The use of filter-feeding fish (*Clarias gariepinus* and *Oreochromis mossambicus*) to remove microalgae from brewery effluent treatment ponds

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Abstract

The removal of microalgae from high rate algal ponds (HRAP) in waste-water treatment systems remains a constraint to their use in effluent treatment systems. Conventional algae harvesting methods often have high energy demands, take up lots of space, are expensive to operate or are time consuming. The aim of the study was to determine if fish such as *Clarias gariepinus* and *Oreochromis mossambicus*, could be used to remove microalgae from waste-water treatment ponds (in the absence/presence of a flocculent in the former and in the absence or presence of pH moderation in the latter), and to investigate the subsequent influence of algae concentration on several water quality parameters. The age of *Clarias gariepinus* (3-12 months) had a positive relationship with the distance between gill rakers (98.27 to 163.34 µm; $y=90.576+4.823*x$: $R^2=0.549$; $F_{(1,18)}=21.867$; $p<0.001$) and these data suggested that these fish might be efficient at removing algae from HRAP effluent. However, this was not the case, even with flocculent application (but this result might have been confounded by very high pH readings, at which flocculation is less likely to occur). *Oreochromis mossambicus* removed some of this algae, but the pH was too high for tilapia culture. It was not possible to moderate the increase in pH by keeping tanks in the dark and thus preventing photosynthesis; but pH fluctuation in HRAP effluent could be moderated using CO_2 sparging in an attempt to make the environment more hospitable for tilapia (the average pH that was moderated with CO_2 was 8.43±0.06, whereas the unmoderated average was 10.65±0.06). However, pH moderation using CO_2 sparging did not increase the rate at which algae were removed by *O. mossambicus*; rather, it compromised O_2 concentration which dropped to 4.17±1.26 mg/l after five hours of CO_2 sparging, whereas it increased to 20.50±1.41 mg/l in treatments with unadjusted pH over the same period. Fish can be used to remove algae from treated effluent, and *Oreochromis mossambicus* remains a recommended species. Future work needs to

investigate moderating fluctuations in pH and O_2 concentration to further facilitate this method of algae removal.

Keywords: Freshwater; aquaculture; waste-water treatment; filter-feeding fish

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1.1 General introduction

Microalgae can be cultured from brewery effluent due to the wastewater being rich in nutrients like phosphorus and nitrogen (Jones *et al*., 2016). However, harvesting the microalgae presents a set of challenges that undermine the application of this process of effluent treatment. Microalgae harvesting technologies either have high energy demands or are not viable economically (Shelef *et al*., 1984; Benemann *et al*., 1980). Filter feeding fish have the potential to be used as a viable mean of microalgae removal (Xie and Yang, 2000; Lu *et al*., 2002); allowing the resultant wastewater to be released to open waters or to be treated further in municipal facilities at a lower cost.

Wastewater in general is an environmental threat which if left untreated can have devastating effects on flora and fauna. A study, conducted by Liu *et al.* (2007), showed that mono-sodium glutamate (MSG) wastewater had elevated chemical oxygen demand (COD), and when used in irrigation this resulted in the germination of certain seed crops being inhibited. In low concentrations, compounds from pharmaceutical effluent may be present in the environment (Archer *et al*., 2017ab). Some of these have been created to induce certain physiological responses, however when unmanaged there exists a potential that they may produce negative undesired effects; for instance the development of abnormalities in human beings or animals (Koplin *et al*., 2002). Sewage treatment works (STW) effluent contains pesticides, diazinon, nonylphenol, chlorfenvinphos; and these amongst other compounds contribute to the toxicity of STW effluents. Sewerage treatment works effluents are toxic to juvenile fish (Murty, 2018); some of the afore-mentioned effluents are as toxic to flora in water-bodies as brewery wastewater.

Brewery effluent, if left untreated and released into the environment, contaminates river waters (Alao *et al*., 2010). According to the World Bank (1997), effluent from the brewery processes of fermentation and filtering is high in organics and accounts for approximately 97 % biochemical oxygen demand (BOD) of the wastewater.

Brewery effluent composition (Table 1) fluctuates with regard to quality and quantity because it is influenced by various processes (Driessen and Vereijken, 2003). Brewery wastewater comprises organic compounds that contribute to COD. In addition to others, these compounds include sugars, soluble starch, volatile fatty acids and ethanol. Brewery effluent also contains total suspended solids (TSS) which are made up of waste yeast, spent grains, ('hot') trub and kieselguhr. The management of the raw material and the quantity of the spent yeast in the wastewater influences phosphorus and nitrogen levels (Driessen and Vereijken, 2003).

As potentially hazardous as it is, brewery effluent is still released into the environment. There are regulations that guide waste disposal processes. Furthermore, an environmental assessment of open water bodies on the impact of a brewery's waste disposal has to be done according to a country's environmental laws (World Bank, 1997; Driessen and Vereijken, 2003). Effluent is a by-product of industrial growth (Browstow *et al*., 2009), which is a vital component of civilization (Meadows *et al*., 2004). Thus, there is the need to devise economic and environmentally friendly disposal strategies. Breweries want to maximize profits by operating at minimum costs at the same time waste disposal legislation is becoming stricter (Fillaudeau *et al*., 2006). Therefore breweries have the options of discharging to a municipal sewer or treating the wastewater before discarding it (World Bank, 1997).

Table 1: Brewery effluent characteristics (Rao *et al*. 2007; Simate *et al*. 2011).

Parameter	**Value**
pH	3-12
Temperature (°C)	18-40
Chemical oxygen demand (mg/l)	2000-6000
Biological oxygen demand (mg/l)	1200-3600
COD:BOD ratio	1.63
Volatile fatty acids (mg/l)	1000-2500
Total Kjeldahl nitrogen (mg/l)	25-80
Phosphate (mg/l)	10-50
Total solids (mg/l)	5100-8750
Total suspended solids (mg/l)	2901-3000
Total dissolved solids (mg/l)	2020-5940

Liu *et al*. (2007) came to the conclusion that when COD was reduced the inhibitory rate of seed germination also decreased. River water is often used for irrigation purposes. There are several factors that influence COD levels and the presence of algae is chief amongst these. If the goal is to operate within certain environmental regulations, algae growth would seem to be an impediment at a first glance, but given the fact that algae assimilates nutrients like nitrogen and phosphorus for growth at a relatively lower cost than the alternatives and COD levels can be

restricted to within acceptable standards to provide water for processes like crop irrigation, algae culture seems appealing. Algae are primary producers in numerous freshwater systems (Addy and Green, 1996) and offer high energy yields (IEA Bioenergy, 2009).

According to Demirbas (2010) and Vandamme *et al*. (2013), microalgal biomass production has associated costs that are even greater than those of crop production. But since microalgae cultures provide a simple solution to tertiary wastewater treatments by assimilation of nitrogen and phosphorus for growth, coupled with the removal of organic toxins and heavy metals (Abdel-Raouf *et al*., 2012), the slightly higher comparative costs are somewhat validated. The advantages and disadvantages of the application of algae in wastewater treatment are outlined in Table 2.

Table 2: The advantages and challenges of the application of algae in wastewater treatment.

Advantages	Disadvantages
Algae use nitrogen and phosphorus to grow which is abundant in brewery effluent.	Method may lead to toxic algae blooms in the open environment.
This is a cost effective method to clean effluent.	Algae harvesting through conventional means has high associated costs.
The photosynthetic materials algae produces are readily available for other trophic levels.	Few organisms can survive in algae rich wastewater.

Microalgae can be removed from the water column by filter-feeding fish. *Clarias gariepinus* is an example of such a fish. This catfish was used firstly because of their ability to withstand harsh environmental conditions and secondly because they are capable of filter feeding. It is a freshwater species often referred to as the sharp-tooth or African catfish. The distribution scatters throughout Africa and other regions including Turkey and the Middle East

(Yalcin *et al*., 2001). In addition to ponds *C. gariepinus* is found in lakes, rivers and seasonal floodplain swamps (Kaunda-Arara *et al*., 2010); it is one of the most vital sources of protein throughout Africa, especially the rural areas (Hecht, 1981).

Clarias gariepinus is a filter feeder and has been reported to be phytoplanktivorous (Skelton, 1993). According to Kadye and Booth (2012), full grown *C. gariepinus* are top predators whereas the juveniles feed on the lower trophic levels. This would seem to suggest that juvenile *C. gariepinus* would be best suited for feeding on algae. However there still remains the question of whether it is structural development or food availability that governs food preference? The same authors later on suggest that since developing *C. gariepinus* have a higher energy demand due to growth, the larger *C. gariepinus* feed more on algae.

Clarias gariepinus can withstand fluctuations in the environment such as changes in pH, oxygen concentration and temperature. The diurnal and nocturnal cycles in a high rate algal pond (HRAP) system present a range of physico-chemical conditions that do not favour the survival of certain fish species. During late summer afternoons the pH is usually elevated due to CO_2 being used upand the production of H^+ by photosynthesis throughout the day (page 74 under Chapter 3' introduction). The pH lowers in the absence of sunlight (Addy and Green, 1996), together with the concentration of dissolved oxygen which gets depleted by the subsequent respiration; however *C. gariepinus* is a robust species capable of survival in challenging conditions owing partly to the presence of the suprabranchial air breathing organ (Hecht and Appelbaum, 1987), and its insensitivity to a wide pH range.

The Mozambique tilapia *O. Mossambicus* is a freshwater species of cichlids that is indigenous to the eastward-flowing rivers of central and southern Africa (Australian Centre Tropical Freshwater Research, 2007). It is omnivorous and has the ability to filter feed (Skelton, 1993). The ability of this fish to interbreed with other cichlid species makes it hard to describe its

appearance as it is not constant. Common features in both males and females include; a dorsal fin that stretches above the gills to the end of the fish and an anal fin continuous towards a rounded tail fin.

Oreochromis mossambicus are categorised as opportunistic omnivores with a diet often consisting of fish, invertebrates, plants and algae; depending on what is available (Australian Centre Tropical Freshwater Research, 2007). Due to this wide diet they can be found in differing environments with varying food sources.

Due to being tolerant of a wide range of conditions they can be found in most unfavourable habitats. According to Allanson *et al.* (1971), *O. mossambicus* can tolerate salt concentrations of 0-120 ppt., making them the most salt tolerant of all the tilapia species.

In regions such as Western Australia, *O. mossambicus* have been reported occupying habitats with salinities of 95 ppt. (Morgan *et al.*, 2004). Since seawater has a salinity of 35 ppt. (Allanson *et al.*, 1971), this suggests that this fish can occupy marine areas (Dange, 1985; Suresh and Lin, 1992; Kültz *et al*., 1995; Nakano *et al.*, 1997).

The ideal temperature range for *O. mossambicus* growth and reproduction is 22-30 °C, albeit this fish can survive in temperatures ranging from 16-39 °C but need 27 °C to breed (Allanson *et al.*, 1971). Therefore, since the present study is not focused on fish growth and temperature fluctuations, and the temperature of the brewery effluent falls within this range, this water parameter should not cause much concern.

There are diseases that are linked to algal blooms which may be of concern. For example pansteatitis is a lethal disease that causes inflammation in animals after the onset of fat cell necrosis (Roberts *et al*., 1979; Herman and Kircheis 1985; Ladds *et al*., 1995; Wong *et al*., 1999; Niza *et al*., 2003; Goodwin 2006; Roberts and Agius 2008; Neagari *et al*., 2011). In

Clarias gariepinus a diet shift from plant matter to fish has been shown to correlate with the development of pansteatitis (Woodborne *et al*., 2012). Woodborne *et al*. (2012) also cited pollution and eutrophication in the Kruger National Park as the indirect cause of pansteatitis in *C. gariepinus*. Pansteatitis has also been reported as widespread in *Oreochromis mossambicus* in Lake Loskop (Oberholster *et al*., 2011). The disease has been linked to eutrophication and increased ingestion of oxidised polyunsaturated fats. In the current study pansteatitis would not be a major factor for the following reasons, the disease develops over time whereas the sampling period in the current study is several hours, secondly due to algae assimilating large proportions of nitrogen and phosphorus, pollution will not be an influencing factor.

pH is an essential water quality parameter that influences the role played by other minerals and elements with regards to overall fish health/stress (Coetzee, 1996). For example exposure of fish to a pH of 5.2 in the presence of the aluminium metal causes the goblet cells to produce mucous on the gills and all over the body of the fish (Lock and van Overbeeke, 1981; Eddy and Fraser, 1982). Of the three phases of reaction to stress (alarm reaction, resistance reaction and exhaustion reaction) mucous secretion represents the resistance reaction phase as the fish is trying to cope with the environmental change brought about by the low pH and aluminium (Coetzee, 1996). Therefore pH levels need to be managed so as to induce as little stress as possible to the fish so that algae removal may proceed efficiently.

Clarias gariepinus were first selected in this study due to their hardiness and ability to withstand the environmental fluctuations in algal ponds. However, preliminary analysis of their gill structure (section 2.4.1), our knowledge of the small size of the algal cells in HRAP (Jones *et al*., 2016; Mogane 2016) and that these fish can feed off the bottom of the tank (Yalcin *et al*., 2001), resulted in the inclusion of a flocculent in the experimental design (section 2.3.2). The findings of Chapter 2 resulted in a decision to investigate the use of an alternative

fish species, *Oreochromis mossambicus* (Chapter 3). This fish was chosen because of its ability to filter feed and because of its hardiness; however, some environmental parameters in HRAP, particularly pH, fell outside of the ideal range for tilapia. For that reason, methods to manipulate HRAP pH were investigated (section 3.3.1), prior to determine the rate that tilapia could remove algae from the water column (section 3.3.2). The objective of the work was not to compare the rate of algae removed between the two fish species that were investigated here.

1.2 Literature review

1.2.1 The value of algae

One of the advantages of algae is that it is rich in diversity and therefore inherently possesses great chemical diversity (Borowitzka, 2009).

- The use of algae in biodiesel production

The efficiency with which algae convert sunlight energy to biomass is 3.0-8.0 %, much more than that of terrestrial crops (0.5 %). Although both types of primary producers utilize the process of photosynthesis, algae produce oils that are more suitable for biodiesel production mainly due to their high oil content and rapid growth rate (Lohrey, 2008).

During the process of oil extraction for biodiesel production, by-products may be recovered from the algal biomass which may be suitable for use in other processes and some of these are proteins, carbohydrates and minerals (Lohrey, 2008).

According to Slade and Bauen (2013), in biodiesel production costs may be reduced by more than 50 % if water, CO_2 and nutrients may be acquired at a low cost. However, the authors

concur that lack of viability (both technical and economic) in available data, concerning algae production systems, creates an uncertain future for the concept.

- The use of algae in the pharmaceutical industry

The application of algae for therapeutical purposes dates back to as far as ~ 70 A.D. (Borowitzka, 2009). The fact that marine algae (*Saccharina* and *Sargassum*) was/is utilized by different communities like the Chinese and Indians, to a certain extent, validates its effectiveness.

Thus far algae have been and most likely will still play a major role in the development of new drugs. In recent times various industries including the pharmaceutical industry have depended on living organisms like algae for the provision of new biologically active compounds.

The application of algae as an antibiotic in modern medicine, due to its potential, has sparked great interest and with technological advances as a catalyst in modern research, breakthroughs seem assured. Algae are also used as a source of anticancer and antioxidant drugs (Pooja, 2014).

However, comparative studies are needed to justify the use of algae as a source of bioactive molecules over animals and other plants, and in order to accomplish this, advances have to be made in algae research (Borowitzka, 2009).

- Algae produces proteins on which animal feeds may be based

As a primary producer algae synthesizes proteins through photosynthesis (Pooja, 2014); these proteins may be used in feed formulations as a supplement (Potts, 1998). Aquaculture for instance has long relied on fishmeal as a protein source but due to factors like cost and limited production, current research is centred on finding a viable alternative (Potts, 1998). Unicellular

algae have great potential in this regard and that has been identified by numerous researchers including (Terao, 1960; Stanley and Jones, 1976; Appler and Jauncey, 1982).

However protein should be sourced with caution from unicellular algae for fish feeds. According to Meske and Pfeffer (1978), stunted growth has been observed when algae has been used as the only protein source in fish feed. Mustafa and Nakagawa (1995), suggest that algae be added in small portions in order to increase growth, improve disease resistance, feed utilization and enhance physical condition. Potts (1998), did a similar study but he used a different fish species and effluent grown algae was added to the fish meal and soya meal instead of completely replacing them; he came to the conclusion that a health effect of the algae on the fish needed to be known and suggested that until such information becomes available fish cultured on effluent grown algae be limited to ornamental fish.

In the present study algae was not used in conjunction with a formulated fish meal, but before this could be done it was imperative that the nutritional requirements of *C. gariepinus* were established/known (Potts, 1998), and since protein is the most vital component in fish meal (Hepher *et al*., 1979), the crude protein content of effluent grown algae had to be investigated.

1.2.2 Conventional algae harvesting techniques

Wastewater has nutrients which are retrievable and re-usable (Kaur and Saxena, 2004). One of the ways to extract these nutrients is through algae growth (Abdel-Raouf *et al*., 2012). Encouraging algal growth is simple; the complex part is harvesting it. It is not that technology to achieve this is not available but rather, the energy and investment used usually surpasses the overall gain, rendering the whole exercise counter-productive.

- Sedimentation

With regard to this solid liquid separation process, solid particle circulation within the liquid medium is unrestricted and bound only by the container (Shelef *et al*., 1984). The driving factor of sedimentation is the difference between the densities of the liquid and the solids (Holdich and Butt, 1997; Carlsson, 1998). Particle size/density is usually directly proportional to particle settling velocity (Shelef *et al*., 1984).

Gravity sedimentation is effective in separating the solids from the liquid, but this is more so when a flocculent is added as even the small/light particles will form aggregates with a decent settling velocity (Agerkvist *et al*., 1990). Gravity sedimentation processes can be achieved through the use of clarifiers and thickeners.

When using clarifiers, the focus is on how clear the overflow is. Koopmann *et al*. (1980) separated the facultative oxidation pond from inflow feed to boost/supplement water clarification. The fill-and-draw method was highly efficient in extracting algae from the facultative oxidation pond effluent. The drawback was that the process was time consuming (Benemann *et al*., 1980).

Within tanks, sloping plates may also be used to encourage contact and settling of solids along the plates. The plates are not erected upright as to promote the downward movement of the solids and their removal (Svarovsky 1979b, Mohn 1980).

Gravity thickening on the other hand concentrates on the thick underflow. However information highlighting use of this device for algae thickening is dearth.

It is possible to apply a flocculent prior to administering gravity clarification as evidenced by Metcalf and Eddy (1974), who managed to extract approximately 85 % of the biomass from a

pond. However artificial flocculants introduce chemicals into the process and this might have knock on effects in terms of changing the aquatic environment.

- Flotation

Air bubbles attach to and transport solids to the surface of the liquid where they form aggregates which can easily be harvested. The instability of the suspended particles is inversely proportional to the air particle contact. Flotation can be achieved through one of three ways: Dispersed air flotation, dissolved air flotation and electrolytic flotation (Shelef *et al*., 1984).

Dispersed air flotation produces large bubbles (one millimetre) through froth flotation or foam flotation. Betzer (1981), succeeded in floating microalgae from high rate oxidation pond effluent by applying ozone flotation. Ozone pumped through the effluent altered the algae cell wall surface and encouraged flotation. However, in order for particles to be transported to the surface they must possess a unique affinity for air bubbles (Svarovsky, 1979b).

Dissolved air flotation is effective to a certain extent although it often depends on the process of flocculation for maximal output (Funk *et al*., 1968); furthermore dissolved oxygen is a limiting factor and its concentration should not drop to below 16 mg/l (Shelef *et al*., 1984).

Electrolytic flotation forms small gas bubbles through electrolysis. This technology has high running costs due to the high energy requirements it has (Shelef *et al*., 1984).

- Filtration and Screening

This technology separates solids from liquids by passing this mixture through a permeable screen with uniform mesh size (Uduman *et al*., 2010). The ideal scenario would be for the screen to restrain the solids whilst allowing the liquid to filter through, all depending on the

size of the openings. Shelef *et al*. (1984) harvested algae with micro-strainers and vibrating screen filters.

Micro-strainers are spinning drums enveloped with screens of desired aperture size which utilize a backwash mechanism for the collection of particles (Shelef *et al*., 1984). The cost of operation of micro-strainers is determined by the flow-through-rate which depends on the mesh size and the size/shape of the algal species being separated (Uduman *et al*., 2010). Even though small mesh size will still restrain large algal species, the best results in terms of achieving high flow-through-rates at minimal costs are attained when mesh size increases with algal species size (Uduman *et al*., 2010). There lies a challenge in balancing algae concentration, as excessive measures may most certainly block the screens and inadequate levels decrease the efficiency of the process (Uduman *et al*., 2010). Either way, the effect would be the same due to the time taken by both "unblocking of the screen" or "the inefficiency" inflating the operation costs. Furthermore according to Golueke and Oswald (1965), micro-strainers have been unable to screen oxidation pond effluents.

Filtration is achieved through application of force in the form of gravity, vacuum, centrifuge or pressure to liquid driving it through a filter (Uduman *et al*., 2010). Filtration can be divided into surface filtration and deep bed filtration. As far as surface filtration is concerned, particles collect as a thin layer on the filter, a stark contrast to deep bed filtration where particles settle within the filter (Mackie and Bai, 1992). Due to filters clogging timeously, backwashes are a necessity and this subsequently dilutes the microalgal concentration. Since a high rate oxidation pond cultures a wide variety of algal species with differing sizes (large microalgae and small microalgae) a challenge is confronted in selecting which driving force to apply as the forces appear to produce the best results when dealing with certain algal sizes (pressure and vacuum are very efficient in recovering species with large cell sizes).

- Centrifugation

Centrifugation utilizes a rotating device to separate solids from liquid (Uduman *et al*., 2010). Separation efficiency is influenced by the characteristics of the smallest particles within the machine (Shelef *et al*., 1984), and also by the density of the particles in relation to the liquid (Taulbee and Maroto-Valer, 2000). Depending on the volume of the liquid handled, centrifuges can be categorized as laboratory centrifuges (1-5000 ml) or preparative centrifuges (<1.0 l). Centrifugation effectiveness is maximized with increasing acceleration and/or mass as the particle is subject to gravitational force (Taulbee and Maroto-Valer, 2000). Although this technique is appealing because it meets the primary objective which is algae recovery, the associated costs in the form of high energy demand and heavy investment render its application unviable for the recovery of low value algae biomass.

1.2.3 Fish as a viable alternative to conventional algae removal technologies

In North America and Europe, zooplankton is usually the preferred option for the elimination of algae and cyanobacteria, but in areas such as the tropics and subtropics zooplankton is less effective and this is where omnivorous filter feeding fish may be utilized (Xie and Liu, 2001). The silver and the bighead carp have been reported to feed on algae (Xie and Liu, 2001), this however might be expected from filter-feeders which constantly feed due to the absence of a stomach. Knowledge is needed on an endemic fish species which can successfully feed on algae. In addition to its high tolerance levels, *C. gariepinus* was preferred for the study because it grows rapidly and its flesh is of high quality. According to Hecht (1985), *C. gariepinus* can grow up to a marketable size of 800-1000 g / fish in its first year. *Oreochromis mossambicus* is a reputable filter feeder that can survive in a wide range of environments.

1.2.4 Nutrient content of algae

Several species of algae have been reported to possess impressive nutritional properties and these include *Chaetoceros calcitrans, Chaetoceros muelleri, Pavlova lutheri, Isochrysis* sp. (T.ISO), *Tetraselmis suecica, Skeletonema costatum* and *Thalassiosira pseudonana* (Enright *et al*., 1986; Thompson, Guo and Harrison, 1993; Brown *et al*., 1997). The nutritional content of algae usually has the following representation: 30-40 % protein, 10-20 % lipid and 5-15 % carbohydrate, provided it is grown to late-logarithmic growth phase (Brown *et al*., 1997; Renaud, Thinh and Parry, 1999). In a study conducted by Potts (1998), the values differed slightly and were as follows: 41.47±0.2 % protein, 4.8±0.4 % lipid and 35.13±0.8 % carbohydrate content. The variation recorded by the researchers on the independent studies might be a result of experimenting on different algal species in differing conditions.

The vitamin content of algae may differ amongst species with vitamin C showing a 1-16 mg g^{-1} range (Brown and Miller, 1992),and vitamin E ranging from 0.07-0.29 mg g^{-1} (Brown, 2002).

1.2.5 Nutritional requirements of *Clarias gariepinus*

In most cases a balanced diet is a consequence of composition similarity between the diet and the consumer. Carnivores for instance are widely regarded to feed on balanced diets (Sterner and Hessen, 1994), probably due to the dominant presence of nutrients like proteins within them and the food they consume. This would explain the piscivorous nature of adult *C. gariepinus*. However, carnivory is not the only type of feeding as it may be substituted by phytoplanktivory depending on fish age or food availability.

Clarias gariepinus' feeding on plant material (algae) demonstrates its capability to break down plant proteins and source energy from carbohydrates (Clay, 1979; Uys, Hecht and Walters, 1987; Van Weerd, 1995).

Throughout the different growth stages (larvae, juveniles, young adults and adults) protein is perhaps the most vital nutrient in *C. gariepinus* diets (Uys and Hecht, 1985; Machiels and Henken, 1985; Uys, 1989; and Degani *et al*., 1989). *Clarias gariepinus* has an optimum dietary levels of crude protein requirement ranging from 25-50 % (Table 3). This has been attributed to certain factors, including variations in fish size, culture conditions (physico - chemical factors), endemic food supply, fish stocking density, quantity of feed, frequency of rations and quality of dietary protein. Toko *et al*. (2008) reported reduction in growth possibly due to a decrease in feed intake and feed efficiency, and poor reproduction performances as a symptom evident in fish cultured on a diet with inadequate protein content.

According to Dupree and Halver (1970), catfish usually require 10 essential amino acids (tryptophan; arginine; histidine; isoleucine; leucine; lysine; methionine; phenylalanine; threonine and valine). Several of these essential amino acids (EAA's) quantitative dietary requirements, roles and deficiencies in *C. gariepinus* have been investigated by Fagbenro *et al*. (1998); Fagbenro *et al.*, (1999); Fagbenro and Nwanna, (1999), in dose-response trials.

The minimum dietary requirement of tryptophan is 11 g/kg (1.1 %) dietary protein (Fagbenro and Nwanna, 1999). In addition to being an EAA, tryptophan assists in the production of various essential compounds (Fagbenro and Nwanna, 1999). Reduced growth, deformed vertebral column (scoliosis) and anorexia are some of the signs of tryptophan deficiency in *C. gariepinus* (Fagbenro and Nwanna, 1999). The quantitative tryptophan requirement for *C. gariepinus* resembles that for *Anguilla japonica* and *Oreochromis niloticus* published by the NRC (1993) (Fagbenro and Nwanna, 1999).

Arginine is an essential amino acid for various fish species (NRC, 1993). In a study conducted by Fagbenro *et al.* (1999), it was established that the minimum dietary arginine requirement in *C. gariepinus* is approximately 45 g/kg (4.5 %) dietary protein. Hird (1986) listed several

metabolic pathways in which arginine plays a role and these include creatine and polyamine synthesis, urea production and, protein synthesis. Reduction in growth responses and increased mortalities are common features in fish fed arginine deficient diets (Fagbenro *et al.*, 1999)

All finfish require lysine in their diets for normal functioning. Fagbenro *et al*. (1998) estimated the dietary minimum requirement of lysine to be 57 g/kg (5.7 %) dietary protein. It precedes carnitine, which facilitates the movement of long chain fatty acyl groups into the mitochondria for beta - oxidation (Fagbenro *et al.,* 1998). Fagbenro *et al.* (1998) observed decreased feed efficiency, poor growth, fin erosion, and increased mortality in lysine deficient fish.

The minimum dietary methionine requirement is 32 g/kg (3.2 %) dietary protein. Methionine also serves as a precursor to carnitine. According to Fagbenro *et al.* (1999), anorexia is a symptom of methionine deficiency. In studies conducted on *Oncorhyncus mykiss* and *Ictalurus punctatus* it was reported that dietary cystine presence lowers the quantity of dietary methionine required for optimal growth (Fagbenro *et al.,* 1999).

Clarias gariepinus obtains vitamin E from its diet and this probably contributes to its tolerance to parasitic infections (Van der Waal, 1972), since vitamin E has antioxidant characteristics. According to Tocher *et al.* (2002), vitamin E addition in fish diet enhances liver health. A decrease in vitamin E however not only results in reduced levels of tissue vitamin E but puts a strain on liver antioxidant enzymes and increases lipid peroxide activities (Tocher *et al.,* 2002).

