, A. G., & Brune, D. E. (2003). Filtration of green algae and cyanobacteria by Nile tilapia, *Oreochromis niloticus*, in the Partitioned Aquaculture System. *Aquaculture, 215*(1), 93-101.

Van Rijn, J. (1996). The potential for integrated biological treatment systems in recirculating fish culture—a review. *Aquaculture, 139*(3), 181-201.

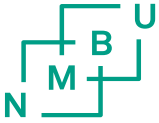
Velichkova, K. (2014). Effect of different nitrogen sources on the growth of microalgae *Chlorella vulgaris* cultivation in aquaculture wastewater. *Agricultural Science and Technology, 6*(3), 337-340.

Widjaja, A., Chien, C.-C., & Ju, Y.-H. (2009). Study of increasing lipid production from fresh water microalgae Chlorella vulgaris. *Journal of the Taiwan Institute of Chemical Engineers, 40*(1), 13-20.

Wurts, W. (2003). Daily pH cycle and ammonia toxicity. *World Aquaculture, 34*(2), 20-21.

Xin, L., Hong-ying, H., Ke, G., & Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus sp*. *Bioresource Technology, 101*(14), 5494-5500.

Yusoff, F. M., Banerjee, S., Khatoon, H., & Shariff, M. (2011). Biological approaches in management of nitrogenous compounds in aquaculture systems. *Dyn Biochem Process Biotech Mol Biol, 5*, 21-31.



Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no