Vitamin C is a vital nutrient in the diet of most fish species (Lovell, 1973; Wilson and Poe, 1973; Andrews and Murai, 1975; Lovell and Lim, 1978). According to Adham *et al.* (2000) and Adewolu and Aro (2009), 50 mg/kg diet is the minimum vitamin C requirement for *C. gariepinus* diets. Vitamin C has antioxidant characteristics that prevent damage to certain catfish tissues (Adham *et al.,* 2000). In a study conducted by Adham *et al.* (2000), shrunken hepatocytes and the presence of yellowish ceroid pigments within the liver gave indication of

liver degeneration in vitamin C deprived catfish. Oedematous separation and telangiectasia of gill lamellae have also been reported in catfish subjected to scorbutic diets (Adham *et al.,* 2000).

Clarias gariepinus has a lipid requirement of nine percent and a carbohydrate requirement of 21 % of the diet in the food they consume (Hecht, 2013).

Table 3: Nutritional requirements of *Clarias gariepinus.*

Nutritional Requirement	**Required percentage within diet (%)**	**Sources**
Protein	>40-42 40	Machiels and Henken (1985) Uys (1989) Degani et al. (1989)
Carbohydrates	21	Hecht (2013)
Lipids	9.0	Hecht (2013)

The nutritional requirements of *C. gariepinus* could be satisfied by the nutrient content of algae based on the aforementioned information.

1.2.6 Feeding behaviour of *Clarias gariepinus*

This opportunistic predator (Potts *et al*., 2008), employs several feeding modes. Depending on food availability (Bruton, 1979), any of the following modes may be applied: individual foraging, individual shovelling, formation feeding and surface feeding. Of particular interest to the current study being the individual shovelling and surface feeding. *Clarias gariepinus* are equipped to feed off the bottom of a water body (Yalcin *et al*., 2001); and are known to filter feed in groups at the water surface using gill rakers (Hecht *et al*., 1988). In addition to a wide mouth which assists in suction, the *C. gariepinus* is equipped with long gill rakers which trap

particles within passing waters. A thin walled and rather short intestine in *C. gariepinus* could be regarded as an indication of the fish's need for a high protein diet (Uys, 1989).

1.2.7 Nutritional requirements of *Oreochromis mossambicus*

Proteins are used continuously for fish maintenance, growth and reproduction functions. There appears to be no specific requirement for carbohydrates, as glucose can be provided by fatty and amino acid precursors via gluconeogenesis but since protein glucose is more expensive, inclusion of carbohydrates in formulated diets has an economic upside. Lipids are a source of essential fatty acids and energy (Table 4). Vitamins and Minerals are essential for normal fish metabolism. However specific requirements are not exactly known for all vitamins.

Table 4: Nutritional requirements of *Oreochromis mossambicus.*

Nutritional Requirement	**Development stage**	**Required percentage within diet (%)**	**Sources**
Protein	Fry	30-56	Winfree and Stickney, 1981; Jauncey, 1982; Al Hafedh, 1999; Siddiqui *et al*., 1988; Twibell and Brown, 1998
	Juveniles	30-40	
	Large tilapia	28-30	
Carbohydrates		30-40	Anderson *et al*., 1984; Teshima *et al*., 1985
Lipids	0.0-2.5 g	5.2	Winfree and Stickney, 1981 and Jauncey, 2000
	2.5-7.5 g	4.4	
	7.5-25 g	8.0-12	
	>25 g	6.0-8.0	

By comparing the nutritional content of algae and the nutritional requirements of *O. mossambicus* it becomes increasingly evident that algae not only satisfy the protein need in diets of these fish but nutrients like lipids, vitamins and carbohydrates as well.

1.2.8 Feeding behaviour of *Oreochromis mossambicus*

Over 24 h *Oreochromis mossambicus* feed the most between 06h30 and 08h30 in the morning and 17h30 and 19h30 in the evening; not much feeding activity occurs during the night (de Moor, 1986).

Oreochromis mossambicus prefer suspension filtering and surface grazing as a means of feeding (GISD, 2012). Plankton rich water enters the mouth via suction as part of respiration. This water then passes through the gills where plankton is trapped through mucous excretion between the gill filaments (Fryer & Iles, 1972).

The ingestion rate is dependent on the nutritional value of the food. A slow ingestion rate was observed in low nutritional value food compared to food with intermediate nutritional value (Taghon and Jumars, 1984).

1.3 Research rational and motivation

Brewery effluent has the potential to result in eutrophication if released into a body of water because of its high nutrient load (Safari *et al*., 2013). Algae have been successfully used to treat this effluent in high rate algal ponds (HRAP) (Jones *et al.,* 2014). However, this results in the accumulation of large volumes of algal biomass made of small algal cells (Jones *et al.,* 2016; Mogane, 2016). The removal of this biomass from the ponds remains a bottle neck in the use of HRAP in effluent treatment. Filter feeding fish could potentially use these algae as a food source and thus algal biomass could be converted into fish biomass, which is more easily harvested. However, this approach to the problem has not been fully investigated.

Clarias gariepinus and *Oreochromis mossambicus* can filter feed on algae (remove algae from water) and survive a broad range of environmental conditions. The optimal conditions under which this feeding process occurs need to be identified so as to maximise algal removal in the treated brewery effluent. This is needed in order to establish whether the concept of using phytoplanktivorous fish to reduce the algal load in integrated algal ponding system (IAPS) is a viable option for polishing this effluent prior to its discharge into the environment. This study however was not designed to compare filtering abilities between the two fish species.

1.4 Research aim

The overall aim of the study was to investigate whether *C. gariepinus* and *O. mossambicus* could be used to remove algae from wastewater treatment ponds, the former in the presence or absence of a flocculent and the latter in the presence or absence of pH manipulation. The aim was also to investigate the influence of algae concentration on several water quality parameters.

Chapter 2: Determining the suitability of *Clarias gariepinus* in removing algae from treated brewery effluent and assessing the influence of flocculation technology in the process.

2.1 Introduction

The functioning of the feeding mechanisms of *C. gariepinus* have been investigated by several authors. Adriaens (2003) stated that some catfish exhibit specialised trophic specialisations, like filter feeding in *C. gariepinus*. For filter feeding in *C. gariepinus* to be understood the morphology of the gills in these fish has to be described.

Basic gill structure

Clarias gariepinus possess four pairs of gills joined into a median inter-branchial septum, plus a fifth rudimentary gill without gill filaments. The lengths and gaps between the gill arches decrease towards the centre (Zayed and Mohamed, 2004). The gill rakers of *C. gariepinus* are long and narrow spaced (Zayed and Mohamed, 2004), similar to those of other planktivorous or detritivorous species (Delariva and Agostinho, 2001), such as *Parapimelodus valenciennis* (planktivorous), *Parapimelodus nigribarbis* (planktivorous) and *Hypostomus commersonii* (detritivorous), that utilise gill rakers as a filter to assist in the retention of small particles (Almeida *et al.,* 2013).

An extra set of specialised gills in the form of bulbous dendritic structures stems from the second and fourth gill arches and these allow the fish to breathe atmospheric oxygen (Zayed and Mohamed, 2004). Oval and elongated cells which carry several micro-plicae cover the surface of gill filaments and lamellae (Zayed and Mohamed, 2004).

Gill micro-structure

Gills are covered by mucous epithelium although mucous cells are found only on the lamellae (Zayed and Mohamed, 2004). Mucous gland openings are located in proximity of the leading edges of gill lamellae which are the first gill structures water passes through during ventilation (Ojha and Hughes, 1988). In Indian major carp, *Cirrhinus mrigala*, mucous cells produce mucous secretions for the purpose of trapping, fixing and lubricating food particles for easy transportation and deglutition. Mucous cells are presumed to play a similar role in *C. gariepinus* (Kumari *et al.,* 2009). There is a constant presence of fixed and free mucous covering the surface of the gills. The fixed mucous timeously secreted by mucous cells is sloughed (Randall *et al.* 1991), but remains in the gill area as free mucous (Eddy and Fraser, 1982). Fixed mucous may act as a binding site for free flowing algae particles and Evans (1989) reported algal spores sticking to substrates through glycoproteins, sulphated fucans and alginates.

Numerous filter feeding animals employ cilia for the transportation of mucous and bound particles to the mouth (Tao *et al.,* 2002), but since the gills of fish lack cilia, Sanderson *et al.* (1991) suggested that directed water-flow acting on aggregated clumps of particles bound together with mucous was responsible for this role.

The gill rakers possess taste buds and infiltrating lymphocytes in the epithelial covering (Zayed and Mohamed, 2004). The taste buds are tasked with selective sorting of palatable food particles (Kumari *et al.*, 2009). Alarm substance cells can also be found within the epithelium (Zayed and Mohamed, 2004)

Atmospheric oxygen breathing

Air breathing organs begin development on the fourth and second gill arches at 3 and 5 cm respectively (Greenwood, 1961). Air breathing ability can be used to identify the end of the larval period (Haylor, 1992a). Under culture conditions *C. gariepinus* start to breathe air 14 days after first feeding at two centimetres in length (Haylor, 1991). From the above it can be derived that after two weeks of feeding *C. gariepinus* gills are sufficiently well developed for air breathing. However the temperature in which the fish are reared ultimately dictates the rate of morphological development (gills included).

Gill rakers for filter feeding

Murray (1975) reported a lack of filter feeding in *C. gariepinus* smaller than 400 mm TL in the natural environment and passive filter feeding in *C. gariepinus* ranging from 400 mm-600 mm in total length. According to Bruton (1979), gill raker numbers increase with fish length and this together with Murray (1975)'s finding would suggest that larger fish are best equipped for filter feeding. Bruton (1979) also reported larger fish filter feeding on phytoplankton; therefore adulthood does not eliminate use of filter feeding.

Murray (1975) observed filter feeding in experimental fish less than 300 mm, however possession of smaller straining apparatus does not necessarily translate to filter feeding being the mode used either, as the habitat of the fish may contribute to a lack of filter feeding in juveniles.

Fish age

In order to estimate the age of fish, hard structures including scales; otoliths; vertebrae; fin rays and spines; opercular bones; cleithra; urohyal bone; and hyomandibular bone have been used (Khan *et al.*, 2011). In a study conducted by Khan *et al.* (2011), amongst vertebrae, pectoral

spines and otoliths, otoliths were considered to be the most suitable structure for ageing *C. gariepinus*. Teleost fishes have three pairs of otoliths, comprising the lapilli, sagittae, and asterisci (Secor *et al.*, 1992). The sagittae are often the otoliths of choice for age determination amongst most fish species most probably due to their large size.

There exist several *C. gariepinus* studies in which at least one of the three otoliths has been incorrectly identified. This is due to the fact that *C. gariepinus* have the lapilli as the largest otolith instead of the sagittae common in other fish (Long and Stewart, 2010). Christensen (1964), who worked on the common sole, is one of the first researchers to implement the use of otoliths in the determination of fish age (Fagade, 1979). Fagade (1979) observed growth markings on the otoliths of *Chrysichthys nigrodigitatus* to assess their growth and age.

It may not be the size (age) of the fish that influences gill development therefore feeding behaviour. Gill development may be influenced by the habitat environment/feeding habit of the fish (Magnuson and Heitz, 1971; Hughes, 1980, 1984; Fernandes and Rantin, 1986; Fernandes *et al.*, 1995; Fernandes, 1996 as cited in Elsheik, 2013), therefore even small *C. gariepinus* (two months old) may be reared in an algal pond and successfully filter feed on algae. The lack of an alternative food source would cause the hardy species to adapt its developing morphology to what is available.

Kadye and Booth (2012) reported a 48 % frequency of occurrence of phytoplankton within *C. gariepinus* stomachs sampled from the Glen Melville dam. Since there was a relative lack of aquatic invertebrates in the Glen Melville Dam, the second most predominant food source was 26.98 % weight content made up of 13.31 % detritus, 9.32 % phytoplankton and 4.35 % algae (fish made up 63.75 % of the total stomach weight content). The *C. gariepinus* in this habitat adapted their feeding mode to the plankton which was abundant.

Since common freshwater species of South African algae cells range from 0.0005-0.2 mm in diameter (van Vuuren *et al.,* 2006), and the mean width between gill rakers of *C. gariepinus* ranges from <0.1 to 0.6 mm depending on fish length (Murray, 1975), the mean width between gill rakers which would be of particular interest is <0.2 mm, in the absence of a flocculent.

Algae flocculation

Several methods of microalgae flocculation have been tested in the past. These include the following:

Bio-flocculation is a poorly understood naturally occurring process (Vandamme *et al*., 2013). Some algae species are more likely to bio-flocculate than others (Vandamme *et al*., 2013). Certain bacteria and fungi possess a positive charge and therefore can interact with the negatively charged microalgae (Zhou, *et al*., 2012; Zhang and Hu, 2012).

Auto-flocculation occurs spontaneously when pH exceeds 9 (Spilling *et al*., 2011). The process relies on CO_2 depletion, calcium or magnesium precipitate formation (Vandamme *et al*., 2013).

Chemical flocculation is a process which employs metal salts like ferric chloride and potassium alum (Vandamme *et al*., 2013). Although it is effective in studying the interaction between flocculants and algal cells, this process has major shortcomings in that the metals contaminate the algae extract (Rwehumbiza *et al*., 2012) and it dramatically decreases pH; rendering it useless for applications such as fish feed.

Natural polymers also result in algal flocculation. A commonly used and most effective type is chitosan, a by –product of shellfish production (Vandamme *et al*., 2013). Chitosan has a positive surface charge which then attracts the negative surface charge of the microalgal cells, which results in floc formation (Vandamme *et al*., 2013). This type of flocculation is ineffective for harvesting seawater microalgae because polymer flocculants undergo coiling at high ionic

strengths (Uduman *et al*., 2010). Flocculation produces algal aggregates and these aggregates might be more easily removed by catfish in the presence of a flocculent.

Gap statement

In *C. gariepinus* that have not been reared in an algae rich environment (i.e. where structural apparatus development has not been influenced by food availability), there still exists the need to tentatively demonstrate which age group possesses the necessary structural apparatus to filter feed best so that this point in development could be referenced as the time filter feeding may be most efficient in *C. gariepinus*. To potentially achieve maximum algae removal rates from algal ponds the age group with the most appropriate gill structure for filter feeding would be used. Furthermore evidence is needed to show whether *C. gariepinus* can be used as a low cost and environmentally friendly means to remove microalgae from brewery effluent.

2.2 Aims and objectives

2.2.1 Determining the relationship between *Clarias gariepinus* age and inter-gill raker width

The aim was to highlight the age group which possessed the structural apparatus to filter feed at optimum levels.

The objectives were to:

- measure the width between the gill rakers and compare with fish age; and
- determine the age that the width between the gill rakers is <0.2mm.

Hypotheses

Null Hypothesis 1: Width between the gill rakers is not related to fish size (age).

2.2.2 Removal of algae by *Clarias gariepinus*

The aim was to investigate whether *C. gariepinus* could be used to remove algae from wastewater treatment ponds in the presence of a flocculent and to determine the influence of algae concentration on several water quality parameters.

The objectives were to:

- assess whether fish remove algae from algal tanks;
- determine if flocculation using chitosan improves algae removal; to
- monitor water quality parameters; and to
- determine whether a relationship exists between algae concentration and the various water quality parameters.

Hypotheses

Null Hypothesis 1: Algae concentration is similar between fish-containing-tanks and fish-absent-tanks.

Null Hypothesis 2: Algae concentration is similar between flocculated tanks and un-flocculated tanks.

Null Hypothesis 3.1: Water quality parameters are similar between fish-containing tanks and fish-absent tanks.

Null Hypothesis 3.2: Water quality parameters are similar between flocculated tanks and un-flocculated tanks.

Null Hypothesis 4: Algae concentration is not related to any water quality parameter.

2.3 Materials and methods

2.3.1 Determining the relationship between *Clarias gariepinus* age and inter-gill raker width

Clarias gariepinus have a wide size range; therefore 5-10 fish within each size range<10 g, 11-50 g, 51-100 g, 101-200 g, 201-300 g, 301-500 g, 501-1000 g, 1001-2000 g, 2001-3000 g, >3000 g were selected from a commercial hatchery (Aquaculture Innovations Pty Ltd, Grahamstown). Some of the larger fish were sourced from the wild in the North West Province. In most populations there is no significant difference between the growth rates of males and females (Hecht, 2013), so there was no need to evaluate sex differences.

Clove oil was added at a ratio of 100 mg/l of water and this induced a state of anaesthesia to the fish (Waterstrat, 1999). Following anaesthesia and euthanasia (through inducing trauma to the brain) the *C. gariepinus* were dissected and the gills from the right side were extracted (Hughes, 1966), and fixed in 10 % neutral formalin. Ethical clearance for fish euthanasia was obtained from Rhodes University before the study commenced (DIFS Jones Nombembe 2016). The gills were washed with water and then put through a series of alcohols of increasing concentration (10, 50 and 70 %) for preservation (Velcheva *et al*., 2010). Since the gill containers were labelled numerically according to size (age), another team member replaced the size (numerical) labels with colour codes and kept this information from the member who performed the analyses for blinding purposes (Kumar and Yale, 2016).

Method for aging *Clarias gariepinus*

The ossicles were removed through a horizontal cut on the head from the snout, over the eyes and to the insertion of the dorsal fin. With the brain and the spinal cord exposed, removal of the otoliths from under the brain tissue was possible (Fagade, 1979). Sagittal otoliths were cleaned, immersed in 70 % ethanol (Khan *et al*., 2011). As each fish contained a pair of sagittal

otoliths, for verification purposes, one was exposed to a flame before sectioning while the other was cut as is. The size of the otolith and its encapsulated geometry influenced the manner in which the initial cutting was performed. Samples were mounted into fibreglass resin and allowed to dry, after which they were transversely sectioned into blocks approximately 400 µm thick using a low speed diamond cutter. The sections were exposed to a DP-ex adhesive, mounted onto a glass cover slip and aerated to dry. Each section was then studied under a light microscope to determine fish age through counting the growth rings.

Method for measuring width between gill rakers

Measurements were taken from the first right gill arch (Macnuson and Heitz, 1971). It was divided into three sections; the upper, middle and lower parts. Five gaps were obtained from each region; these were added and divided by five to obtain an average for that gill area. An average for the whole gill arch was calculated by adding the three gill area averages and dividing by three. Five averages belonging to the same size class were added and divided by five for an average for that size class. The width of the gap between rakers was measured to the nearest 0.1 µm with an Olympus SX16 dissecting microscope connected to a personal computer operating on Stream Motion software. Due to the presence of a film of mucous on the gill rakers, the visually measured gap was consistently zero on all but the largest *C. gariepinus*. For this reason, each gill arch was softly brushed with soap and water, if brushed too hard the epidermis striped away. Whenever this accidentally occurred, the row of rakers was discarded (Murray, 1975).

Statistical methods

A linear regression was used whereby fish age was the independent variable and width between gill rakers was the dependent variable. The hypotheses were tested at $p \leq 0.05$.

2.3.2 Removal of algae by *Clarias gariepinus*

Site and operation overview

The investigation was carried out at the Project Eden site at IBhayi Brewery (SAB Ltd) in Port Elizabeth. High rate algae ponds (HRAP's) (Park *et al*., 2011), ranging from 1.8-3.7 m^3 in volume (Jones *et al*., 2016), were used to culture algae on anaerobically digested brewery wastewater rich in nutrients (e.g. phosphorous and nitrogen). This algae culture was used in the subsequent *Clarias gariepinus* feeding experiments. Specimens of *C. gariepinus* were introduced into tanks filled with this algae rich wastewater.

Integrated algal ponding effluent treatment system

Brewery effluent flowed through a system made up of various components collectively termed integrated algal ponding system (IAPS). The anaerobic digester (AD) (Figure 1) was tasked with breaking down organic matter by employing bacteria and also released nitrogen and phosphorus in the process. Through gravity flow the effluent was transported into the primary facultative pond (PFP, 17 m^3) (Figure 2) where opportunistic algal growth was observed, leading to a decrease in chemical oxygen demand (COD) (Jones *et al.*, 2011).

The effluent passed through a splitter box and was directed to two high rate algal ponds (HRAP's, 5.4 m^3 per series; Jones *et al*., 2016; Figure 3). In order to meet the Department of Water and Environmental Affairs of South Africa general limit for COD (75 mg/l), HRAP flow rates were adjusted so as to lower the COD (Jones *et al.*, 2011). The high rate algal ponds

(HRAP's) were operated in continuous culture in order to improve wastewater treatment and enhance conditions for *Micractinium* growth, which is a reputable animal feed supplement (Johnson, 2012). The algae complex found in the HRAPs is dynamic and constantly changes and formed the basis for another study (Mogane, 2016; Jones *et al*., 2016) and therefore algae species were not identified as part of the current study. A pump channelled the microalgae rich effluent from the HRAPs (where most algal assimilation occurred) into six treatment fish tanks, replicated three times (18 tanks).

The positions of the treatments were randomised amongst the 12 tanks.

Aquaculture system

The system was all assembled in a greenhouse tunnel and consisted of two rows with identical features: There were two identical biological filters and aeration pumps supplying tanks with oxygen. Two mechanical filters on each row lay attached to pumps and these were tasked with receiving municipal water together with recirculating the water from the tanks. In the middle of each row on the right there were twelve 500 l capacity tanks which contained 100 l of algal water (Figure 4).

- Fish introduction into tanks

Clarias gariepinus were sourced from a fish farm (Aquaculture Innovations, Pty Ltd) in Grahamstown. Fish of similar size were transported in oxygenated tanks from the farm to the Project Eden site at SAB's IBhayi Brewery facility in Port Elizabeth.Fish of a particular age (four months) were introduced into 6 of the 12 tanks, the other six tanks contained no fish. Since six tanks were used for the *C. gariepinus* and were to contain 12 fish; the treatments were randomly allocated to the tanks. The fish acclimated for two weeks.

- Chitosan solution preparation

The goal was to make a ten percent chitosan solution. One gram of chitosan powder (Table 5) was dispersed in 50 ml of water with a high-speed stirrer, after-which 50 ml of two percent hydrochloric acid were added. The concoction was then stirred until dissolution was reached, producing 1 % chitosan solution (Czechowska-Biskup *et al*., 2007). Eleven litres of this solution was then added to the tanks intended for flocculation (this accumulated to a 10 % chitosan solution in the 100 l tanks). Chitosan solution was applied an hour before sampling in three of the six fish-containing-tanks to determine whether sizable aggregates of algae would be formed. The other three tanks were utilised as a control.

Adding chitosan was meant to assist catfish in algae removal by forming aggregates of algae which would be easier to filter by the fish. Each tank was then observed to check what effect, if any, flocculation had on algae concentration. This process was repeated in the tanks with no fish and hence was replicated three times.

The algal biomass within the tanks was estimated by direct measurement of total suspended solids (TSS) (Ramaraj *et al.*, 2013). The tank water was stirred before 250 ml samples were taken. Water samples were taken for testing hourly from hour zero to the fifth hour.

Figure 1: The anaerobic digester (Cilliers, 2012).

Figure 2: The primary facultative pond.

Figure 3: The splitter box directing brewery effluent from the primary facultative pond to the high rate algae ponds (Cilliers, 2012).

Figure 4: The aquaculture system showing tanks used for the experiments.

Table 5: Certificate of chitosan analysis (Beijing Wisapple Biotech Co., Ltd).

Product	**Water soluble Chitosan (Food Grade)**		**(Source: Crab Shell)**	
Batch No.	Batch Size	Mfg. Date	Report Date	Exp. Date
WA20150603	400kg	20150603	20150604	20170602

Items	**Specifications**	**Results**
Appearance	Light white powder	Complies
Viscosity	>10	27
Deacetylation degree	>90 %	91.2 %
Indiscerptible	<1.0 %	0.5 %
Moisture	<10 %	8.35 %
Particle size	>40	80
pH	4.0-6.0	4.5
Heavy metal	<10 ppm	500 ppm
Total plate count	<1000 cfu/g	NMT 100 cfu/g
Storage	Sealed, Kept in a shady & dry place	
Shelf life	Two years	

Laboratory Processes

- Methods to test whether fish remove algae from algal ponds (total suspended solids)

The algal biomass within the tanks was determined and recorded by checking total suspended solids (TSS) (Ramaraj *et al.,* 2013), every 2 hours for six hours. Sampling began at 10 am and flocculation was applied an hour before sampling. Each 250 ml water sample was poured into a filtering cup. The filtering cup was clamped onto a filtering head and between these two there was one fourth of an 8.0 µm filter paper (Whatman 40 Ashless Circles, 125 mm diameter, Cat no. 1440 125). The filtering head was inserted into a 1000 ml conical flask. The conical flask was connected to a vacuum pump by a rubber tube. The vacuum pump was switched on to commence the filtering process and it was only switched off once the entire sample had passed through the filter paper and collected in the conical flask. The filter papers were rolled and put in sterile 50.00 ml plastic tubes with screws caps. Aluminium foil was used to wrap each of the tubes. The tubes were stored away from sunlight in a cooler box filled with ice.

The filter papers containing the algae biomass retentate were weighed on a digital scale to two decimal places within two days after sampling was performed. After filtration wet samples were weighed before being oven dried at 80 °C for 24 hours and weighed again.

Turbidity

Since the algae tanks were shallow, application of a sechi disk was not practical so turbidity measurements were made using a sechi tube. A fluorescent glass tube light cover was purchased; this transparent tube came with end caps. A disk was cut from a white plastic lid and measured to fit inside one of the end caps. The disk was divided into four quarters and alternating quadrants were coloured with a black permanent marker. The disk was glued to the bottom of the tube with the coloured inside facing upwards. A scale was made on the side of

the tube. A centimetre stick and a marker were used to highlight every 0.5 cm, with 0 cm being at the base of the upright tube. From each tank, water was poured into the turbidity tube, and at the point at which the coloured in disk was not visible a depth reading was taken. The lower the depth the higher the turbidity was. This was done at the start of the experiment and at hourly intervals.

Methods to test water quality parameters

a) Chemical oxygen demand (COD)

A medium range COD test kit was utilised to determine the COD of the brewery effluent (VARIO reagent, Product number 420722, range: 0 - 1500 mg/l O2, Dortmund, Germany). The method used was the potassium dichromate solution, whereby a water sample is oxidised with potassium dichromate and catalysed by silver sulphate addition. Mercury sulphate conceals the chloride. Unconsumed $Cr_2O_7^{2-}$ ions concentration was determined with a spectrophotometer (Cilliers, 2012).

Sample preparation: 250 ml water samples were filtered through an 8.0 µm filter paper (Whatman 40 Ashless Circles, 125 mm diameter, Cat no. 1440 125); 2.0 ml of the filtered sample was added to a vial. Vials were sealed and shaken before heating for two hours in a preheated digester reactor at a temperature of 150 °C. After heating, the vials were shaken again. The spectrophotometer (Merck Pharo 100 Spectroquant, product number 100706, Darmstadt, Germany) was used to analyse the samples using colorimetric determination (Cilliers, 2012).

b) Ammonia Low Range (0-2.5 mg/l N)

A low-range ammonium test kit was used to test the ammonia concentration of the brewery effluent (Merck Pty Ltd, product: 1.14752.0001). Ammonia nitrogen (NH_3 - N) exists in the form of ammonia and as ammonium ions. A pH-dependent equilibrium is found between the two. Ammonium nitrogen takes the form of ammonia in extremely alkaline solution and it reacts with hypochlorite ions to form monochloramine which then reacts with a substituted phenol to form a blue indophenol derivative that can be measured with a spectrophotometer (Cilliers, 2012).

Sample preparation: 250 ml water samples were filtered through an 8.0 µm filter paper (Whatman 40 Ashless Circles, 125 mm diameter, Cat no. 1440 125); 5.0 ml portions were pipetted to 10 ml vials, together with 0.6 ml of Reagent 1 and one level blue micro spoon of Reagent 2. Vials were sealed and shaken to mix contents. A five minute standing period was allowed before four drops of Reagent 3 were included and the sample left to react for a further five minutes (Cilliers, 2012). The same spectrophotometer was used to analyse the samples using colorimetric determination (Cilliers, 2012).

c) Nitrate

A nitrate test kit was used to test the nitrate concentration of the brewery effluent (Merck Pty Ltd, product: 1.09713.0001). Sample preparation: 250 ml water samples were filtered through an 8.0 µm filter paper (Whatman 40 Ashless Circles, 125 mm diameter, Cat no. 1440 125); 0.5 ml of post filter sample water was pipetted into an empty 10 ml glass vial after 4 ml of Reagent 1 was added. 0.4 ml of Reagent 2 was also pipetted into the same vial, which was shaken and allowed to react for 10 minutes (Cilliers, 2012). The same spectrophotometer was used to analyse the samples using colorimetric determination (Cilliers, 2012).

d) Nitrite

A nitrite test kit was utilised to test the nitrite concentration in the brewery effluent (Merck Pty Ltd, product: 1.14776.0001). When sulphanilic acid is added to an acidic solution, diazonium salt is formed due to nitrite ions. Diazonium salt reacts with N·(1-naphtyl) ethylenediamine dihydrochloride to produce a red-violet azo dye (Cilliers, 2012).

Sample preparation: 250 ml water samples were filtered through 8.0 µm filter paper (Whatman 40 Ashless Circles, 125 mm diameter, Cat no. 1440 125); 5 ml of the filtered water sample was pipetted into a 10 ml glass vial before one level micro spoon of Reagent 1 was poured into the vial, which was shaken and allowed to react for 10 minutes. The same spectrophotometer was used to analyse the samples through colorimetric determination (Cilliers, 2012).

e) Phosphate

A phosphate test was used to test phosphate concentration in the water (Merck Pty Ltd, product: 1.14842.0001). Orthophosphate ions within sulphuric solutions react with ammonium vanate/heptamolybdate to produce molybdovanadophosphoric acid (orange-yellow colour) (Cilliers, 2012).Two hundred and fifty millilitre water samples were filtered through a quadrant of an eight micrometre filter paper. Five millilitre proportions of the filtered water sample were pipetted into a 10 ml glass vial after-which 1.2 ml of Reagent 1 was added (Cilliers, 2012). This was done at the start and at hourly intervals.

f) Physical parameters

Multi-parameter probes (Figure 5) were used to measure the following: temperature, electrical conductivity (Hannah, HI98311; Woonsocket, United States of America), pH (Hanna, HI98127; Woonsocket, United States of America) and dissolved oxygen (Oxyguard, Handy

Polaris; Farum, Denmark). A CO_2 meter was used to measure CO_2 (Oxyguard, GO2P, portable CO_2 meter, Farum, Denmark) and a sechi tube was used was used for turbidity measurements.

Statistical methods

Interactions between factors were compared using repeated measures multifactor analysis of variance (ANOVA) at $p \leq 0.05$. If there were no interactions between factors, then treatment means within each factor were compared at $p < 0.05$. In all cases, data were first checked for equality of variance (Levene, 1960) and that the residuals were normally distributed (Shapiro and Wilk 1965). A linear regression was also used to analyse the relationship between algae concentration and water quality parameters.

Figure 5: Multi parameter probes for pH (Hanna, HI98127; Woonsocket, United States of America) and temperature, electrical conductivity (Hannah, HI98311; Woonsocket, United States of America).

2.4 Results

2.4.1 Determining the relationship between *Clarias gariepinus* age and gill raker width

The *C. gariepinus* ages had a directly proportional relationship with the first right inner gill arch width between the gill rakers, meaning the width between the gill rakers increased with fish age but age only explained 55 % of the relationship. The width between gill rakers ranged from 98.27 µm to 132.93 µm for fish of 3 to 6 months in age, 124.62 µm to 163.34 µm for fish of 9 to 12 months old. ($y=90.576+4.823*x$: $R^2=0.549$; $F_{(1,18)}=21.867$; $p<0.001$; Figure 6).

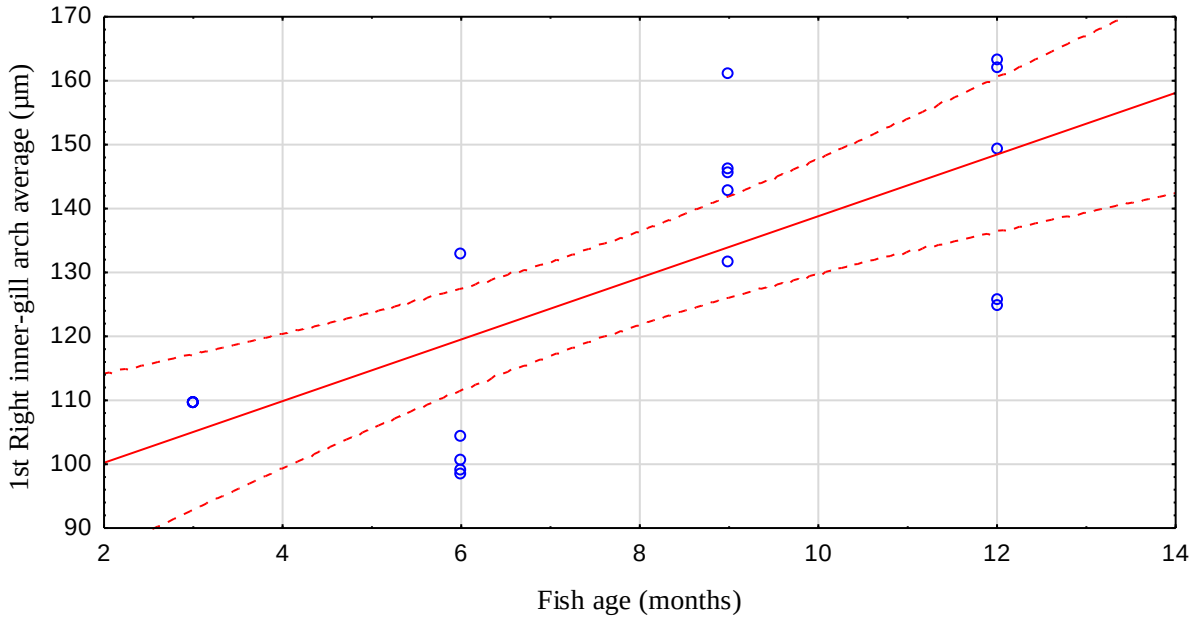

Figure 6: The relationship between *Clarias gariepinus* age (months) and the width between the gill rakers on the first right inner gill arch (µm) of fish less than a year old ($y=90.576+4.823*x$: $R^2=0.549$;$F_{(1,18)}=21.867$; $p<0.001$).

2.4.2 Removal of algae by *Clarias gariepinus*

Dry total suspended solids

There was no interaction in algae concentration (presented as total suspended solids, TSS in mg/ml; particle size >8.0 µm) amongst all the factors (fish/no fish and flocculent/no flocculent) over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.734; p=0.542; Figure 7). There was no significant difference in algae concentration between fish-containing-tanks and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=2.431; p=0.090; Figure 8). A significant difference was observed in algae concentration over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=5.152; p=0.007; Figure 9). No significant difference in algae concentration was observed between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.280; p=0.840; Table 6).

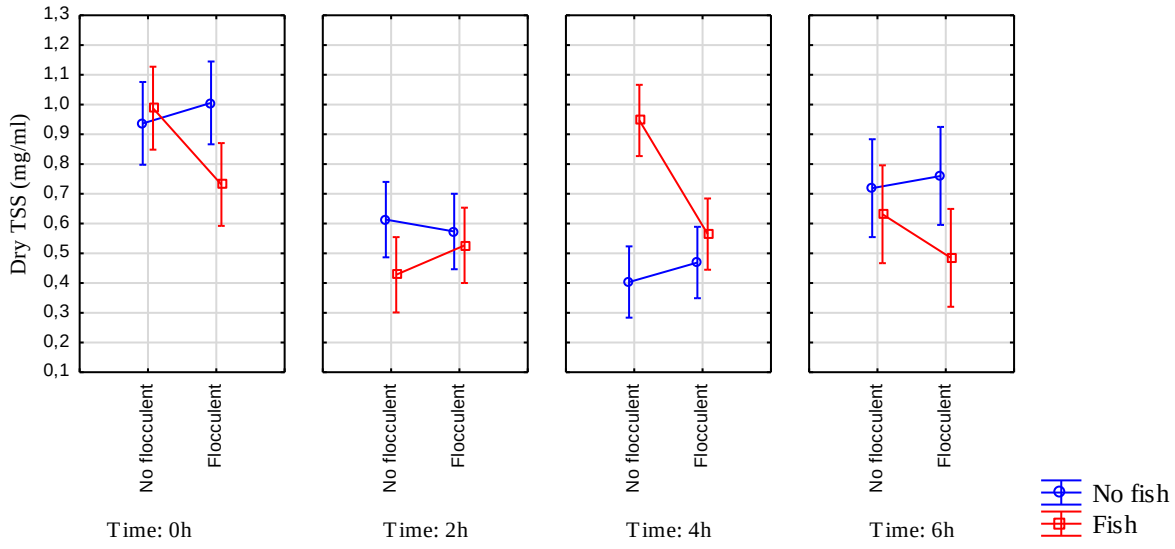

Figure 7: The mean (± standard error) algae concentration (presented as total suspended solids, TSS in mg/ml; particle size >8.0 µm) in tanks of post-high-rate-algal-pond effluent in the presence and absence of fish, that either include flocculent or no flocculent over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.734; p=0.542).

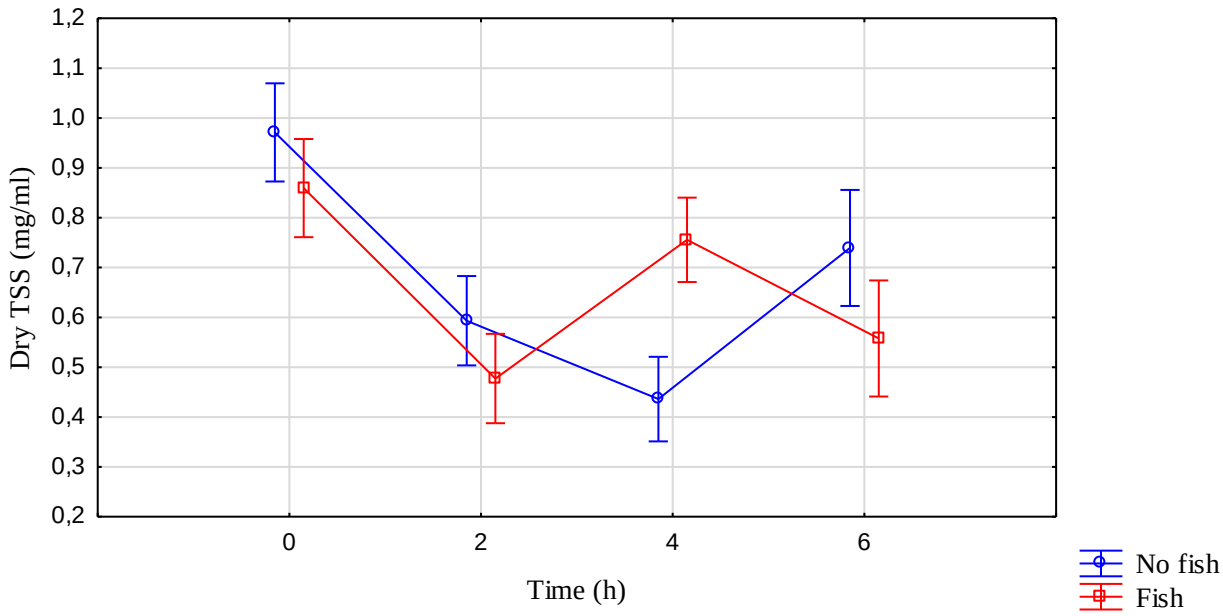

Figure 8: The mean (± standard error) algae concentration (mg/ml) (presented as total suspended solids, TSS in mg/ml) in tanks of post-high-rate-algal-pond effluent in the presence and absence of fish over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=2.431; p=0.090).

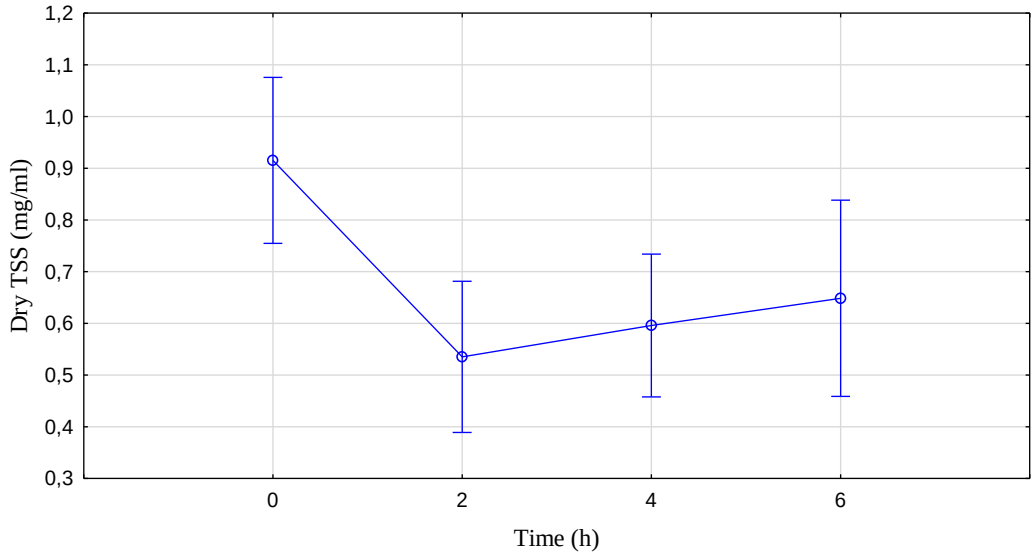

Figure 9: The mean (± 95 % confidence interval) algae concentration (presented as total suspended solids, TSS in mg/ml; particle size >8.0 µm) in tanks of post-high-rate-algal-pond effluent, over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=5.152; p=0.007).

Table 6: The mean (± standard error) algae concentration (TSS in mg/ml) in the absence and presence of a flocculent over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.280; p=0.840).

Time (h)	0	2	4	6		
					$F_{(3, 24)}$	p
Flocculent	0.87±0.10	0.55±0.09	0.52±0.09	0.62±0.12		
					0.28	0. 84
No flocculent	0.96±0.10	0.52±0.09	0.68±0.09	0.68±0.12		

Turbidity

There was no interaction in turbidity (cm) amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.182; p=0.681; Figure 10). A significant difference was observed in turbidity over time, where visible distance increased from about 8.025±0.312 cm at the start of the experiment to 11.767±0.616 cm after four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=24.138; p=0.001; Figure 11). No significant difference in turbidity was observed between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.201; p=0.665; Table 7). There was no significant difference in turbidity between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=2.247; p=0.172; Table 7).

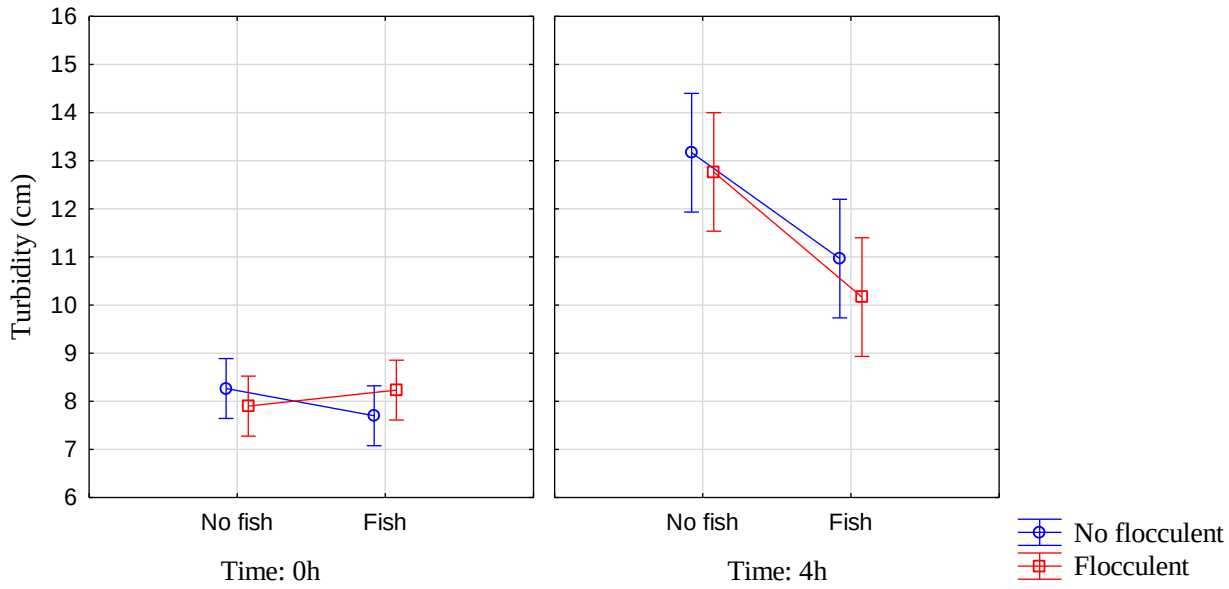

Figure 10: The mean (± standard error) turbidity (cm) in tanks of post-high-rate-algal-pond effluent in the presence and absence of fish, that either include flocculent or no flocculent over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.182; p=0.681).

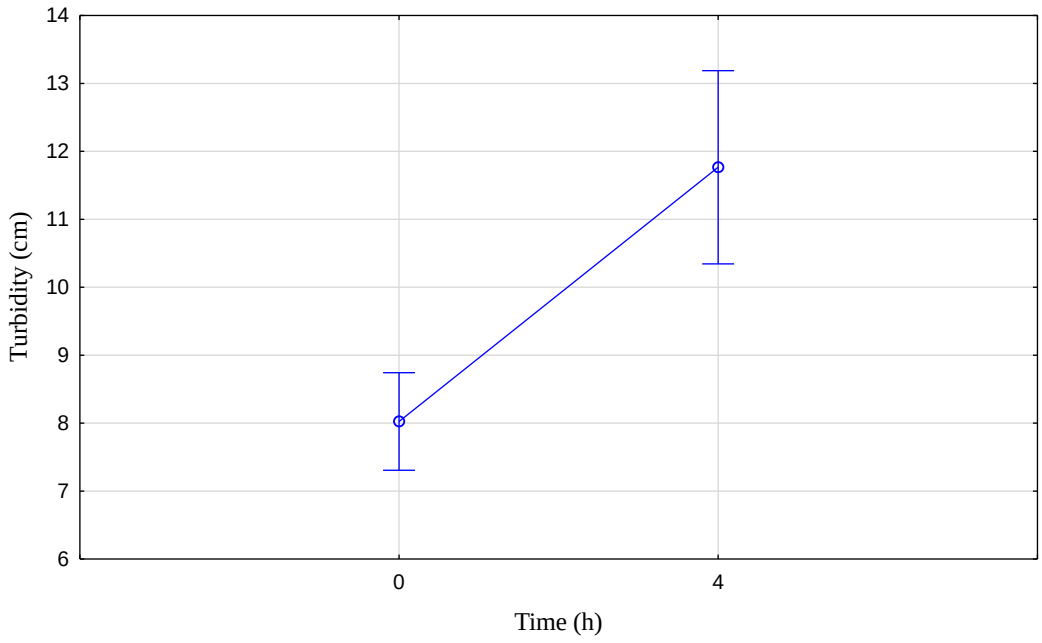

Figure 11: The mean (± 95 % confidence interval) turbidity (cm) in tanks of post-high-rate-algal-pond effluent, over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=24.138; p=0.001).

pH

There was no interaction in pH amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.715; p=0.422; Figure 12). A significant difference was observed in pH over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=7.096; p=0.029; Figure13). There was no significant difference in pH between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.655; p=0.442; Table 7). There was no significant difference in pH between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=4.881; p=0.058; Table 7).

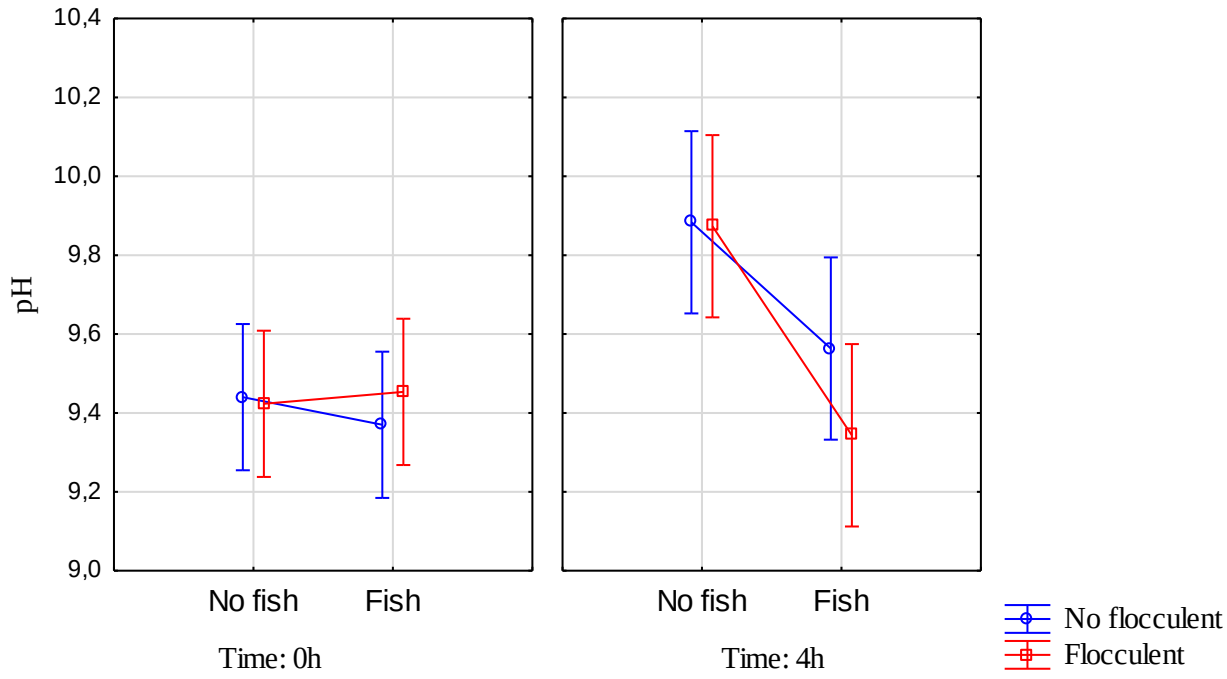

Figure 12: The mean (± standard error) pH in tanks of post-high-rate-algal-pond effluent, that either include flocculent or no flocculent and fish or no fish over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.715; p=0.422).

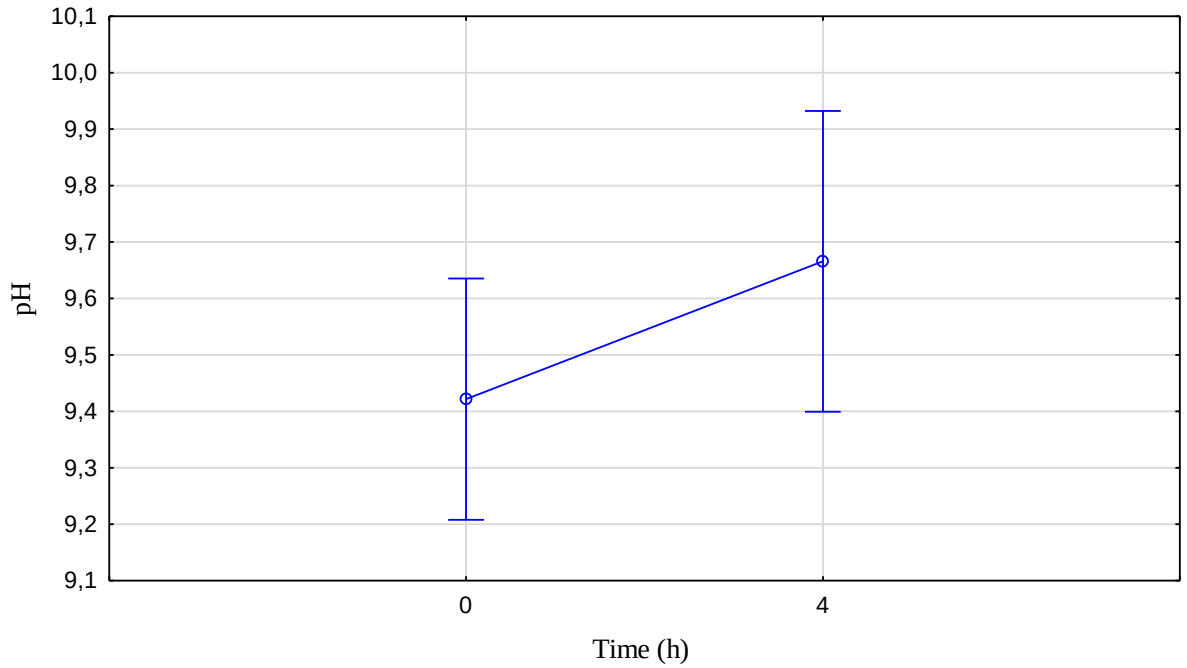

Figure 13: The mean (± 95 % confidence interval) pH in tanks of post-high-rate-algal-pond effluent, over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=7.096; p=0.029).

Dissolved oxygen

There was no interaction in dissolved oxygen amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=1.375; p=0.275; Figure 14). Dissolved oxygen was significantly different between fish-containing tanks and fish-absent tanks. Dissolved oxygen was markedly higher in the fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=7.228; p=0.028; Figure 15). No significant difference in dissolved oxygen was observed between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.073; p=0.794; Table 7). There was no significant difference in dissolved oxygen between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.153; p=0.706; Table 7).

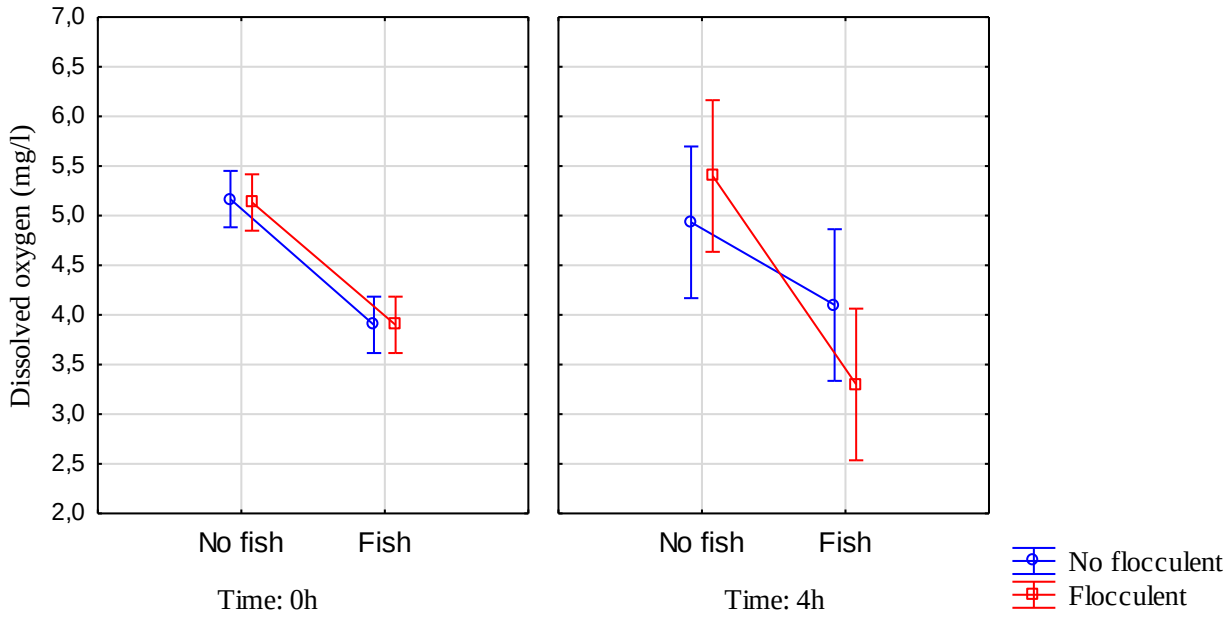

Figure 14: The mean (± standard error) dissolved oxygen (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include flocculent or no flocculent and fish or no fish from hour zero to hour four (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=1.375; p=0.275).

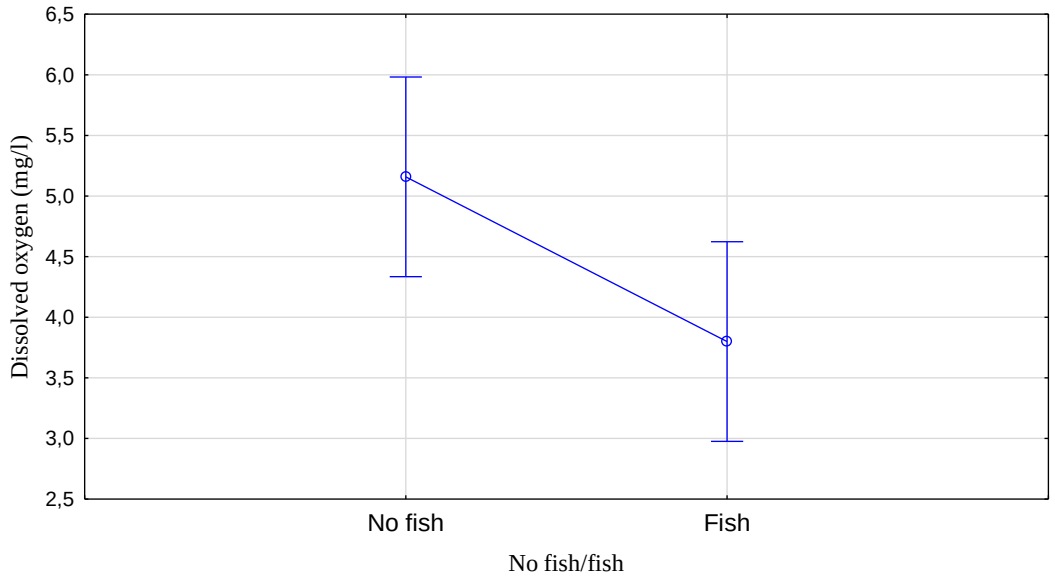

Figure 15: The mean (± 95 % confidence interval) dissolved oxygen (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include fish or no fish (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=7.228; p=0.028).

Temperature

There was no interaction in temperature amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.035; p=0.855; Figure 16). There was no significant difference in temperature between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.055; p=0.820; Table 8). There was no significant difference in temperature between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=1.276; p=0.291; Table 8).

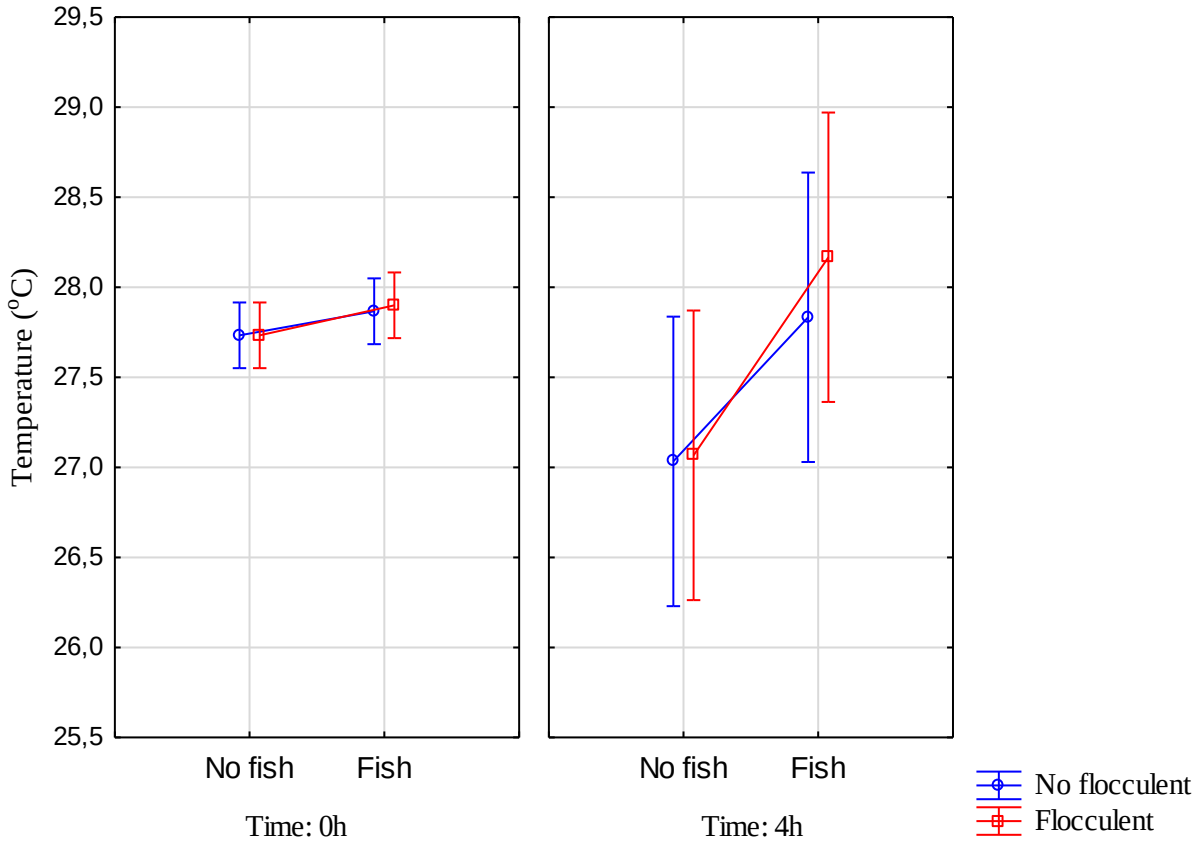

Figure 16: The mean (± standard error) temperature (˚C) in tanks of post-high-rate-algal-pond effluent, that either include flocculent or no flocculent and fish or no fish from hour zero to hour four (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.035; p=0.855).

Electrical conductivity

There was no interaction in electrical conductivity amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.120; p=0.738; Figure 17). Electrical conductivity was significantly different over four hours. Electrical conductivity was lower at the start of the experiment compared to the final hour of experimentation (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=6.349; p=0.036; Figure 18). No significant difference in electrical conductivity was observed between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.393; p=0.548; Table 8). There was no significant difference in electrical conductivity between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.287; p=0.107; Table 8).

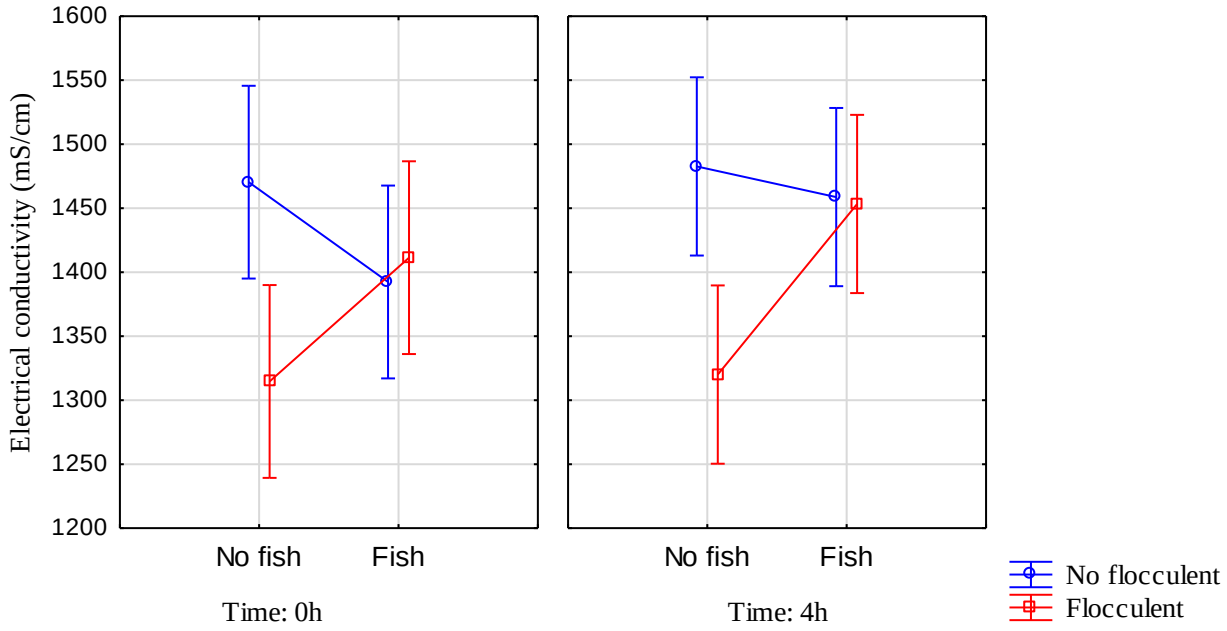

Figure 17: The mean (± standard error) electrical conductivity (mS/cm) in tanks of post-high-rate-algal-pond effluent, that either include flocculent or no flocculent and fish or no fish over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.120; p=0.738).

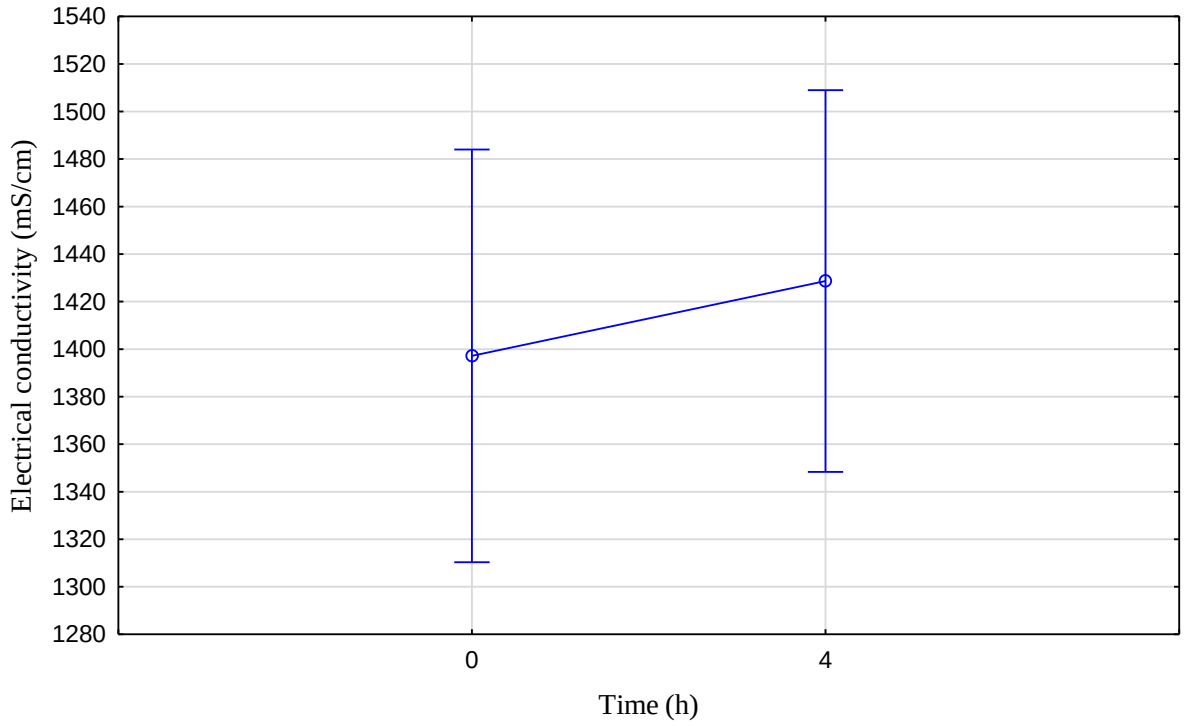

Figure 18: The mean (± 95 % confidence interval) electrical conductivity (mS/cm) in tanks of post-high-rate-algal-pond effluent, over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=6.349; p=0.036).

Ammonia

There was no interaction in ammonia amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.639; p=0.597; Figure 19). There was a significant difference in ammonia over four hours. Ammonia spiked during the fourth hour (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=3.870; p=0.022; Figure 20). There was no significant difference in ammonia between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.821; p=0.495; Table 9). No significant difference in ammonia was observed between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.373; p=0.773; Table 9).

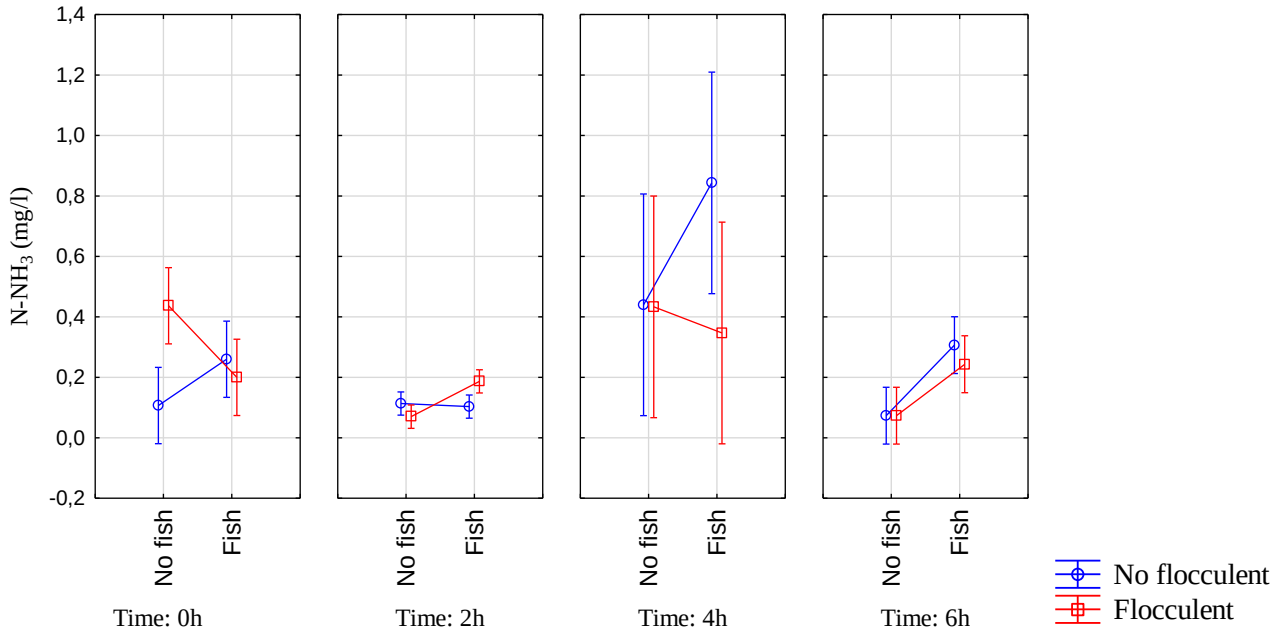

Figure 19: The mean (± standard error) ammonia (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include flocculent or no flocculent and fish or no fish over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.639; p=0.597).

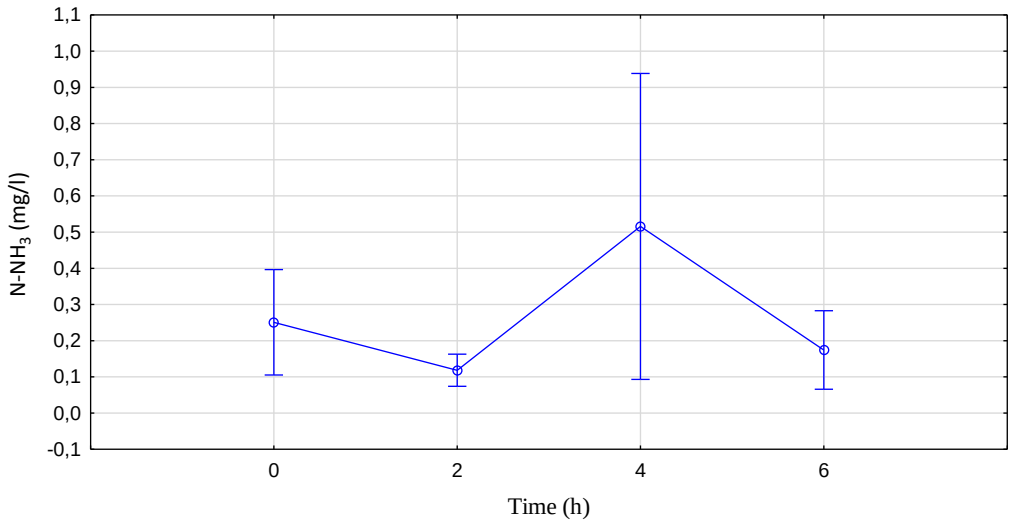

Figure 20: The mean (± 95 % confidence interval) of ammonia (mg/l) in tanks of post-high-rate-algal-pond effluent, over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=3.870; p=0.022).

Nitrite

There was no interaction in nitrite amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.299; p=0.826; Figure 21). No significant difference in nitrite was observed between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.606; p=0.618; Table 9). There was no significant difference in nitrite between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=1.312; p=0.294; Table 9).

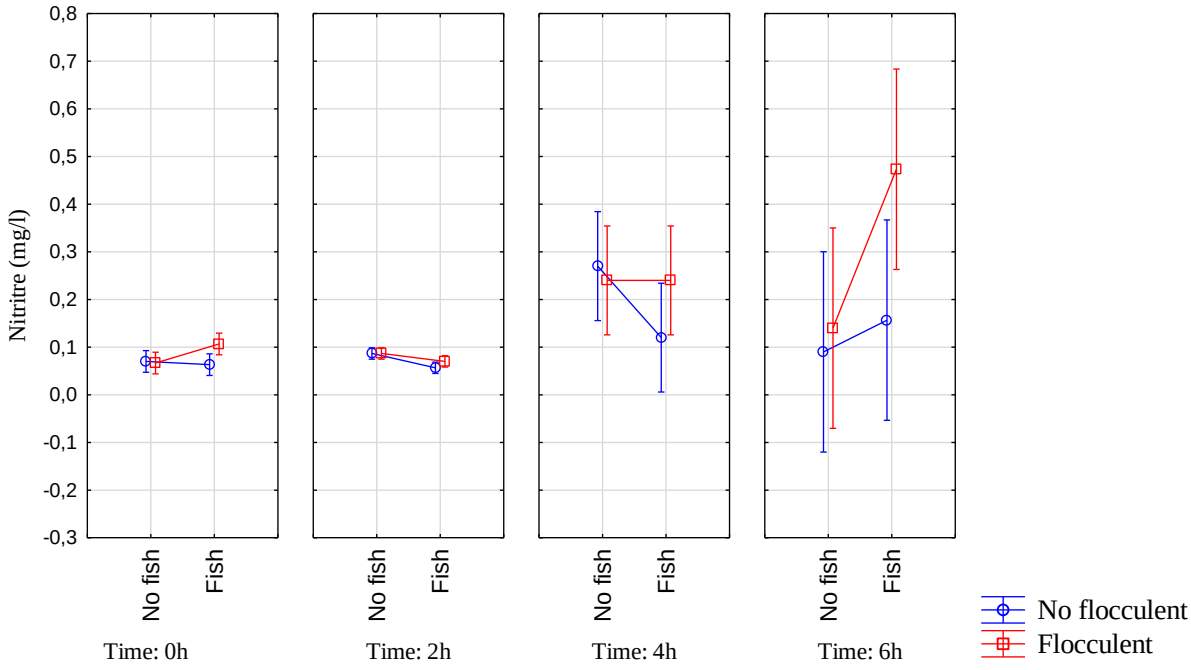

Figure 21: The mean (± standard error) nitrite (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include flocculent or no flocculent and fish or no fish over six hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.299; p=0.826).

Phosphate

There was no interaction in phosphate amongst all the factors (fish/no fish and flocculent/no flocculent) over four hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.278; p=0.612; Figure 22). There was no significant difference in phosphate between flocculated and un-flocculated tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.205; p=0.663; Table 9). No significant difference in phosphate was observed between tanks which included fish and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.867; p=0.379; Table 9).

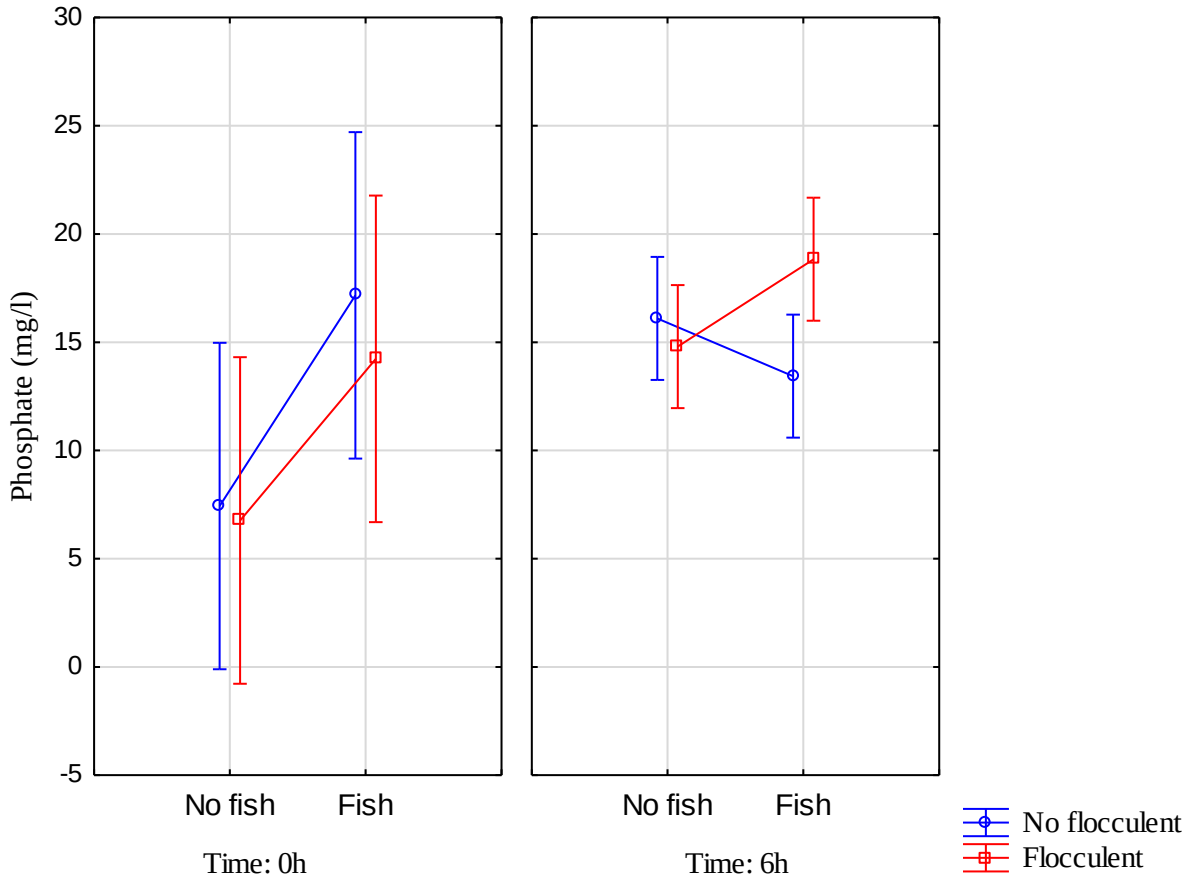

Figure 22: The mean (± standard error) phosphate (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include flocculent or no flocculent and fish or no fish over six hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.278; p=0.612).

Turbidity, pH and dissolved oxygen, non-significant variances

Table 7: The mean (± standard error) turbidity, pH and dissolved oxygen which included fish or no fish and in the absence or presence of a flocculent over four hours (Multifactor Repeated Measures ANOVA: $p>0.05$).

	Time (h)			
	0	4	F	p
Turbidity (cm)				
Flocculent	8.07±0.44	11.47±0.87	0.20	0.67
No flocculent	7.98±0.44	12.07±0.87		
Fish	7.97±0.44	10.57±0.87	2.25	0.17
No fish	8.08±0.44	12.97±0.87		
pH				
Flocculent	9.43±0.13	9.61±0.16	0.66	0.44
No flocculent	9.41±0.13	9.72±0.16		
Fish	9.41±0.13	9.45±0.16	4.88	0.06
No fish	9.43±0.13	9.89±0.16		
Dissolved Oxygen (mg/l)				
Flocculent	4.52±0.20	4.35±0.54	0.07	0.79
No flocculent	4.53±0.20	4.52±0.54		
Fish	3.90±0.20	3.70±0.54	0.15	0.71
No fish	5.15±0.20	5.17±0.54		

Temperature and electrical conductivity non-significant variances

Table 8: The mean (± standard error) temperature and EC, which included fish or no fish and in the absence or presence of a flocculent over four hours (Multifactor Repeated Measures ANOVA: $p>0.05$).

	Time (h)			
	0	4	F	p
Temperature (˚C)				
Flocculent	27.82±0.13	27.62±0.57	0.06	0.82
No flocculent	27.00±0.13	27.43±0.57		
Fish	27.88±0.13	28.00±0.57	1.28	0.29
No fish	27.73±0.13	27.05±0.57		
Electrical conductivity (mS/cm)				
Flocculent	1363.00±53.26	1386.67±49.26	0.39	0.55
No flocculent	1431.33±53.26	1470.67±49.26		
Fish	1401.83±53.26	1456.00±49.26	3.23	0.11
No fish	1392.50±53.26	1401.33±49.26		

Ammonia, nitrite and phosphate non-significant variances

Table 9: The mean (± standard error) ammonia, nitrite and phosphate which included fish or no fish and in the absence or presence of a flocculent over four/six hours (Multifactor Repeated Measures ANOVA: $p>0.05$).

	Time (h)					
	0	2	4	6	F	p
N-NH_3 (mg/l)						
Flocculent	0.32±0.09	0.13±0.03	0.39±0.26	0.16±0.07	0.82	0.50
No flocculent	0.18±0.09	0.11±0.03	0.64±0.26	0.19±0.07		
Fish	0.23±0.09	0.15±0.03	0.60±0.26	0.28±0.07	0.37	0.77
No fish	0.27±0.09	0.09±0.03	0.44±0.26	0.07±0.07		
Nitrite(mg/l)						
Flocculent	0.09±0.02	0.08±0.01	0.24±0.08	0.31±0.15	0.61	0.62
No flocculent	0.07±0.02	0.07±0.01	0.20±0.08	0.12±0.15		
Fish	0.09±0.02	0.06±0.01	0.18±0.08	0.32±0.15	1.31	0.29
No fish	0.07±0.02	0.09±0.01	0.26±0.08	0.12±0.15		
Phosphate (mg/l)						
Flocculent	10.50±5.33			16.82±2.01	0.21	0.66
No flocculent	12.30±5.33			14.77±2.01		
Fish	15.70±5.33			16.13±2.01	0.87	0.38
No fish	7.10±5.33			15.45±2.01		

Relationship between total suspended solids and turbidity, various water quality parameters.

Dry TSS particle size >8µm (Algae concentration) had a positive relationship with turbidity values (Figure 23) and chemical oxygen demand (Figure 24); however a negative relationship was observed with pH (Figure 25).

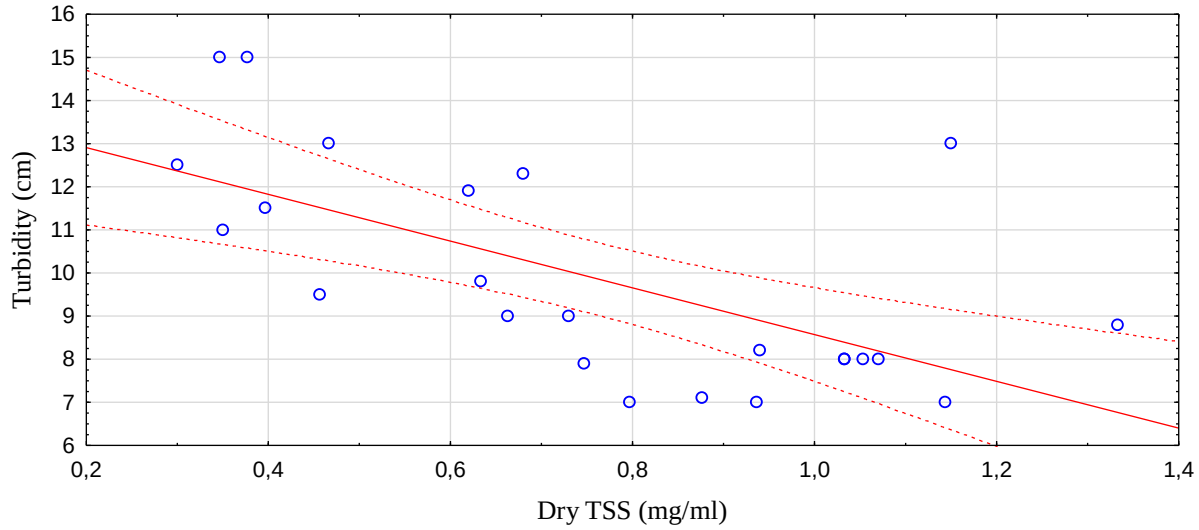

Figure 23: The relationship between algae concentration (mg/ml) and turbidity (cm) (y=13.991-5.42*x: R^2=0.414; p<0.001).

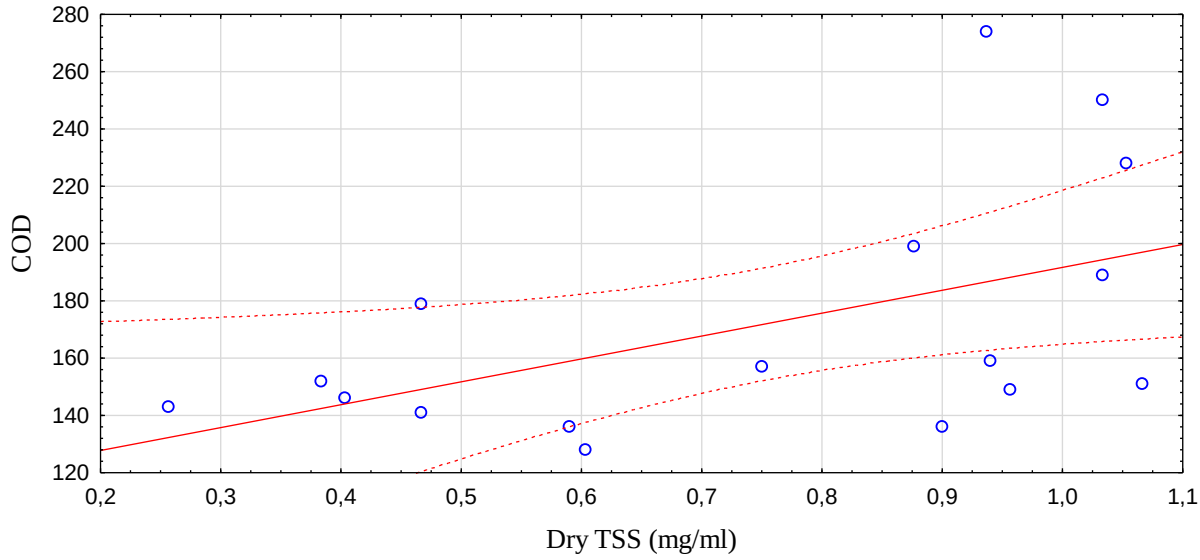

Figure 24: The relationship between algae concentration (mg/l) (measured as total suspended solids, TSS; particle size >8.0 µm) and chemical oxygen demand (COD) (y=111.812+79.910*x: R^2=0.262; $F_{(1,15)}$=5.337; p<0.036).

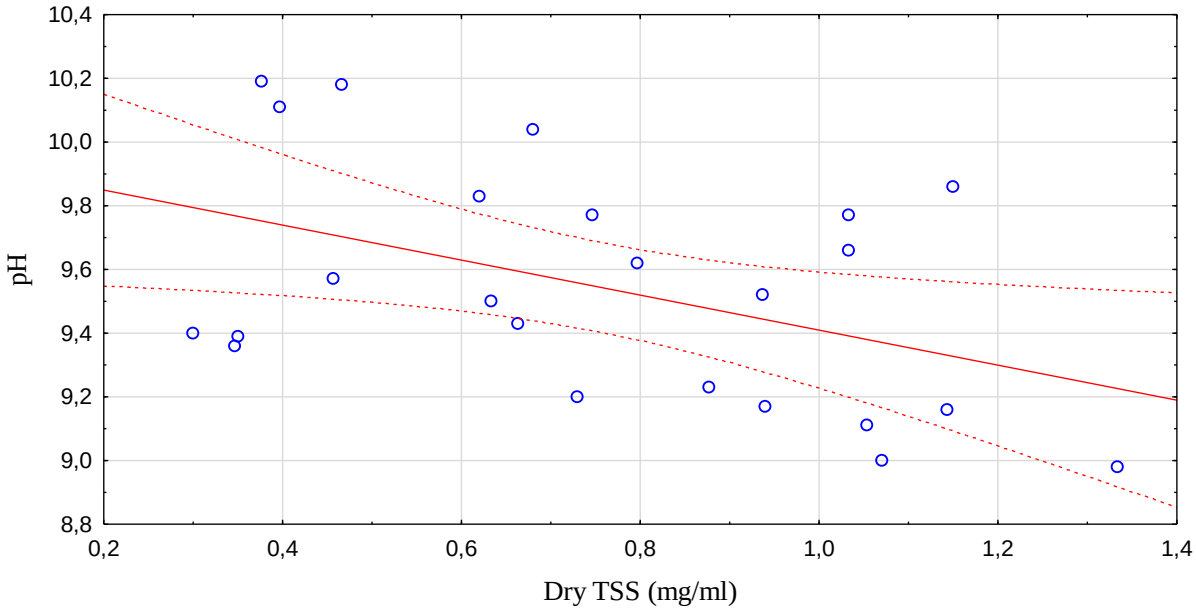

Figure 25: The relationship between algae concentration (mg/ml) (particle size >8.0 µm) and pH (y=9.959-0.550*x: R^2=0.205; $F_{(1,22)}$=5.676; p=0.026).

A positive relationship was observed between turbidity (cm) and dry TSS particle size >8.0 µm (mg/ml) (Figure 26), pH (Figure 27), ammonia (Figure 28) and phosphate (Figure 29).

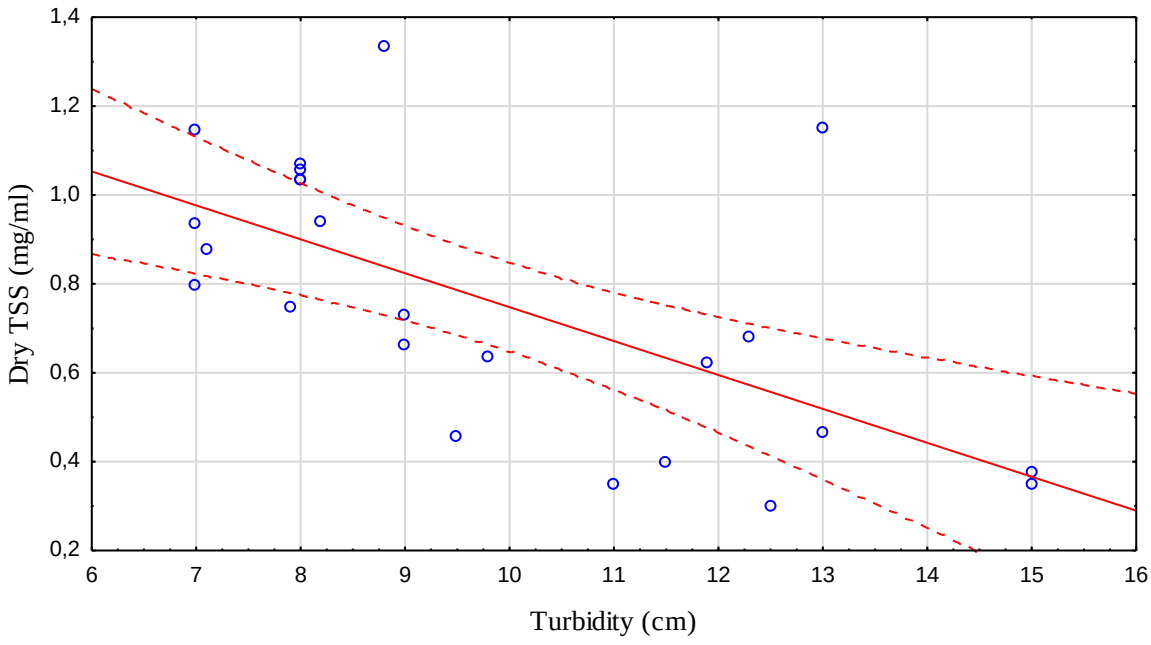

Figure 26: The relationship between turbidity (cm) and algae concentration (mg/l) (particle size >8.0 µm; mg/ml) (y=1.511-0.076*x: R^2=0.414; $F_{(1,22)}$=15.525; p=0.0007).

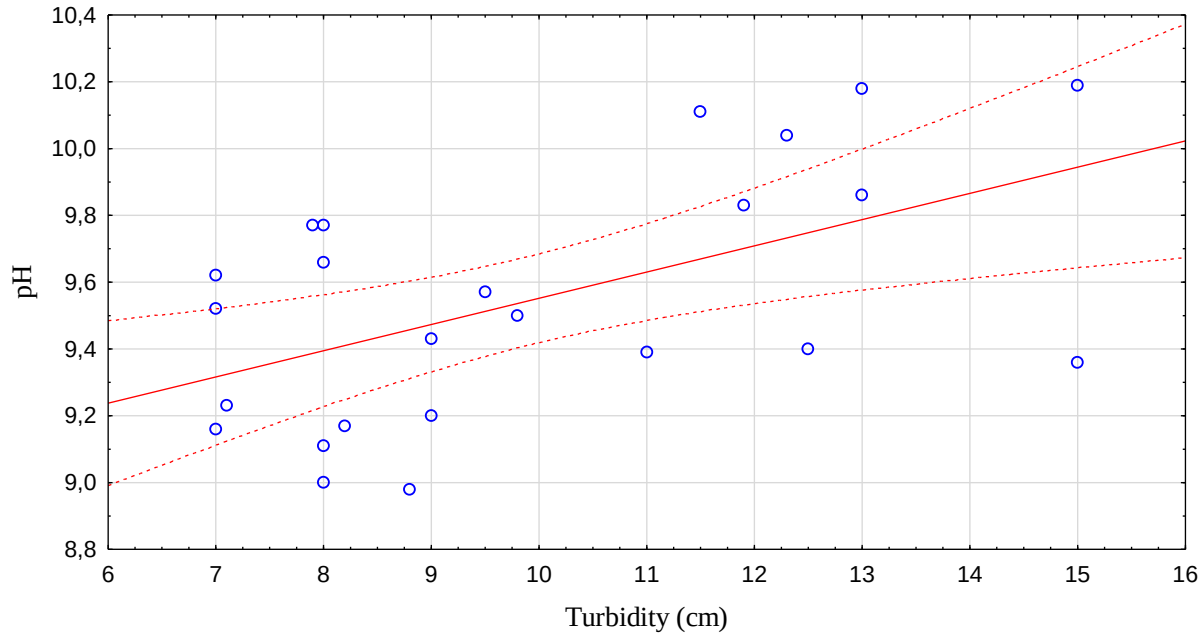

Figure 27: The relationship between turbidity (cm) and pH (y=8.767+0.079*x: R^2=0.297; $F_{(1,22)}$=9.288; p=0.006).

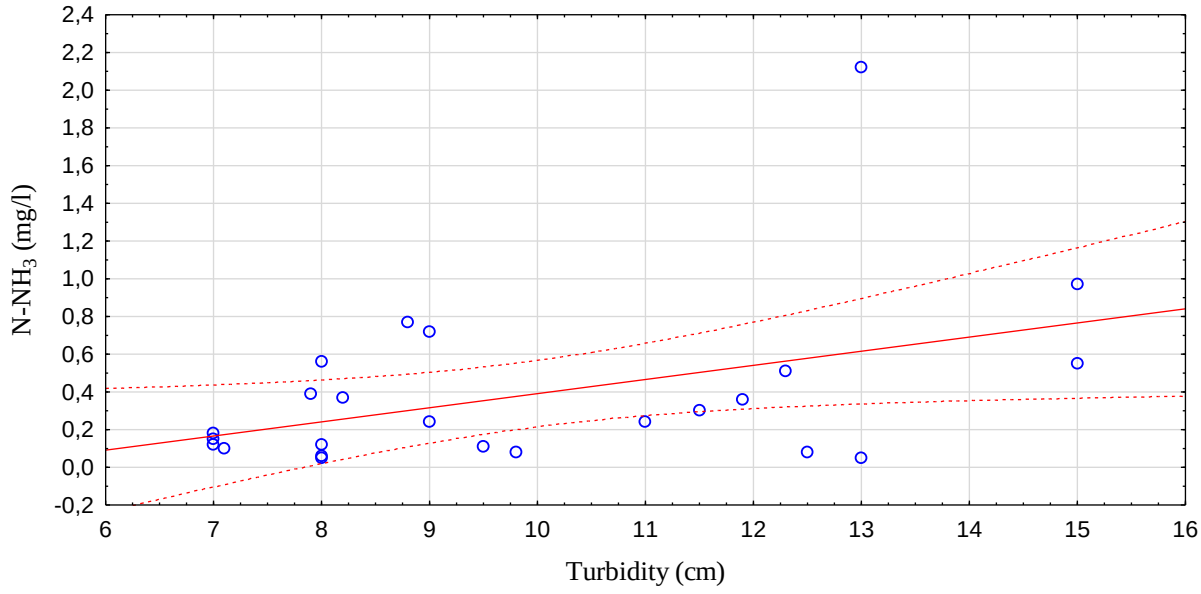

Figure 28: The relationship between turbidity (cm) and ammonia (mg/l)(y=-0.358+0.075*x: R^2=0.180; $F_{(1,22)}$=4.823; p=0.039).

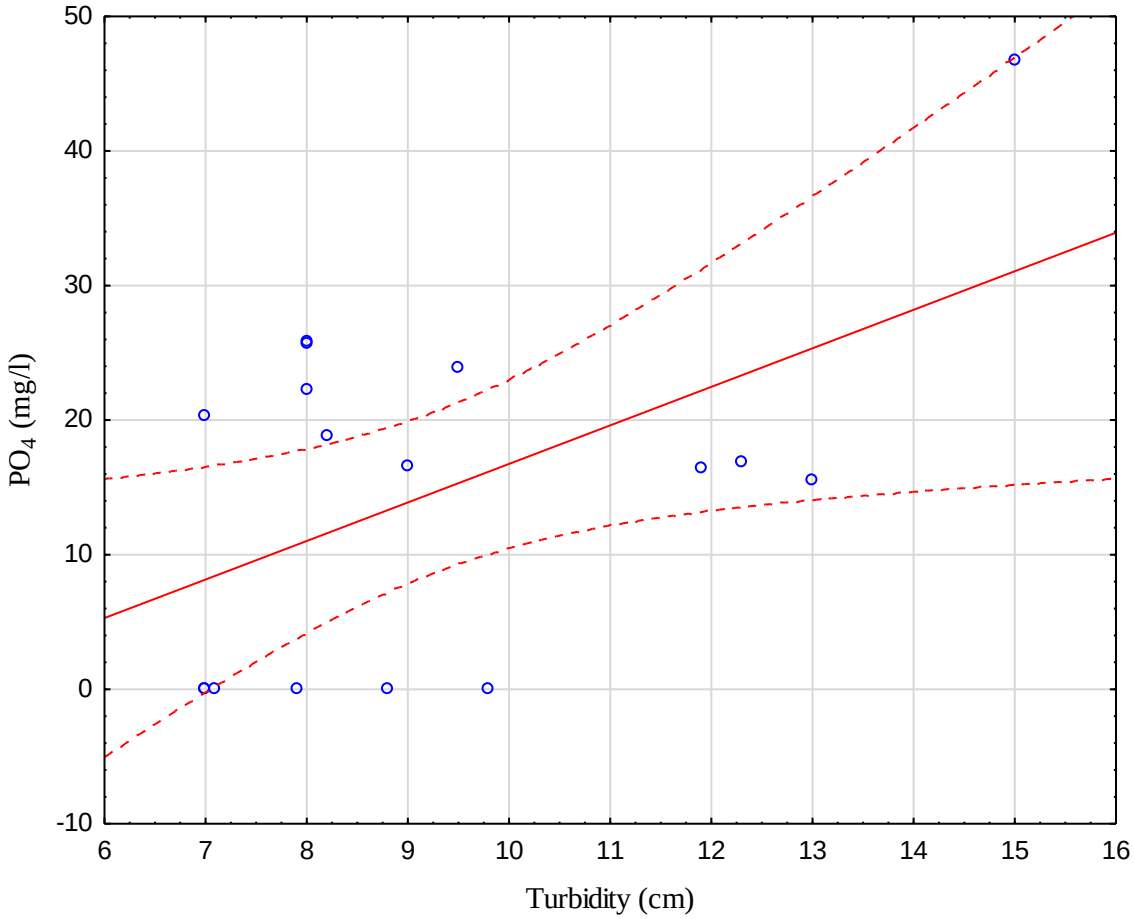

Figure 29: The relationship between turbidity (cm) and phosphate (mg/l) (y=-11.899+2.865*x: R^2=0.271; $F_{(1,15)}$=5.586; p=0.032).

Non-significant relationships between dry total suspended solids and water quality parameters (dissolved oxygen, nitrite, ammonia, temperature, electrical conductivity and phosphate)

There was no significant relationship between dry total suspended solids (mg/ml) (particle size >8.0 µm) and water quality parameters (dissolved oxygen, nitrite, ammonia, temperature, electrical conductivity and phosphate) (Table 10).

Table 10: The relationship between dry total suspended solids (mg/ml) (particle size >8.0 µm) and water quality parameters (dissolved oxygen, nitrite, ammonia, temperature, electrical conductivity and phosphate) in post-high-rate-algal-pond measured over six hours. These data are the combined means of all treatments as there were no slopes to signify a relation with dry TSS particle size >8.0 µm (Regression: $p>0.05$).

	F	R^2	p-value
Dry TSS vs. Dissolved Oxygen	$F_{(1,22)}=0.021$	0.001	0.887
Dry TSS vs. NO_2	$F_{(1,32)}=2.339$	0.068	0.136
Dry TSS vs. N-NH3	$F_{(1,46)}=2.429$	0.050	0.126
Dry TSS vs. Temperature	$F_{(1,22)}=2.246$	0.093	0.148
Dry TSS vs. EC	$F_{(1,22)}=0.398$	0.018	0.534
Dry TSS vs. Phosphate	$F_{(1,27)}=3.490$	0.115	0.073

Non-significant relationships between turbidity and water quality parameters (dissolved oxygen, CO_2, ammonia, temperature, pH and chemical oxygen demand)

There was no significant relationship between turbidity and water quality parameters (dissolved oxygen, CO_2, ammonia, temperature, pH and chemical oxygen demand) (Table 11).

Table 11: The relationship between turbidity and water quality parameters such as dissolved oxygen, nitrite, electrical conductivity, temperature and chemical oxygen demand (COD) in post-high-rate-algal-pond measured over six hours. These data are the combined means of all treatments as there were no slopes to signify a relation with turbidity (Regression: $p>0.05$).

	F	R^2	p-value
Turbidity vs. Dissolved Oxygen	$F_{(1,22)}=0.613$	0.027	0.442
Turbidity vs. NO_2	$F_{(1,17)}=1.049$	0.058	0.320
Turbidity vs. EC	$F_{(1,22)}=0.059$	0.003	0.810
Turbidity vs. Temperature	$F_{(1,22)}=3.499$	0.137	0.075
Turbidity vs. COD	$F_{(1,3)}=0.238$	0.073	0.659

2.5 Discussion

Due to lack of knowledge on the age group of *Clarias gariepinus* which filter feeds best, this had to be investigated in a pre-study format. This knowledge gave way to the main-study of this chapter which was to determine if *Clarias gariepinus* with the aid of a flocculent could filter-feed on algae. The findings of both studies are discussed below.

2.5.1 The effect of *Clarias gariepinus* age on inter-gill raker width

The width between gill rakers is directly proportional to fish age. In the present study it was observed that inter-gill raker width steadily increased from about 100 µm at two months of age to over 160 µm at 12 months. In a study conducted by Macnuson and Heitz (1971), on Scombridae fish, there was a considerable variation in gill raker gap amongst fish of different size. According to Murray (1975), the mean width between gill rakers ranged from <0.1 to 0.6 mm depending on fish size. Generally, there is a link between fish age and fish length. Usually fish increase in length as they get older; given conditions are favourable (Conover and Munch, 2002). Therefore, the observations made by Macnuson and Heitz (1971) and Murray, (1975) are consistent with those of the present study.

Clarias gariepinus less than a year old are well suited to filter feed on algae (based on gill development). Murray (1975) observed that fish less than 300 mm filter feed on algae. Furthermore according to Willoughby and Tweddle (1978), as the *C. gariepinus* grow older they exhibit a specific dietary shift towards fish. However, *C. gariepinus* immersed in an environment dominated by microalgae will filter feed, particularly if it is the only source of food (Murray, 1975).

2.5.2 The removal of algae by *Clarias gariepinus*

Clarias gariepinus require sufficient time to acclimatize to new conditions including the available diet, which is why *C. gariepinus* less than a year old, kept in brewery wastewater with microalgae for six hours do not significantly reduce the algae concentration. In the current study, *C. gariepinus*-containing tanks had algae concentrations which were less than those observed in the *C. gariepinus*-absent tanks at hour zero, the second hour and the sixth hour of experimentation, but both treatments followed a similar trend, resulting in a non-significant p-value of 0.090. Furthermore during the fourth hour of experimentation the tanks with *C. gariepinus* had a higher algae concentration compared to the tanks without fish, hence algae concentration between these two treatments (*C. gariepinus*/no *C. gariepinus*) was not significantly different. In a study conducted by Refaey *et al.* (2017), channel catfish recovered after 168 hours from stressful conditions. Catfish need to be kept in uniform conditions for an extended period of time if they are to be experimented upon outside of their habitats. Willoughby and Tweddle (1978) conducted a study in Malawi in the Lower Shire River and reported that catfish feed on plant matter especially filamentous algae. That study was done in a natural environment (in the wild) and over a two-year sampling period. The time it takes for catfish to fully acclimatize and switch diet to microalgae wastewater needs further study.

Regardless of the factors driving the change, algae concentration in a water body decreases over time (Divakaran and Pillai, 2002). There was less algae at the end of the experiment compared to at the start.

2.5.3 The effect of a flocculent, chitosan

A low pH value 7 is needed for a flocculent (chitosan) to be most efficient and assist the fish in algae removal (Vandamme *et al*., 2013). The effect of chitosan on algae concentration was negligent in the present study, probably because of the high pH value of the high rate algal

pond (HRAP). Had the researcher in the current study been aware of the need for a lower pH to improve the efficiency of the chitosan in the current study, a different result might have been obtained. In a study conducted by Divakaran and Pillai (2002), "chitosan reduced the algal content effectively by flocculation and settling," and the maximum pH levels of 7 were reached for freshwater algae species. In the present study the pH values were generally high (ranging from just above 9 to a little over 10), which is typical of a microalgal culture environment (Chang and Lee, 2012), and which could possibly account for the current result.

2.5.4 Water quality parameters

a) pH

Phytoplankton (herewith made synonymous to algae) influences various water quality components such as pH (National Statistical Coordination Board, 1999). An algae rich environment is often associated with an alkaline pH within a water body (Carpenter and Lodge, 1986; Ceci, 2015). Since algae concentration was the same between *C. gariepinus*-containing and *C. gariepinus*-absent tanks, and flocculated and un-flocculated tanks, the pH values were also similar. However pH did increase over the experimentation period.

b) Dissolved oxygen

Fish presence is usually linked to a decrease in dissolved oxygen. In the current study dissolved oxygen was higher in *C. gariepinus*-absent tanks compared to *C. gariepinus*-present tanks. The decrease in oxygen was due to fish respiration (Mallya, 2007) and could have affected the fish's normal functioning, like feeding (Bagherzadeh Lakani *et al*., 2013). The amount of oxygen contained in water also depends on temperature and salinity (Mallya, 2007). For example, warm seawater tends to hold less oxygen than cold freshwater (Mallya, 2007). A similar effect on dissolved oxygen by these two parameters (temperature and salinity) would also be expected

in brewery effluent which when recycled is known to have elevated salinity concentrations (Jones *et al*., 2016), although not as high as the salinity of seawater.

Chitosan has no influence on water dissolved oxygen concentration levels. In the present study dissolved oxygen was similar between flocculated and un-flocculated tanks. Gylienė *et al.,* (2015), however, reported chitosan adsorbing dissolved oxygen from a solution. The water medium used by Gylienė *et al.* (2015) was bidistilled water which contained dissolved oxygen instead of brewery wastewater containing microalgae. This could have played a role into the realisation of differing outcomes.

c) Temperature

Temperature is a water parameter mostly dictated by the external environment. "Heat inputs and outputs including solar radiation, convection from air, and conduction from soil influence water temperature by either increasing or decreasing it" (Khan *et al.*, 2011). Due to the fact that all of the treatments were subjected to the same conditions this parameter was uniform between tanks which included fish and fish-absent tanks and also between flocculated and un-flocculated tanks.

d) Electrical conductivity

The presence of various ions in water increases its electrical conductivity levels (Olajumoke *et al.,* 2010). Nitrates, phosphates and chlorides are some of the inorganic dissolved solids which possess negatively charged ions (United States Environmental Protection Agency, 2012), and they can be found in catfish faeces (Rubio *et al*., 2016). These ions have been reported to cause an increase in water electrical conductivity (River Watch Network, 1997). Wastewater electrical conductivity differed significantly over time in the

present study; however no significant difference was observe between fish-containing-tanks and fish-absent-tanks, and flocculated and un-flocculated tanks.

e) Ammonia

Ammonia is released from decaying fish waste (Francis-Floyd *et al*., 2009). In the present study ammonia in brewery wastewater significantly differed over time. Ammonia was elevated during the fourth hour of experimentation. It was expected that fish-containing tanks have higher levels of ammonia compared to fish-absent tanks; however this was not the case as the absence or presence of fish had no significant effect on brewery wastewater ammonia concentration. Perhaps it takes an extended period for the ammonia to diffuse into the water or the algae resident time within the fish gut was extensive, delaying ammonia leaching from fish waste. Flocculation has minimal if any effect on ammonia concentration. Concentrated ammonia on the other hand was utilised by Kusuma *et al*. (2015) to form a chitosan nano-particle.

f) Nitrite and phosphate

The other sampled water quality parameters (NO_2 and PO_4) across all treatments showed no significant differences ($p>0.05$). Nitrite concentration does not fluctuate much in brewery wastewater (Simate *et al*., 2011). Nitrite concentration in the effluent tanks did not fluctuate based on presence or absence of catfish and presence or absence of flocculation; neither did it significantly vary over time. In brewery wastewater, nitrite is formed from nitrogen oxide, but this too is further oxidised into nitrate ions. Therefore the nitrite state seldomly increases (Simate *et al*., 2011). Phosphate concentration also appears to be un-affected by fish presence or absence and flocculent absence or presence. In the short term (6.0 h) there was no apparent temporal influence on phosphate concentrations in the treatments.

2.5.5 The relationship between physico-chemical parameters and algae concentration

Algae concentration may be reliably inferred from turbidity and total suspended solids measurements (Bilotta and Brazier, 2008). In the present study these two water parameters shared a positive relationship with each other. "Suspended solids" include the mass of organic material held within a water column (Bilotta and Brazier, 2008), and this accounted for the majority of the matter in brewery wastewater.

Dry total suspended solids have a positive relationship with chemical oxygen demand. Dry total suspend solids were directly proportional to chemical oxygen demand. Awang and Shaaban (2016) reported suspended solids as major contributors to chemical oxygen demand.

Algae concentration appears to have a negative relationship with pH. An increase in algae concentration (turbidity and total suspended solids) was accompanied by a decrease in pH levels although the water medium remained relatively alkaline. The National Statistical Coordination Board (1999), reported turbidity and total suspended solids as having increasing trends whereas pH had a decreasing trend in a study conducted in the Philippines.

A positive relationship exists between turbidity and ammonia. A decrease in turbidity was followed by a decrease in ammonia. Lawler *et al*. (2006) also observed a directly proportional relationship between these factors. Immediately after wet-weather episodes, large increases in water turbidity were simultaneous with increases in ammonia in The River Tame (Lawler *et al*., 2006). Webster *et al*. (2001) also observed an association between turbidity peaks and ammonia spikes in The River Tame.

Turbidity and phosphate are positively related. Fluctuations in turbidity were mirrored by phosphate fluctuations. Jin *et al*. (2013) came to a similar conclusion. "In the upper reaches a 50 % reduction of phosphate loads results in a strongly reduced primary production" (DeGroodt and de Jonge, 1990). There is a clear link between these two factors suggesting co-dependence or one influencing the other.

2.6 Conclusion

Clarias gariepinus filter feed best when they are less than a year old, based on the development of their gill rakers and the size of algae species found in South Africa. In the current study the fish did not feed significantly on the algae and this may be attributed to the fish requiring sufficient time to acclimatize to differing conditions.

Flocculation using chitosan did not assist in algae removal. Chitosan is most efficient in low pH environments and the desired effect of forming algae aggregates which could be easily filtered by the catfish could not be achieved in an alkaline solution. However, algae concentration was significantly different when only time was taken into account and this is attributed to algal settling which was independent of the presence or absence of the flocculent.

Most of the monitored physico-chemical parameters remained similar amongst the different treatments. Dissolved oxygen is the only water quality parameter that differed between fish-containing and fish-absent tanks; the fish used the oxygen for respiration. There is a relationship between algae concentration and several water quality parameters. Taking into account algae concentration and/or turbidity and the interactions with the various water quality parameters (turbidity, dry TSS, chemical oxygen demand and pH, ammonia and phosphate); algae filtration by *C. gariepinus* is influenced by and/or influences these water parameters.

Since *C. gariepinus* did not efficiently remove microalgae by filter feeding, alternative fish that can withstand fluctuations in water quality should be considered for future work; for example: *Oreochromis mossambicus*. Furthermore, the fluctuation in water quality might have influenced the ability of the fish to remove algae so work is needed to develop methods to mitigate fluctuations such as pH.

Chapter 3: The removal of microalgae from brewery wastewater by *Oreochromis mossambicus* and the influence brewery wastewater pH moderation has on this process.

3.1 Introduction

Changes in physical and chemical characteristics of the water that fish inhabit may be beneficial or detrimental to the fish. These changes may boost or impede processes like feeding (Serafy and Harrell, 1993). Due to photosynthesis, algae consume CO_2 from the water, which leads to elevated hydroxide ion levels resulting in an increase in pH (Carpenter and Lodge, 1986; Ceci, 2015). Fish deaths have been reported in water bodies with alkaline levels of up to pH 10 (Jordan and Lloyd, 1964). Furthermore (Daye and Garside, 1975; 1976; 1980; Mallat, 1985), reported surface tissue damages leading to stress reactions, such as the over production of gill mucus from fish in water bodies, with a pH over nine. Dissolved CO_2 decreases ocean water pH (Brennan, 2017).

Due to their physical features (mucus secretion and compact gills) and a wide diet range, *O. mossambicus* has the potential to filter feed on algae (Skelton 1993). The removal of microalgae from wastewater by *O. mossambicus* has been reviewed in the first chapter. The purpose of the first experiment was to investigate whether CO_2 decreased water pH in freshwater bodies filled with algae. The purpose of the second experiment was to investigate whether *O. Mossambicus* could successfully remove algae by filter feeding.

3.2 Aims and objectives

3.2.1 Developing a method to moderate pH in algal ponds

The aim was to find a method to lower pH in wastewater treatment ponds.

The objective was to compare the most efficient method to reduce water pH by comparing the pH between (a) light deprived and treatments subject to sunlight and (b) carbon dioxide sparged treatments (i.e. pH moderated) and treatments that did not receive additional carbon dioxide (i.e. no pH moderation).

Hypotheses

Null hypothesis 1: Water pH is similar between CO_2 sparged and non CO_2 sparged tanks.

Null hypothesis 2: Water pH is similar between light and dark tanks.

3.2.2 Removal of algae by *Oreochromis mossambicus*

The aim was to study the efficacy of algae removal by *Oreochromis mossambicus* under different conditions, to determine if the algae were being consumed by the fish and to determine the influence of algae concentration on several water quality parameters.

The objectives were to:

- assess whether fish remove algae from algal tanks;
- assess the fish gut weights at the end of the experiment, and describe the fish gut contents.
- ascertain the role of pH moderation in algae removal;
- monitor water quality parameters; and to
- determine whether a relationship exists between algae concentration and the various water quality parameters including pH modification.

Hypotheses

Null Hypothesis 1: Algae concentration is similar between fish-containing tanks and fish-absent tanks.

Null Hypothesis 2: Fish gut weights are similar in all the fish containing tanks

Null Hypothesis 3: Algae concentration is similar between pH moderated tanks and unmoderated tanks.

Null Hypothesis 4.1: Water quality parameters are similar between fish-containing tanks and fish-absent tanks.

Null Hypothesis 4.2: Water quality parameters are similar between pH moderated tanks and unmoderated tanks.

Null Hypothesis 5: Algae concentration is not related to any water quality parameter.

3.3 Materials and methods

3.3.1 Developing a method to moderate pH in algal ponds

Treatments

There were four treatments: light exposed and dark treatments, both with and without CO_2 sparging. Each treatment was replicated three times. Tanks one, two and four were treatment one (light exposed tanks without CO_2). Treatment two was made up of tanks three, five and six (dark tanks without CO_2). Tanks seven, eight and ten formed treatment three (light exposed tanks with CO_2). Treatment four consisted of tanks nine, eleven and twelve (dark tanks with CO_2). Carbon dioxide was sparged from a gas cylinder to intended tanks through a network of rubber tubes over the duration of the experiment. Light exposed treatments were left uncovered, whereas the dark treatments were covered with dark plastic.

Water quality parameters

Temperature, dissolved oxygen, pH, CO_2 and turbidity were monitored in post-high-rate-algal-pond effluent, over five hours starting from 10 am. Temperature (Hannah, HI98311. Woonsocket, United States of America), pH (Hanna, HI98127. Woonsocket, United States of America) and dissolved oxygen (Oxyguard, Handy Polaris. Farum, Denmark) were recorded using electronic probes. Similarly, an electronic CO_2 meter was used to measure CO_2 (Oxyguard, GO2P, portable CO_2 meter. Farum, Denmark) and a sechi tube was used was used for turbidity measurements (Myre and Shaw, 2006).

Statistical methods

Interactions between factors were compared using repeated measures multifactor analysis of variance (ANOVA) at $p < 0.05$. If there were no interactions between factors, then treatment means within each factor were compared at $p<0.05$. In all cases, data were first checked for equality of variance (Levene, 1960) and that the residuals were normally distributed (Shapiro and Wilk 1965).

3.3.2 Removal of algae by *Oreochromis mossambicus*

Experimental facilities

This investigation was also carried out at the Project Eden site at IBhayi Brewery (SAB Ltd) in Port Elizabeth using the same integrated algal ponding effluent treatment system used in the *Clarias gariepinus* study (Section 2.3.2; Chapter 2).

The aquaculture system was also similar to that used in the *C. gariepinus* study, except that it contained a biological filter and an aeration pump supplying 40 l tanks with water and oxygen respectively. A mechanical filter was included in series to a pump, which received a top-up from the municipal water system, together with recirculating the water from the tanks. There

were also twelve 500 l capacity tanks which were filled with isolated municipal water and two 40 l capacity tanks were suspended via tubes in each of these tanks.

Experiment design

There were four treatments (Table 12): fish containing and fish absent treatments; both with and without CO_2 sparging (CO_2 sparging was included to decrease water pH). Each treatment was replicated three times. Tanks eight, ten and twelve were treatment one (fish containing tanks with CO_2). Treatment two was made up of tanks seven, nine and eleven (fish absent tanks with CO_2). Tanks two, four and six formed treatment three (fish containing tanks without CO_2). Treatment four consisted of tanks one, three and five (fish absent tanks without CO_2). Carbon dioxide was pumped from a compressed carbon dioxide (CO_2) gas cylinder connected to a CO_2 regulator (Figure 30) into the CO_2 sparged treatments only.

Table 12: *Oreochromis mossambicus* experiment design highlighting the different treatments.

Treatment number	Factor 1: Sparging	Factor 2: Fish	Tank number
T1	Carbon dioxide	Fish	8,10,12
T2	Carbon dioxide	No fish	7,9,11
T3	No carbon dioxide	Fish	2,4,6
T4	No carbon dioxide	No fish	1,3,5

Figure 30: Compressed carbon dioxide gas cylinder connected to a carbon dioxide regulator (Mogane, 2016).

Experimental animals

Oreochromis mossambicus were sourced from a commercial fish farm (Rivendell Hatchery, Pty Ltd) in Grahamstown. The fish which were similar in size and were transported in oxygenated tanks from the farm to the Project Eden. Upon arrival the fish were placed in 500 l capacity tanks in the recirculating aquaculture system and were fed a commercial feed at 10 % body weight once a day during the acclimation period of 14 days.

Water and fish introduction into tanks

Fourteen fish were randomly stocked into each of six of the 40 l tanks, seven days before the experiment. Clove oil was added at a ratio of 100 mg/l of water and this induced a state of anaesthesia to the fish (Waterstrat, 1999). The fish were then scooped with a net, weighed on a scale to one decimal place (3.5±2.6 g) and total length measured with a ruler (46.0±39.2 mm).

Another six of the 40 l tanks in the same recirculating aquaculture system were left with no fish in them. Four days before experimentation, fish feeding was stopped and during those four days the faeces were siphoned from the tanks. The recirculating aquaculture system supplying the tanks was detached and remained so throughout the experiment. However aeration was not turned off. The 40 l tanks were drained and 10 l recirculating system water left in the tank, after which they were weighed and stocking with fish.

The same water levels were achieved in the tanks without fish too, so algae concentration at the start of the experiment was the same in all treatments. Thirty litres of algae containing treated brewery effluent was pumped directly from the second ponds in the HRAP trains into each of the 12 tanks.

Data collection

Algal samples were taken from both second HRAP ponds and each tank at the start of the experiment, and again at hourly intervals for the duration of the trial (five hours). The algal biomass within the tanks was determined by using the total suspended solids and turbidity methods employed in the *C. gariepinus* study (Section 2.3.2; Chapter 2).

At the end of the experiment all the tilapia were terminated following anaesthesia by inducing trauma on the head and dissected before the guts were removed and weighed. Ethical clearance for fish euthanasia was obtained from Rhodes University before the study commenced (DIFS Jones Nombembe 2016). The fish guts were then stored in labelled vials, wrapped in foil and frozen. At the Microbiology Laboratory Unit at Rhodes University the guts were photographed using a stereo microscope connected to a computer. The presence or absence of algae was determined based on the colour of the gut content, where a green gut indicated the presence of algae.

Chemical oxygen demand (COD), ammonia (0-2.5 mg/l N), and phosphate were determined using the same methods described earlier (Section 2.3.2; Chapter 2).

A chloride test kit was used to test the chloride concentration of the water (Merck Pty Ltd, Product: 1.14897.0001). Chloride ions react with mercury (II) thiocyanate to produce semi separated mercury (II) chloride. Iron (III) ions then react with the released thiocyanate to produce red iron (III) thiocyanate (Cilliers, 2012). Two hundred and fifty millilitre water samples were filtered through a quadrant of an eight micrometre filter paper. Since the chloride concentration exceeded the optimum range of 250 ml/l, the chloride samples were diluted 1:2, before 2.00 ml was added into a glass vial. Then 2.50 ml of Reagent 1 was pipetted into the same vial followed by 0.50 ml of Reagent 2 before the contents were sealed, mixed and allowed to react for one minute (Cilliers, 2012). This was done at the start and at the end of the experiment.

Temperature, EC and pH data were collected using the same method described in section 2.3.2; Chapter 2 over a five hour period starting from 10 am. The water in the tank was agitated by increasing the air bubbling in all of the tanks prior to taking the algal and water samples.

Trial termination

Trial termination was pre-determined to be either when tank algae concentration stopped decreasing or when toxic free-ammonia concentration levels of 0.5 mg/l (Tucker, 1988 in Boyd, 1990) were reached, which ever occurred first.

Statistical methods

Interactions between factors were determined using repeated measures multifactor analysis of variance (ANOVA) at $p \leq 0.05$. If there were no interactions between factors, then treatment means within each factor were compared at $p \leq 0.05$. In all cases, data were first checked for

equality of variance (Levene, 1960) and that the residuals were normally distributed (Shapiro and Wilk 1965). A linear regression was also used to analyse the relationship between algae concentration and water quality parameter.

A one way ANOVA was used to compare the weight of the fish guts between treatment means at $p \leq 0.05$; a multifactor ANOVA could not be applied here since it was not possible to collect gut samples from treatments that included no fish. Again, data were first checked for equality of variance (Levene, 1960) and that the residuals were normally distributed (Shapiro and Wilk 1965). For algae presence, qualitative analysis was applied where only algae presence or absence was observed.

3.4 Results

3.4.1 Developing a method to moderate pH in algal ponds

pH

A significant difference was observed in pH between CO_2 sparged tanks and tanks where no CO_2 was sparged; pH was lower in CO_2 sparged tanks. Over the five hour experimentation period in both treatments (CO_2 and no CO_2) pH decreased after the first hour before increasing in the fourth and fifth hour (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=211; p<0.001; Figure 31). There was no significant difference in pH between light and dark tanks over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=8.938; p=0.050; Appendix 1).There was no interaction amongst all the factors over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=1.864; p=0.326; Appendix 1).

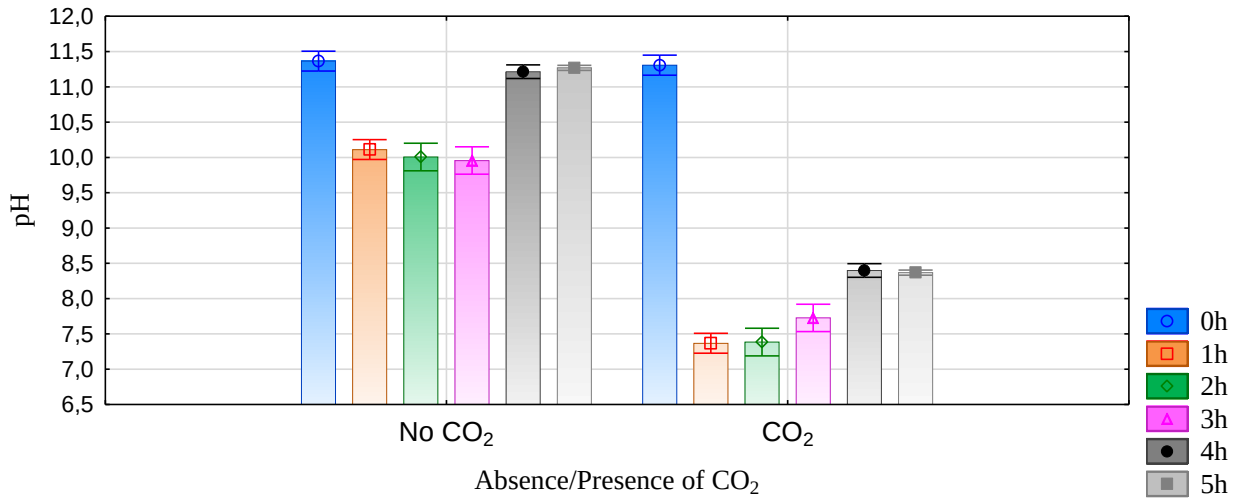

Figure 31: The mean (± 95 % confidence interval) pH between carbon dioxide and non-carbon dioxide treatments over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=211; p<0.001).

Carbon dioxide

There was a significant difference in CO_2 concentration between light and dark tanks in the first three hours. Carbon dioxide was higher in dark than in light exposed tanks. In the dark tanks CO_2 concentration started to decrease in the fourth and fifth hour and became similar to that observed in the light tanks over the same period (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=12.977; p=0.030; Figure 32). There was a significant difference in CO_2 concentration between CO_2 bubbled and tanks where no CO_2 was bubbled in over five hours. Carbon dioxide was higher in tanks in which CO_2 was bubbled. Carbon dioxide was only similar between the two treatments (CO_2 and no CO_2) at hour zero (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=7993. 9; p<0.001; Figure 33). There was no interaction amongst all the factors over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=2.882; p=0.207; Appendix 1).

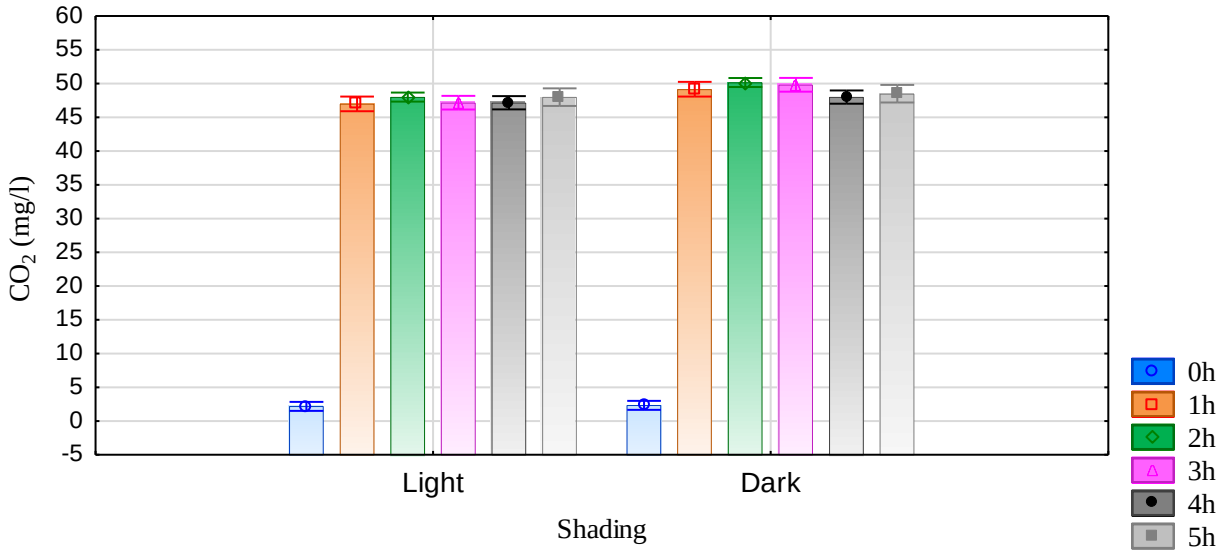

Figure 32: The mean (± 95 % confidence interval) carbon dioxide (mg/l) between light and dark treatments over a five hour period (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=12.977; p=0.030).

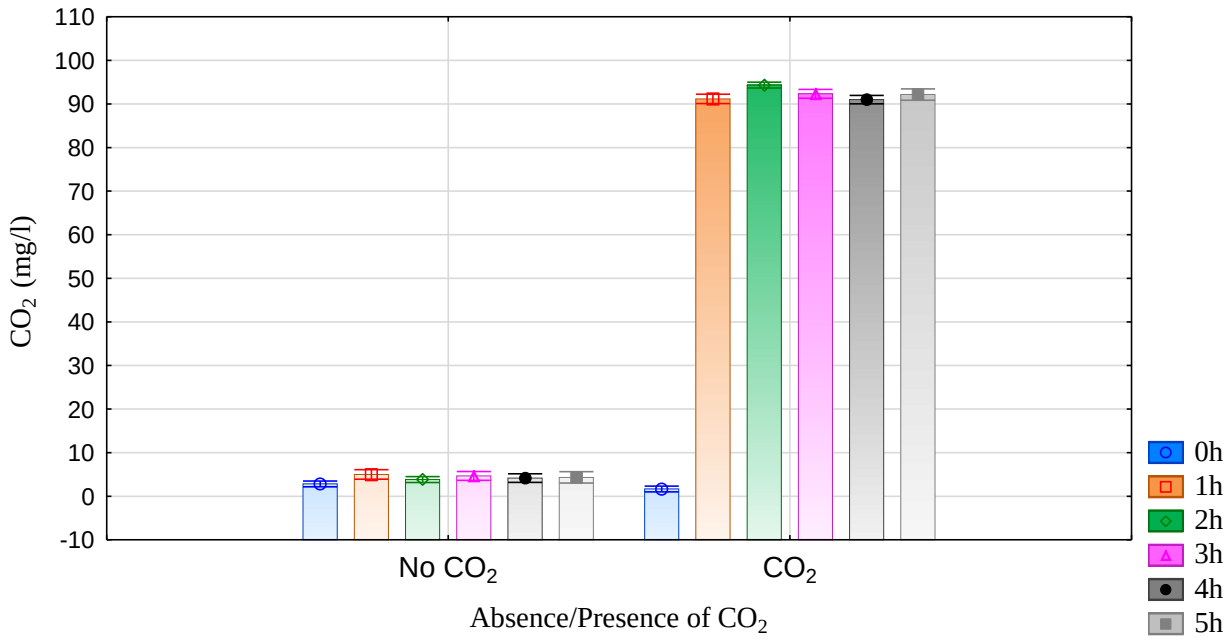

Figure 33: The mean (± 95 % confidence interval) carbon dioxide (mg/l) between carbonated and non-carbonated treatments over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=7993.9; p<0.001).

Dissolved oxygen

There was a significant difference in dissolved oxygen between light and dark tanks over five hours. Dissolved oxygen was similar between treatments at the start and it dropped in the dark tanks, whereas it increased in the light tanks (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=44.126; p=0.005; Figure 34). There was no significant difference in dissolved oxygen between CO_2 sparged and tanks without sparging over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=5.751; p=0.090; Appendix 1). There was no interaction amongst all the factors over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=8.214; p=0.056; Appendix 1).

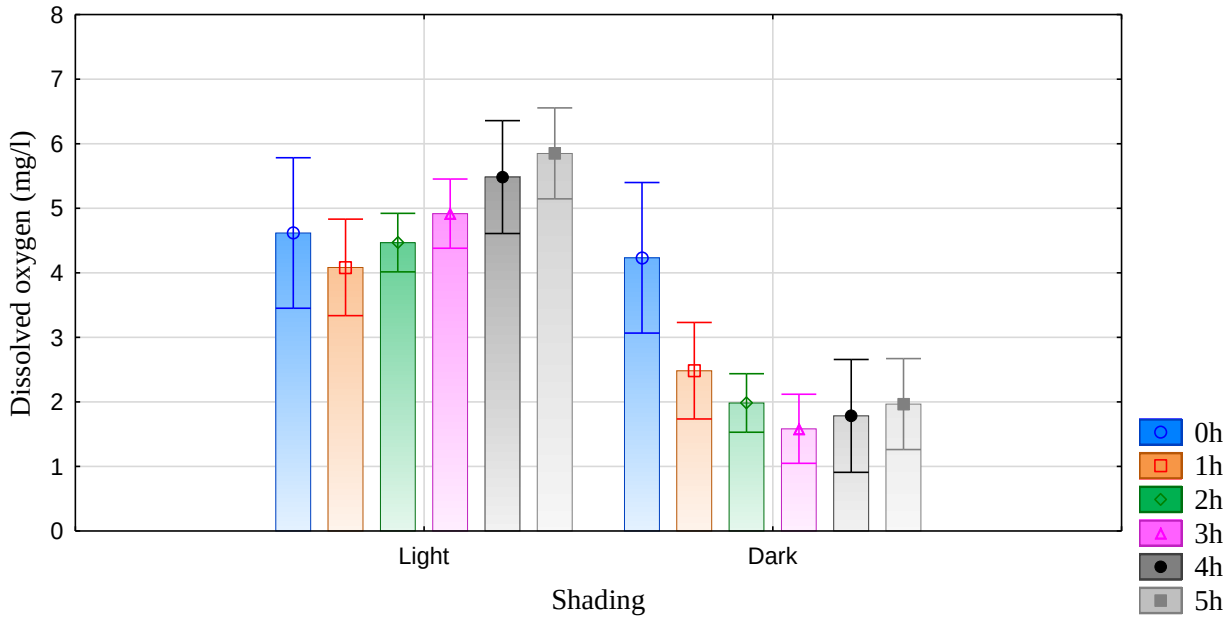

Figure 34: The mean (± 95 % confidence interval) dissolved oxygen (mg/l) between light (environment exposed) and dark (covered) treatments over five hours (Multifactor Repeated Measures ANOVA: $F_{(6, 3)}$=44.126; p=0.005).

Turbidity

There was a significant difference in turbidity between light and dark tanks over five hours. Turbidity was visibly higher in the light exposed tanks during fourth hour (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=9.127; p=0.049; Figure 35). There was no significant difference in turbidity between CO_2 sparged tanks and tanks that were not sparged over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=1.964; p=0.310; Appendix 2). There was no interaction amongst all the factors over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=1.903; p=0.319; Appendix 2).

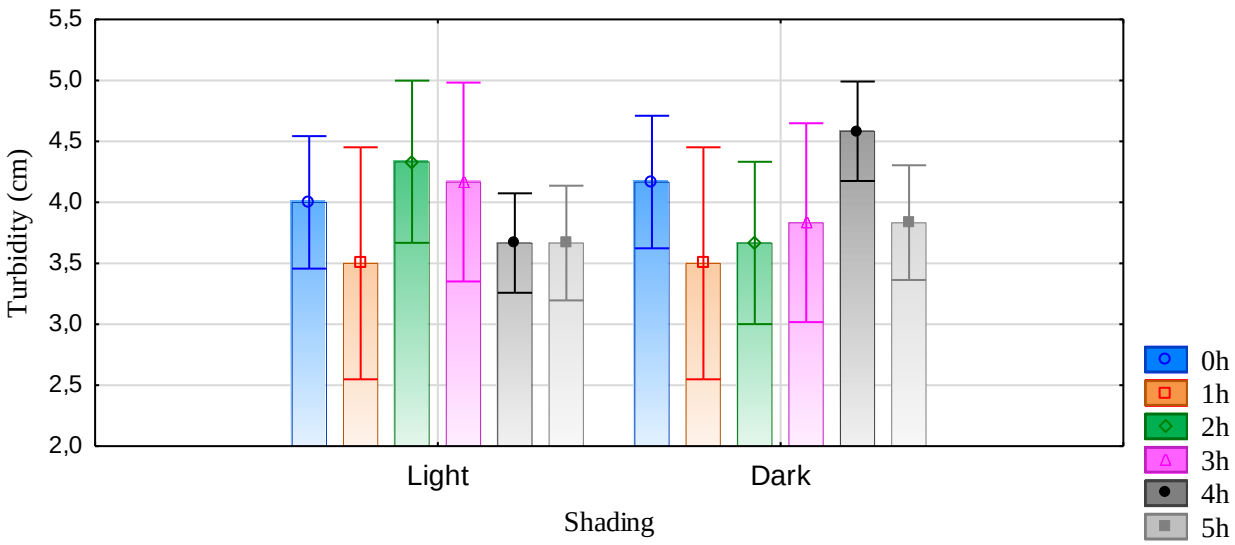

Figure 35: The mean (± 95 % confidence interval) turbidity (cm) between light and dark treatments over five hours (Multifactor Repeated Measures ANOVA: $F_{(6,3)}$=9.127; p=0.049).

3.4.2 Removal of algae by *Oreochromis mossambicus*

3.4.2.1 Algal removal

Dry total suspended solids

There was no interaction in algae concentration amongst all the factors (fish/no fish and pH moderation/no pH moderation) over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=1.38; p=0.25; Figure 36). There was a significant difference in algae concentration between fish-containing tanks and fish-absent tanks (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=2.35; p=0.05; Figure 37). No significant difference in algae concentration was observed between pH moderated and un-moderated tanks (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.25; p=0.94; Table 13).

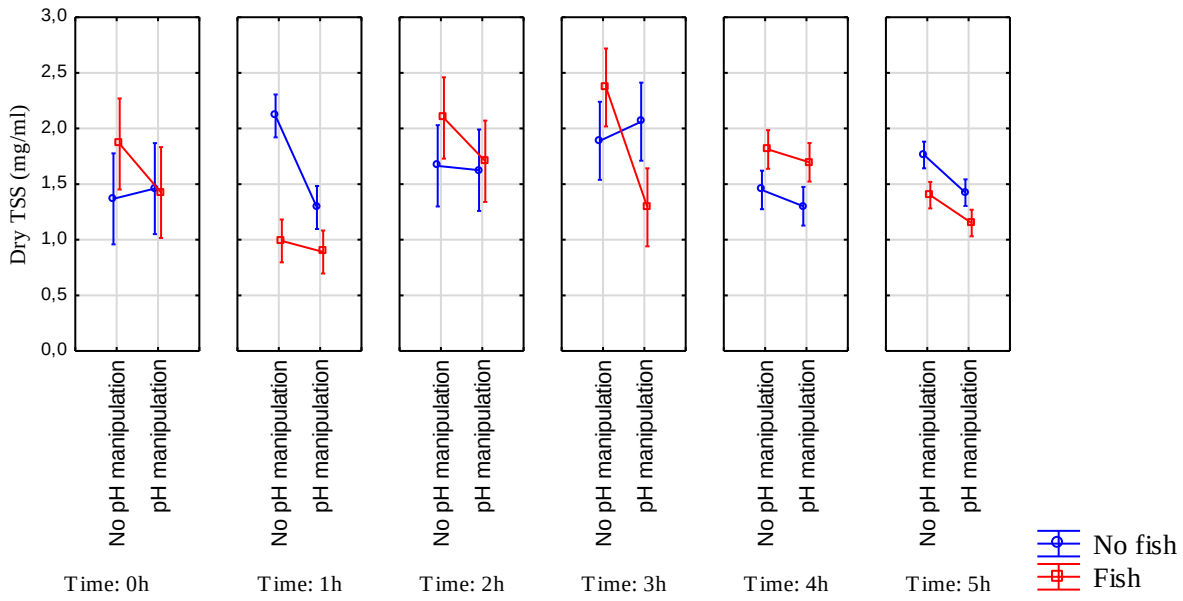

Figure 36: The mean (± standard error) algae concentration (measured as dry total suspended solids, TSS in mg/ml; particle size >8.0 µm) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=1.38; p=0.25).

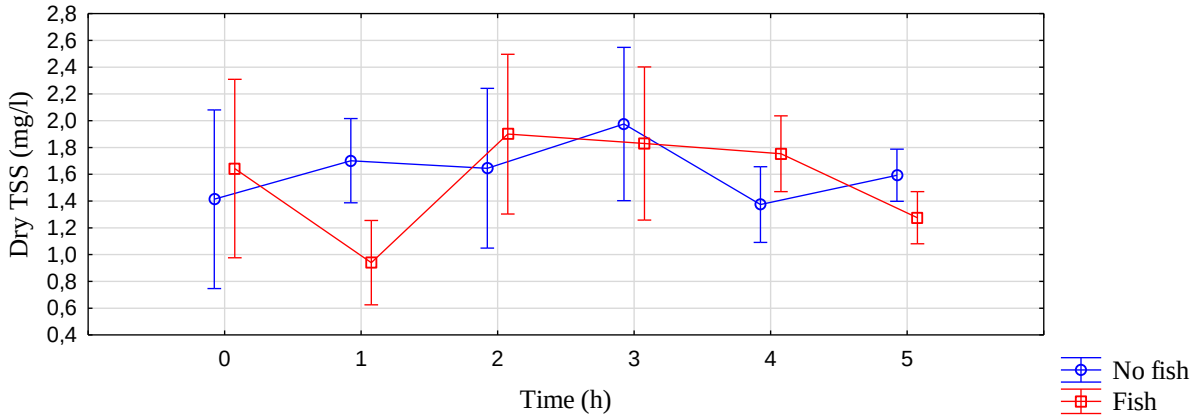

Figure 37: The mean (± 95 % confidence interval) algae concentration (measured as dry total suspended solids, TSS in mg/ml; particle size >8.0 µm) in tanks of post-high-rate-algal-pond effluent, that either include fish or no fish over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=2.35; p=0.05).

Table 13: The mean (± standard error) algae concentration (measured as dry total suspended solids, TSS in mg/ml; particle size >8 µm) in the absence and presence of pH moderation over six hours (Multifactor Repeated Measures ANOVA: $F_{(5, 40)}$=0.25; p=0.94).

	Time (h)						F (5, 40)	P value
	0	**1**	**2**	**3**	**4**	**5**		
pH moderation	1.44±0.29	1.09±0.14	1.67±0.26	1.68±0.25	1.50±0.12	1.29±0.09	0.25	0.94
No pH moderation	1.61±0.29	1.55±0.14	1.88±0.26	2.13±0.25	1.63±0.12	1.58±0.09		

Turbidity

There was no interaction in turbidity amongst all the factors (fish/no fish and pH manipulation/no pH manipulation) over five hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.048; p=0.986; Figure 38). A significant difference in turbidity was observed between tanks with fish and tanks without fish over five hours. The turbidity increased with time for treatments both with and without fish; however, this increase in turbidity was less when fish were present in the tank (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=3.401; p=0.034; Figure 39). There was a significant difference in turbidity between pH manipulated and unmanipulated tanks. Turbidity was higher in pH manipulated tanks (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=6.850; p=0.031; Figure 40).

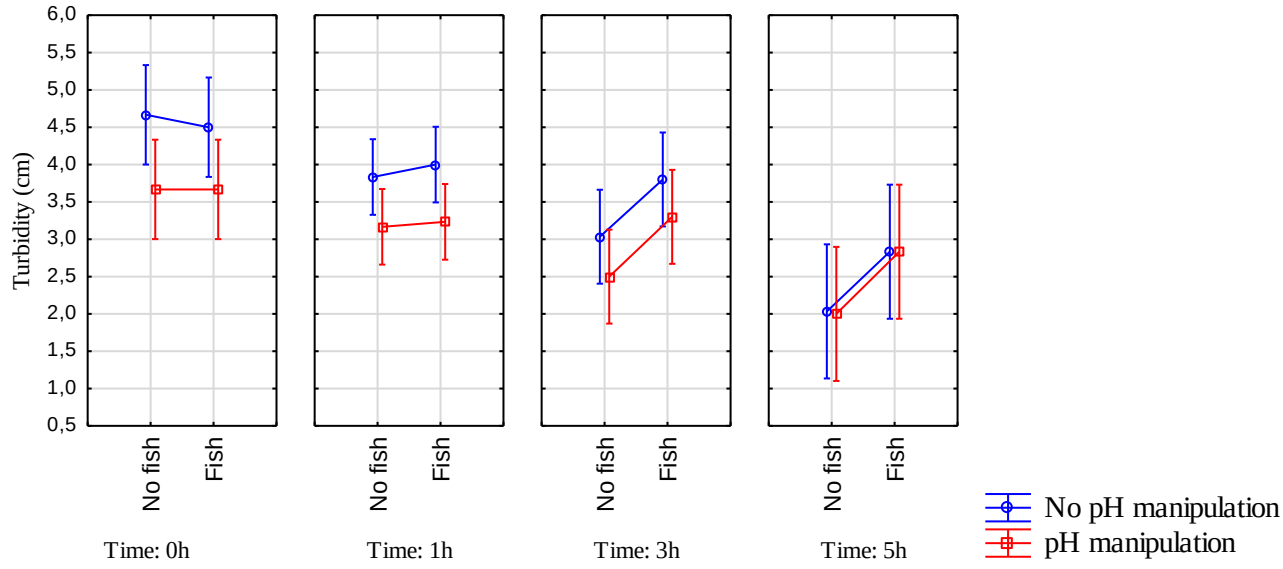

Figure 38: The mean (± standard error) turbidity (cm) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish over five hours indicating a lack of a significant difference amongst the factors (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=0.048; p=0.986).

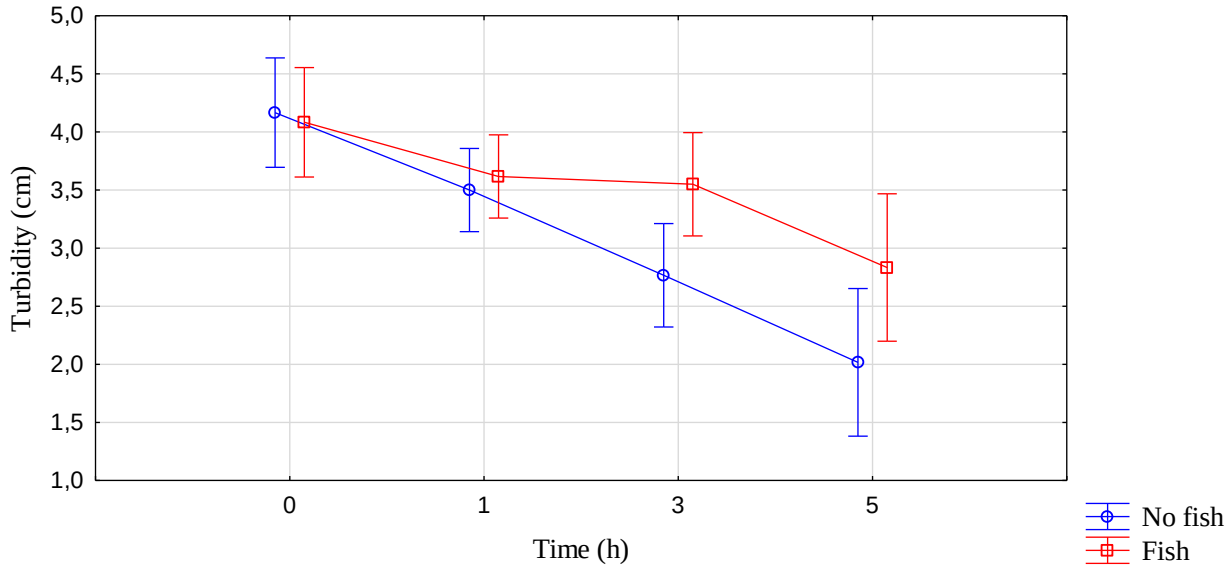

Figure 39: The mean (± 95 % confidence interval) turbidity (cm) in tanks of post-high-rate-algal-pond effluent, that either include fish or no fish over five hours (Multifactor Repeated Measures ANOVA: $F_{(3,24)}$=3.401; p=0.034).

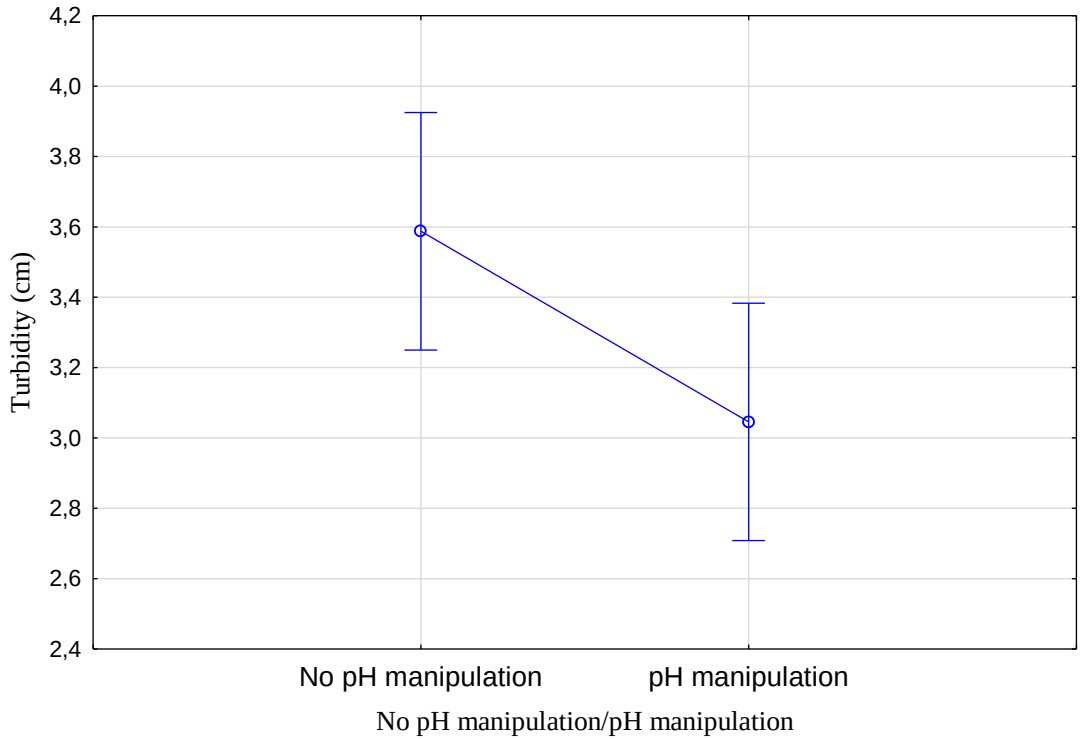

Figure 40: The mean (± 95 % confidence interval) turbidity (cm) in tanks of post-high-rate-algal-pond effluent that either include pH manipulation or no pH manipulation (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=6.850; p=0.031).

Fish gut weights and algae presence

The fish gut weights (g) were not significantly different amongst the treatments (Single Factor ANOVA: $F_{(5,78)}$=0.277; p=0.924; Table 14). Algae were present in *Oreochromis mossambicus* guts (Figure 41).

Table 14: The mean (± standard error) fish gut weights (g) from tanks containing fish at the end of the experiment (Single Factor ANOVA: $F_{(5,78)}$=0.277; p=0.924).

Groups	n-value	Sum (g)	Mean (g)	Standard error
No-pH manipulation	14	3.38	0.24	0.02
No-pH manipulation	14	3.26	0.23	0.02
No-pH manipulation	14	3.12	0.22	0.01
pH manipulation	14	3.75	0.27	0.02
pH manipulation	14	3.60	0.26	0.01
pH manipulation	14	3.20	0.23	0.01

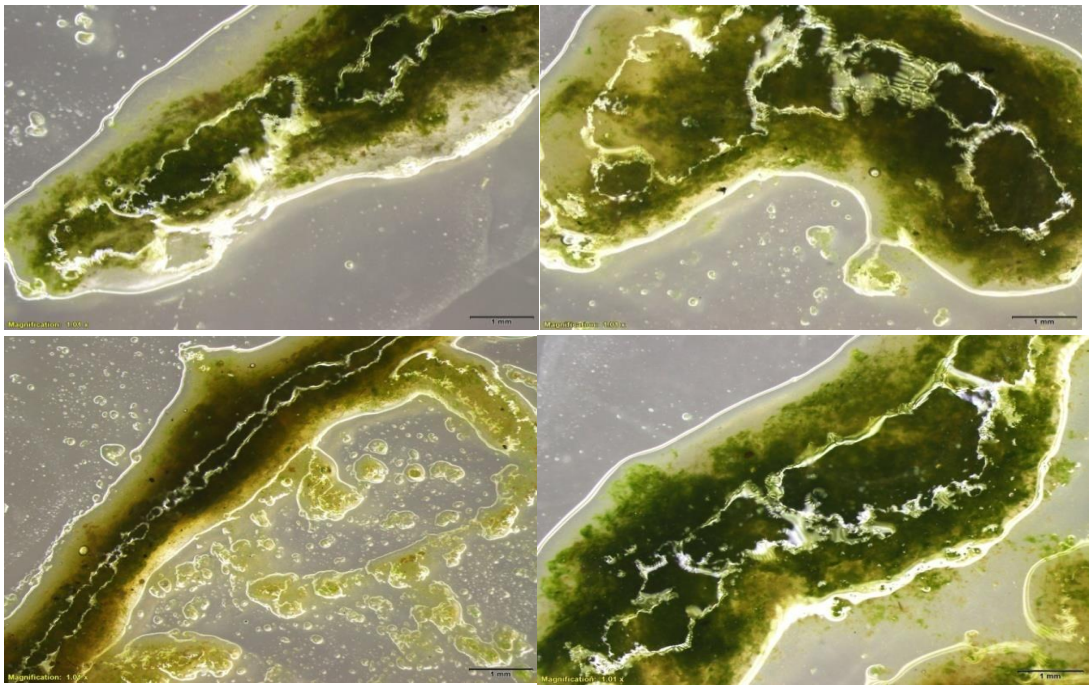

Figure 41: Algae in the guts of *Oreochromis mossambicus.*

3.4.2.2 Water Quality Parameters

pH

There was no interaction in pH amongst all the factors (fish/no fish and pH manipulation/no pH manipulation) over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.392; p=0.851; Figure 42). There was a significant difference in pH between pH manipulated and unmanipulated tanks over five hours. pH was lower in the manipulated tanks and a similar trend was observed in both treatments (pH manipulation and no pH manipulation) whereby pH decreased in the first few hours before increasing again towards the end of the experiment (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=392.95; p<0.001; Figure 43). There was no significant difference in pH between tanks which included fish or no fish (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.239; p=0.943; Appendix 3).

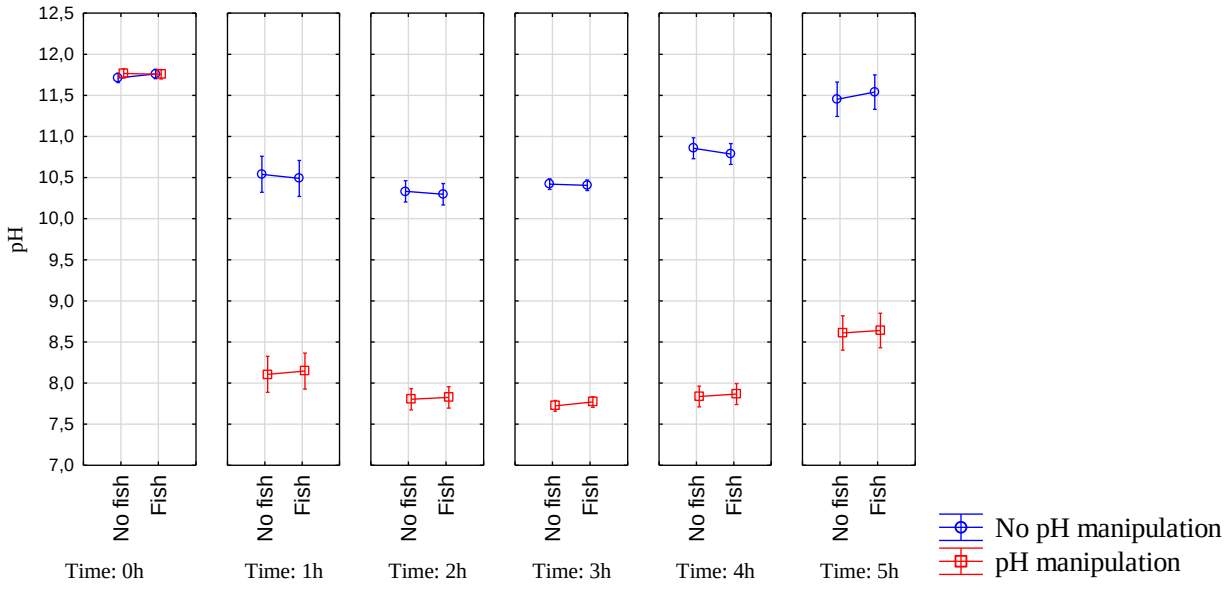

Figure 42: The mean (± standard error) pH in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish over five hours highlighting the lack of a significant difference amongst the factors (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.392; p=0.851).

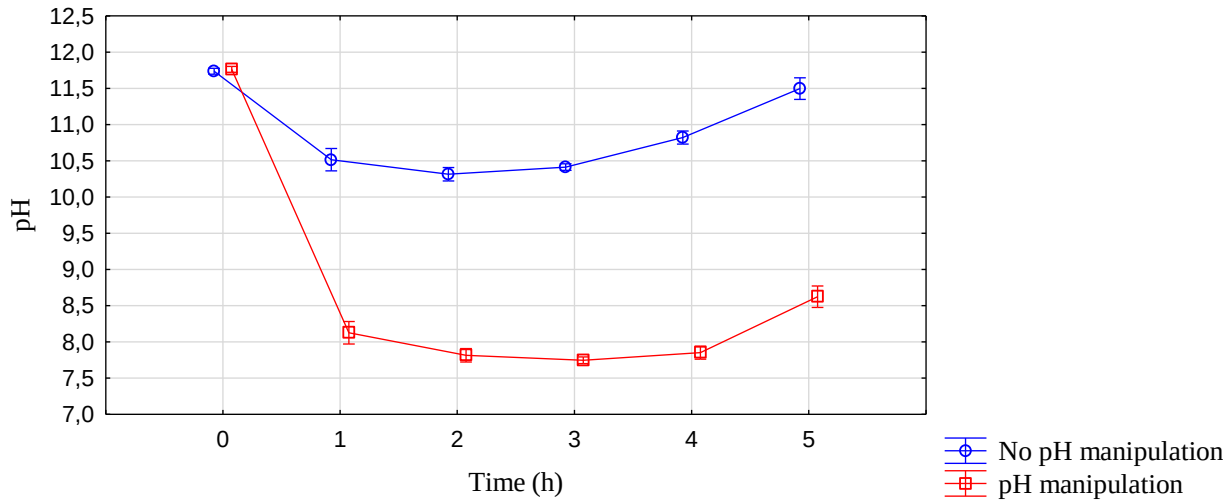

Figure 43: The mean (± 95 % confidence interval) pH in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=392.95; p<0.001).

Carbon dioxide

No significant interaction was observed in CO_2 (mg/l) amongst all the factors (fish/no fish and pH manipulation/no pH manipulation) over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.695; p=0.630; Figure 44). There was an interaction in CO_2 between pH manipulated and unmanipulated tanks. Carbon dioxide was similar at hour zero (0 mg/l), then it increased sharply at hour-one (90 mg/l) in pH manipulated tanks and remained relatively the same till the fifth hour whereas no noticeable increase was observed in unmanipulated tanks (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=7391.1; p<0.001; Figure 45). There was no significant difference in CO_2 between tanks which included fish or no fish (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.777; p=0.572; Appendix 3).

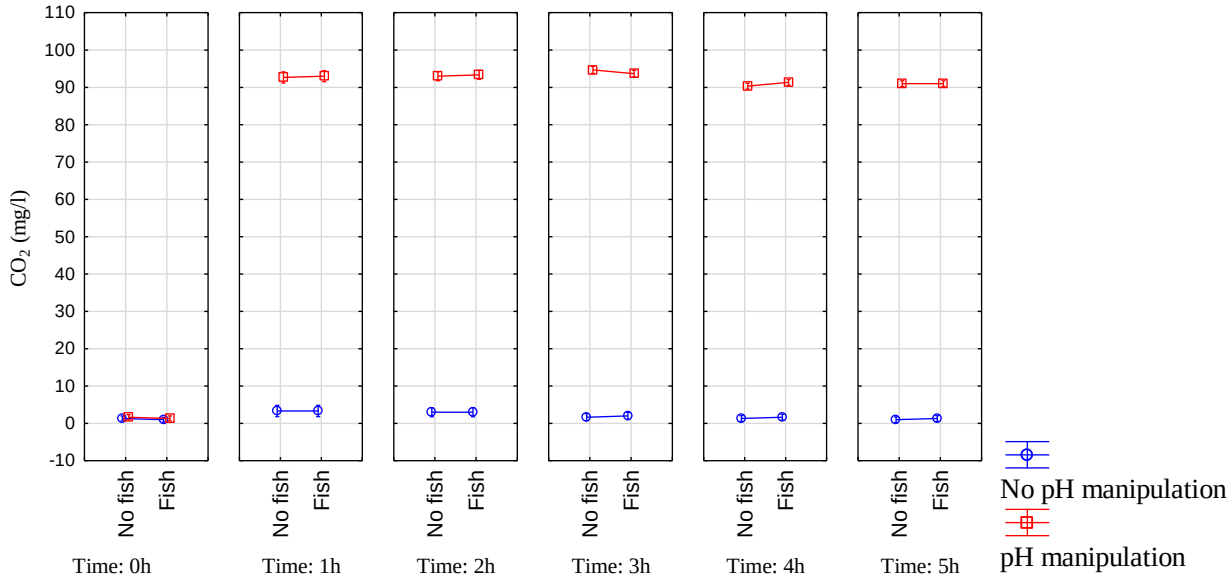

Figure 44: The mean (± standard error) carbon dioxide(mg/l) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish over five hours showing the lack of a significant difference amongst the factors (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.695; p=0.630).

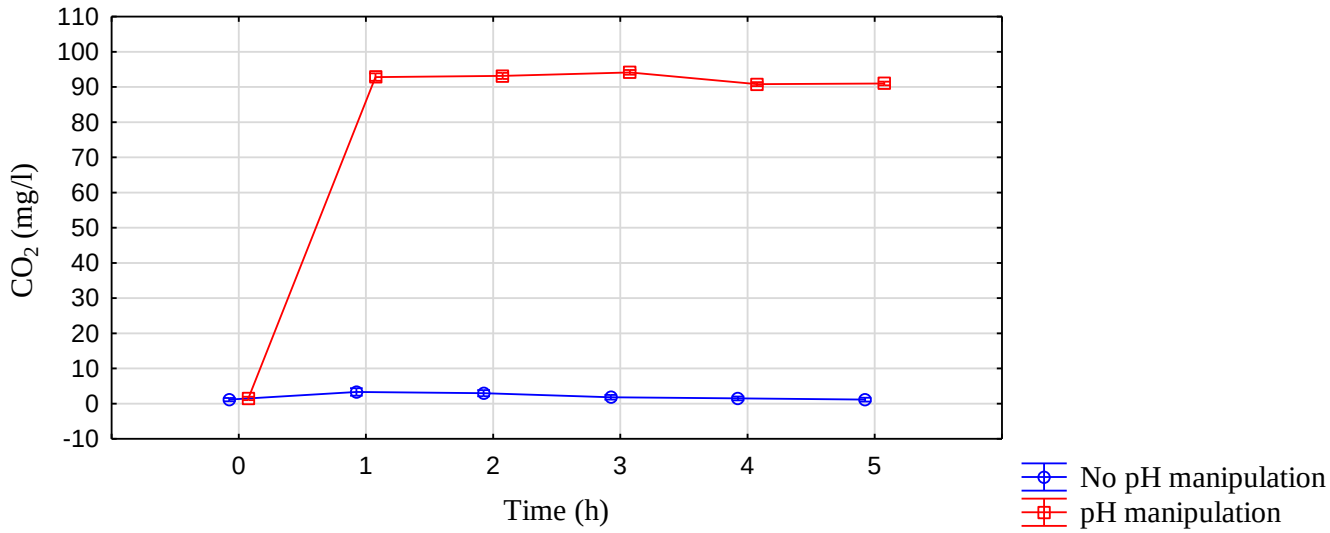

Figure 45: The mean (± 95 % confidence interval) carbon dioxide (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=7391.1; p<0.001).

Dissolved Oxygen

There was no interaction in dissolved oxygen amongst all the factors (fish/no fish and pH manipulation/no pH manipulation) over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.599; p=0.701; Figure 46). However, dissolved oxygen differed significantly between pH manipulated and unmanipulated tanks over five hours. Dissolved oxygen was higher in the unmanipulated tanks and generally had an upward trend throughout the experimentation period, whereas the opposite was observed in the pH manipulated tanks (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=74.965; p<0.001; Figure 47). There was no significant difference in dissolved oxygen between tanks which included fish or no fish (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.262; p=0.931; Appendix 3).

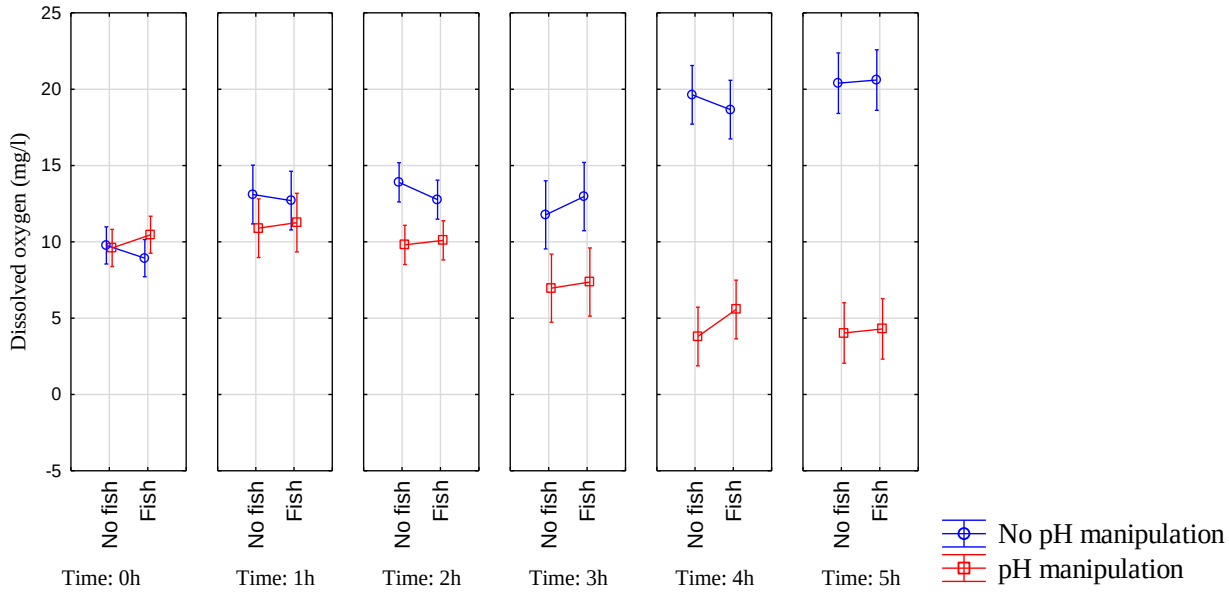

Figure 46: The mean (± standard error) dissolved oxygen (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish over five hours highlighting the lack of a significant difference amongst the factors (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.599; p=0.701).

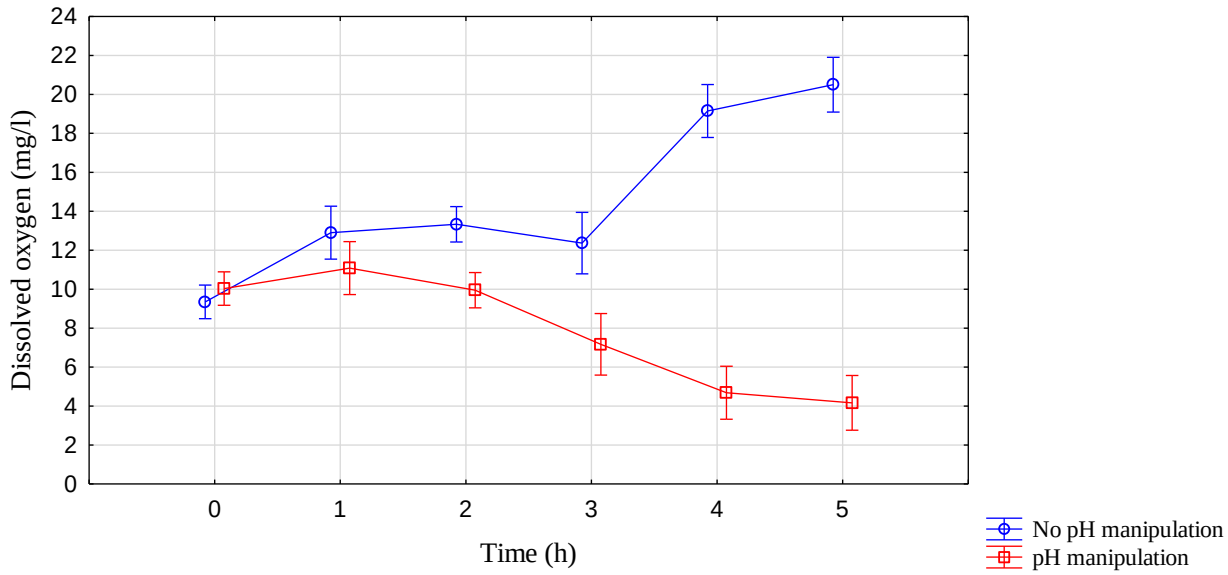

Figure 47: The mean (± 95 % confidence interval) dissolved oxygen (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=74.965; p<0.001).

Temperature

There was no interaction in temperature amongst all the factors (fish/no fish and pH manipulation/no pH manipulation) over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.470; p=0.797; Figure 48). Temperature in the unmanipulated tanks increased sharply after the start of the experiment, where-after it levelled off; whereas it increased progressively over the course of the experiment in the pH manipulated tanks (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=6.244; p<0.001; Figure 49). There was no significant difference in temperature between tanks which included fish or no fish (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=1.016; p=0.421; Appendix 3).

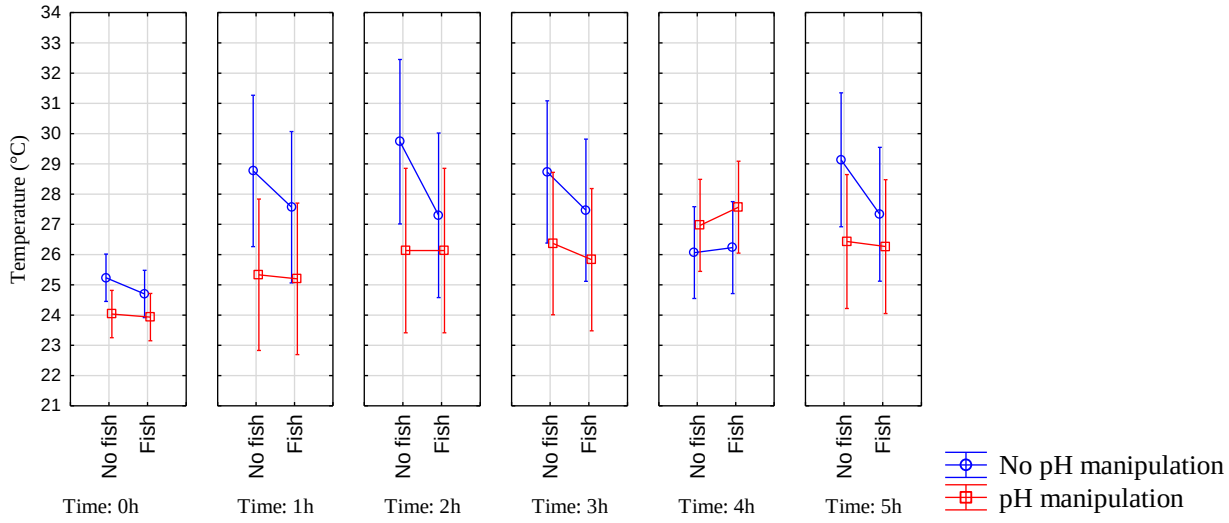

Figure 48: The mean (± standard error) temperature (°C) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish over five hours outlining the lack of a significant difference amongst the factors (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.470; p=0.797).

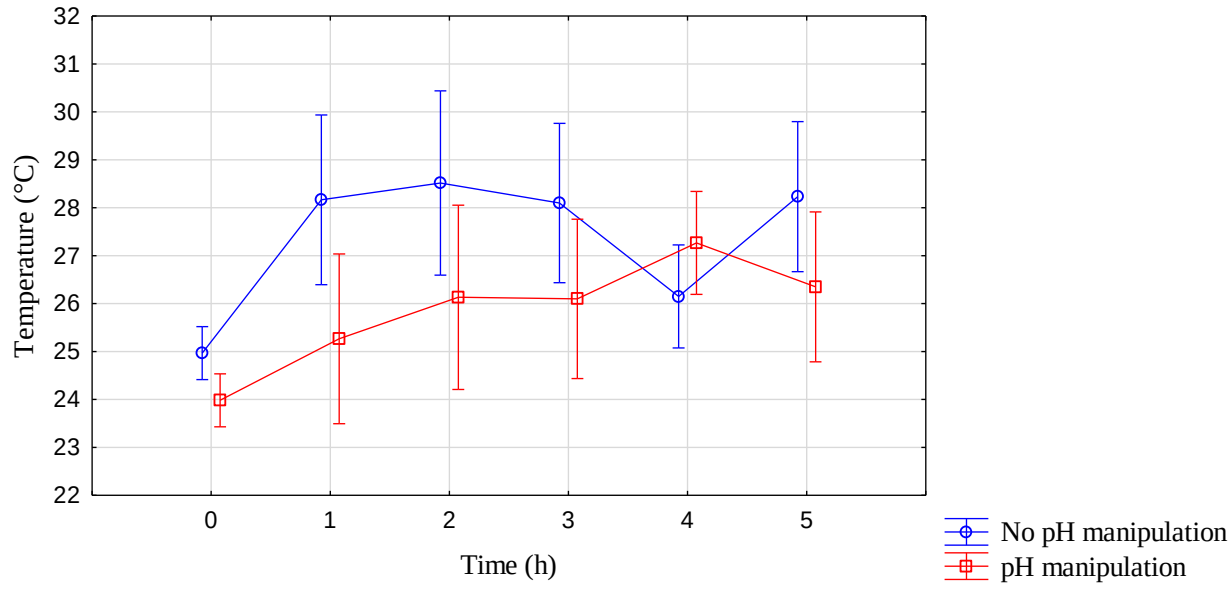

Figure 49: The mean (± 95 % confidence interval) temperature (°C) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=6.244; p<0.001).

Ammonia

No significant difference in ammonia was observed amongst fish/no fish and pH manipulation/no pH manipulation over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=1.359; p=0.260; Figure 50). There was a significant interaction in ammonia amongst factors (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=8.764; p=0.018; Figure 50); when there were fish in the tank, pH manipulation had no effect on ammonia whereas pH manipulated treatments had a significantly higher ammonia concentration when fish were absent (Figure 51). No significant difference in ammonia was observed between pH manipulated and unmanipulated tanks over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.384; p=0.857; Appendix 4). There was no significant difference in ammonia between tanks which included fish or no fish over five hours (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=0.660; p=0.656; Appendix 4).

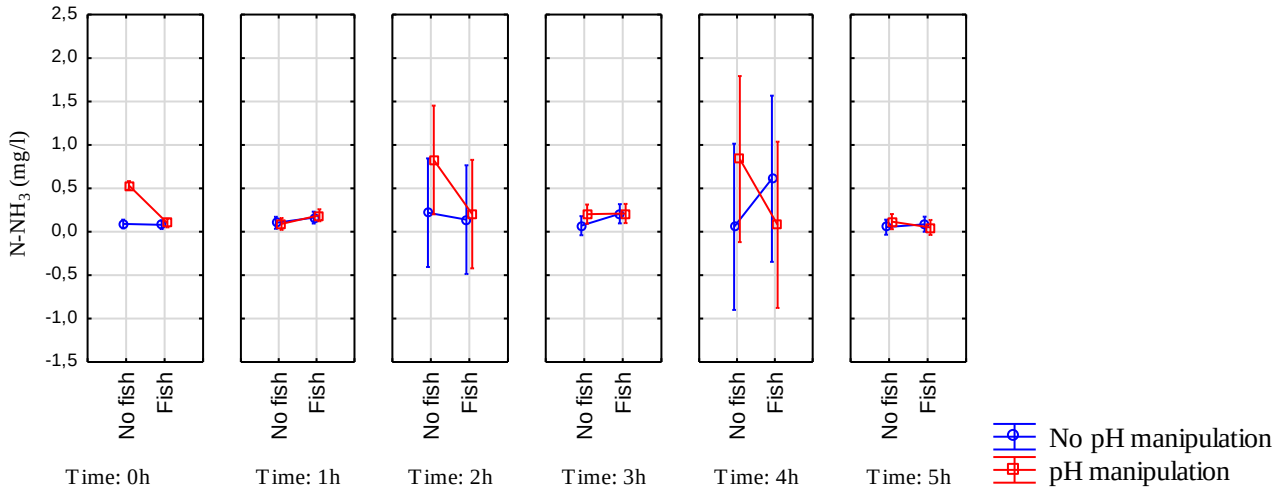

Figure 50: The mean (± standard error) ammonia (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish over five hours highlighting the lack of a significant difference amongst the factors (Multifactor Repeated Measures ANOVA: $F_{(5,40)}$=1.359; p=0.260).

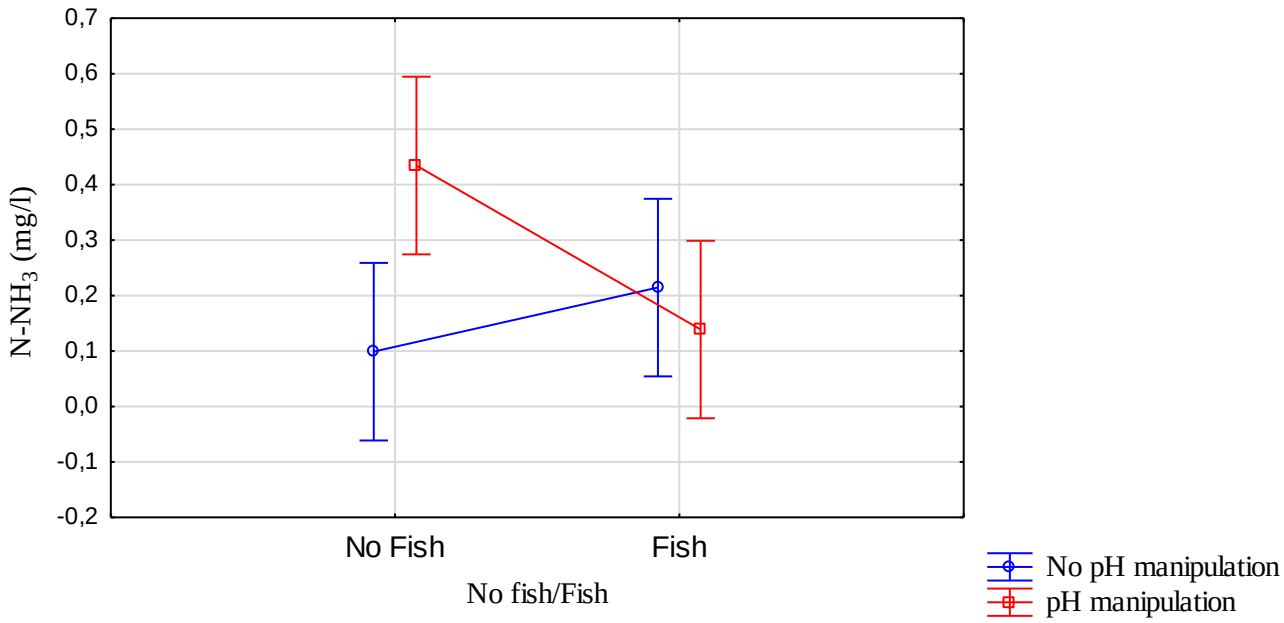

Figure 51: The mean (± 95 % confidence interval) ammonia (mg/l) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=8.764; p=0.018).

Chemical oxygen demand

There was no significant interaction in chemical oxygen demand amongst all the factors (fish/no fish and pH manipulation/no pH manipulation) over five hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.002; p=0.969; Figure 52). No significant difference in chemical oxygen demand was observed between pH manipulated and unmanipulated tanks over five hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.860; p=0.381; Appendix 4). There was no significant difference in chemical oxygen demand between tanks which included fish or no fish over five hours (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.717; p=0.422; Appendix 4).

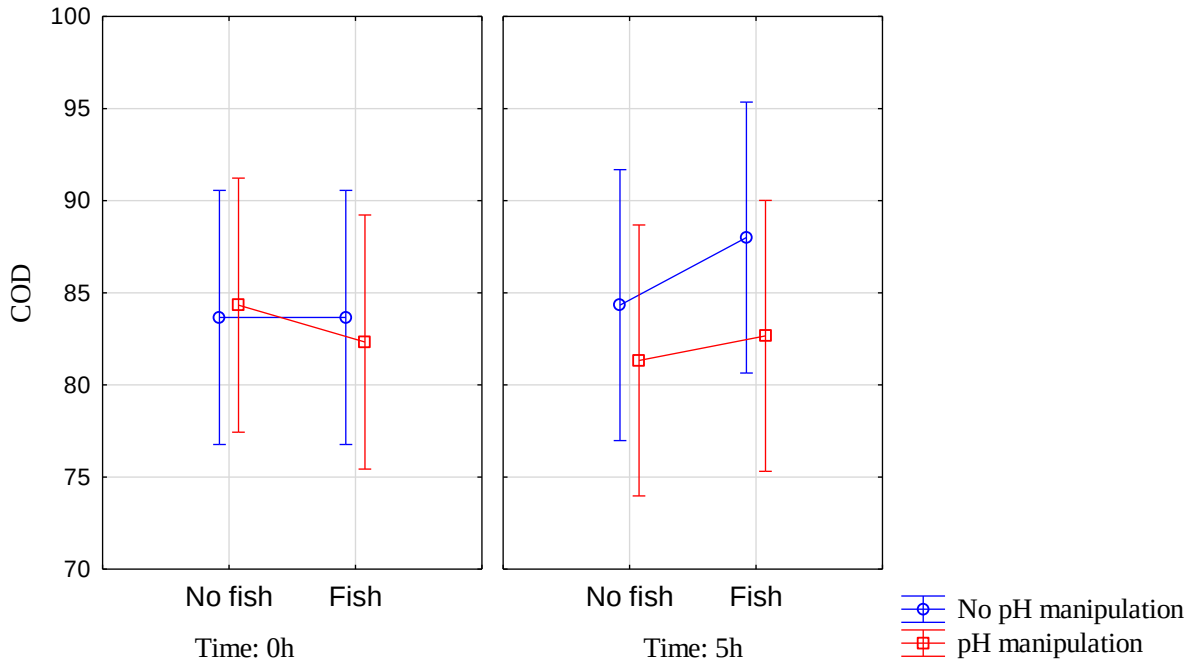

Figure 52: The mean (± standard error) chemical oxygen demand (COD) in tanks of post-high-rate-algal-pond effluent, that either include pH manipulation or no pH manipulation and fish or no fish at the beginning and at the end of the experiment (Multifactor Repeated Measures ANOVA: $F_{(1,8)}$=0.002; p=0.969).

3.4.2.3 Relationships between turbidity and several water quality parameters (pH and CO_2).

There was a significant relationship between turbidity and pH (y=7.689+0.713*x: R^2=0.157; $F_{(1,46)}$=8.540; p=0.005; Figure 53). A significant relationship was also observed between turbidity and CO_2 (y=107.185-21.501*x: R^2=0.178; $F_{(1,46)}$=9.952; p=0.003; Figure 54).

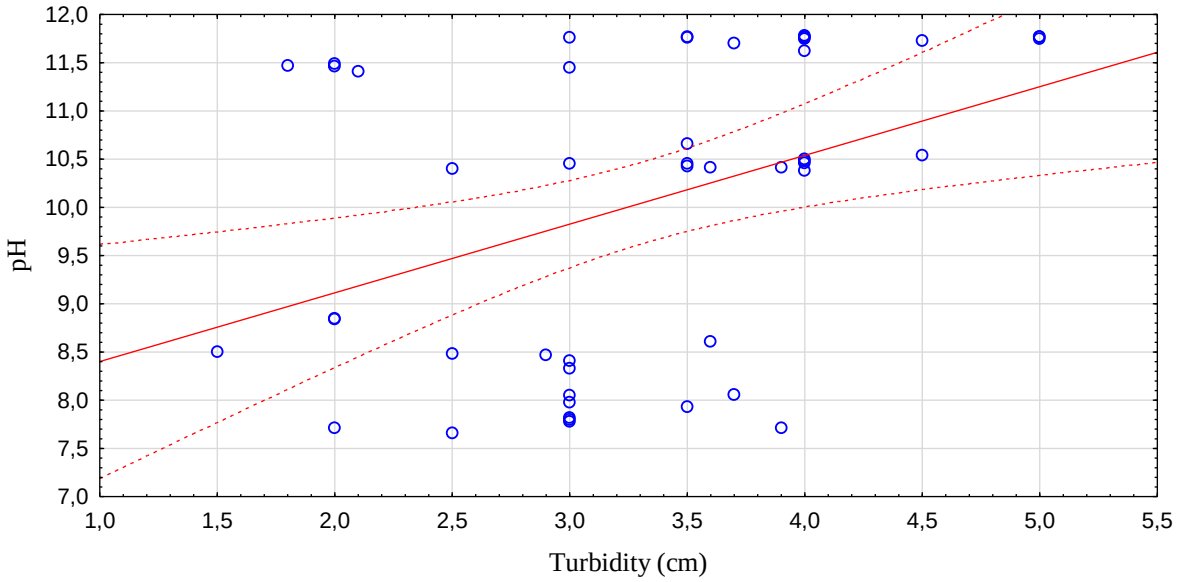

Figure 53: The relationship between turbidity (cm) and pH (y=7.689+0.713*x: R^2=0.157; $F_{(1,46)}$=8.540; p=0.005).

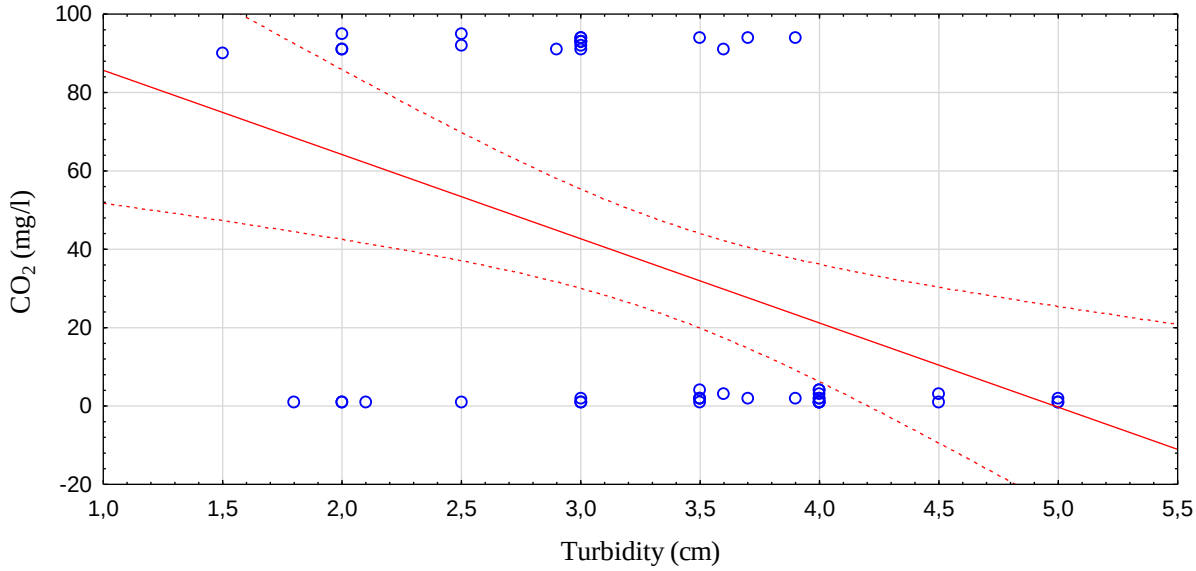

Figure 54: The relationship between turbidity (cm) and CO_2 (mg/l) (y=107.185-21.501*x: R^2=0.178; $F_{(1,46)}$=9.952; p=0.003).

Non-significant relationships between turbidity and water quality parameters (dissolved oxygen, ammonia, temperature, and chemical oxygen demand).

There was no relationship between turbidity and all the measured water quality parameters (Regression $p>0.05$; Table 15).

Table 15: The relationship between turbidity and water quality parameters (dissolved oxygen, ammonia, temperature, and chemical oxygen demand) in post-high-rate-algal-pond measured over six hours. These data are the combined means of all treatments as there were no slopes (when a regression was plotted) to signify a relation with turbidity (Regression: $p>0.05$).

	F	R^2	p-value
Turbidity vs. Dissolved Oxygen	$F_{(1,46)}=0.016$	0.0003	0.900
Turbidity vs. $N\text{-}NH_3$	$F_{(1,46)}=1.123$	0.024	0.295
Turbidity vs. Temperature	$F_{(1,46)}=1.860$	0.039	0.179
Turbidity vs. COD	$F_{(1,22)}=0.091$	0.004	0.766

Non-significant relationships between total suspended solids and water quality parameters (dissolved oxygen, carbon dioxide, ammonia, temperature, turbidity, pH and chemical oxygen demand).

There was no relationship between algae concentration total suspended solids (TSS) and all the measured water quality parameters (Regression $p>0.05$; Table 16).

Table 16: The relationship between dry total suspended solids (TSS in mg/ml; particle size >8.0 µm) and water quality parameters (dissolved oxygen, carbon dioxide, ammonia, temperature, turbidity, pH and chemical oxygen demand) in post-high-rate-algal-pond measured over six hours. These data are the combined means of all treatments as there were no slopes to signify a relation with dry total suspended solids (TSS in mg/ml; particle size >8 um) (Regression: $p>0.05$).

	F	R^2	p-value
Dry TSS vs. Dissolved Oxygen	$F_{(1,70)}=0.624$	0.009	0.432
Dry TSS vs. CO_2	$F_{(1,70)}=3.513$	0.047	0.065
Dry TSS vs. N-NH_3	$F_{(1,70)}=0.009$	0.0001	0.923
Dry TSS vs. Temperature	$F_{(1,70)}=0.979$	0.014	0.326
Dry TSS vs. Turbidity	$F_{(1,46)}=0.015$	0.0003	0.902
Dry TSS vs. pH	$F_{(1,70)}=1.107$	0.016	0.296
Dry TSS vs. COD	$F_{(1,22)}=0.970$	0.042	0.335

3.5 Discussion

In the previous chapter flocculation was applied as a means of assisting algae removal; this however did not produce the desired results. In this chapter, an alternative fish species was used and chitosan was not applied; however due to *Oreochromis mossambicus* being less hardy than *Clarias gariepinus*, the brewery wastewater environment had to be manipulated. pH manipulation through several means had to be explored in a pre-study before algae filtration by *Oreochromis mossambicus* could be investigated.

3.5.1 pH reduction through carbon dioxide sparging

Carbon dioxide sparging reduces water pH (Al-Mutazand Al-Ghunaimi, 2001). In the present study it was observed that at hour zero the water pH was relatively similar between CO_2 bubbled and treatments where no CO_2 was bubbled in. The pH levels dropped noticeably and became neutral after the introduction of CO_2 through sparging. The same effect of CO_2 on water albeit sea water was reported by Brennan (2017). Carbon dioxide dissolved in water reacts to form carbonic acid. Through loss of a hydrogen ion, the carbonic acid becomes bicarbonate. After losing another hydrogen ion, the carbonic acid becomes carbonate (Wurts and Durborow, 1992). The released protons are the ones responsible for lowering the water pH (Brennan, 2017). It appears that irrespective of the source, CO_2 has a common effect on water which is the reduction of pH.

Closed systems with organic matter tend to have more CO_2 than open ones. Since the dark tanks were closed not much CO_2 could escape through the atmosphere and there was little if any photosynthetic activity in dark tanks hence the CO_2 could not be used up in the light driven process (Lambers *et al*., 2008).

The natural environment (air and direct sunlight) is generally known as the main source of dissolved oxygen in water bodies. Consequently, light treatments had more dissolved oxygen than dark treatments in the current study. Atmospheric oxygen may have efficiently been absorbed by the water surface (He *et al*., 2003), and due to photosynthesis by the algae more oxygen may have been produced as a by-product (Levine, 2011). The uncovered (light) treatments were exposed to various oxygen sources hence they had higher oxygen content.

Interpreting the response of turbidity to light and dark treatments is problematic. The dark treatments were more turbid than the light treatments. One of the possible explanations for this would be more CO_2 being trapped in the covered tanks and this allowing the algae to multiply at an increased rate. According to Strain (2016), green pigmented flora photosynthesize and produce green material at a rate that far exceeds that observed in the 1980's and 1990's and Strain (2016), further cited excess atmospheric CO_2 as the driver of this process. The challenge with this theory when bringing it to the current study is that in the dark tanks there was no sunlight for the algae to photosynthesize efficiently. With that stated, the light independent stage of the photosynthesis process could have taken place (Raines, 2003). An alternative reason for the higher turbidity observed in dark treatments could be the sample containing more fish waste material and/or suspended algae.

3.5.2 The removal of algae by *Oreochromis mossambicus*

3.5.2.1 Algae removal

Tilapias are generalist feeders that can filter feed and utilise microalgae as a food source (Skelton 1993). After the first hour of the experiment dry total suspended solids as an indication of algae biomass, was lower in the fish-containing tanks compared to the fish-absent tanks and this trend was also seen in the fifth hour of experimentation. Turbidity readings during the third and fifth hour of the experiment indicated the tanks with fish as having a lower algae

concentration compared to the fish-absent tanks (algae concentration was inferred from turbidity readings). Studies by Caulton (1982), Dempster *et al*. (1995), McDonald (1985a, b), Robinson *et al*. (1990, 1995) and Northcott *et al*. (1991) reported *O. mossambicus* to filter feed on algae. The difference in turbidity in the present study could have been due to the filter feeding activity of the fish and this is further supported by the presence of algae in fish intestines at the end of the study.

Tilapias filter feed similarly at both alkaline and neutral pH levels. There was no significant difference in dry total suspended solids between pH moderated and non pH moderated tanks. Turbidity was also similar, with averages between both treatments ranging from just above three centimetres for pH manipulated tanks to less than 3.6 cm for non-pH manipulated tanks. The optimal pH for *Oreochromis niloticus* survival is pH 7-8 (El-Sherif and El-Feky, 2009). It would be expected that at a similar pH range *O. mossambicus* would perform the same since these fishes are related; this however was not so and this was probably because of the high carbon dioxide content that was used to lower the pH.

It appears that algae size does play a role in algae filtration by tilapia. In a study conducted by Turker *et al*. (2003), it was easier for *O. niloticus* to filter feed on large algae particles. It would have been interesting to see the effect of flocculation in algae filtration by tilapia; however, since the effect of this process was more or less negligible in the *C. gariepinus* study due to a relatively high pH (Chapter 2), its application was not tested here.

Algae filtration by fish also influences the nature of the algae community. According to Xie and Yang (2000), and Lu *et al*. (2002), in lakes filter feeding fish play a major role in influencing the size of algae. The current study and the aforementioned studies are similar in that algae removal by fish remains the central theme and therefore the same in terms of algae community structure being influence by fish would be expected.

Some plant cell walls tend to interfere with the digestion of the cells by fish. Work on *Oreochromis niloticus* revealed that this fish is capable of creating an extremely acidic environment within the digestive system (pH 1) by producing gastric acid, and this assists in the breakdown of these cell walls (Fryer and Iles, 1972; Moriaty and Moriaty, 1973a, b). In the present study, the *O. mossambicus* had an average length of 45.96 mm. According to Lu *et al*. (2002), algae assimilation is directly proportional to fish gut length. Therefore it would seem that in *O. niloticus* the older the fish (from 15 to 34 mm) the more it would filter feed on algae. Since these fish are closely related it appears that the fish used in the current study were at a reasonable age for algae assimilation and well equipped to digest the algae.

pH moderation was not a major factor in tilapia filter feeding on algae. There were no significant differences in fish gut weights. The fish in tanks without carbon dioxide sparging were immersed in an environment that was too alkaline for the fish to function normally. In carbon dioxide sparged tanks, the low pH effect was countered by increased carbon dioxide concentration. The carbon dioxide used to lower the pH displaced dissolved oxygen within the water column. Therefore it would be expected that other fish species normal functioning, including feeding, be disrupted by pH moderation through carbon dioxide sparging. In solving the high pH challenge an unintended effect of dissolved oxygen depletion was observed and this resulted in fish in both treatments (pH moderated and unmoderated treatments) to feed similarly as they were both stressed.

Where algae are the predominant food source, *O. mossambicus* will filter feed on them. Algae were present in all the fish guts. In a study by Wakil *et al.* (2014), *O. niloticus* stomach content comprised of algae such as *Chlorella*, *Volvox*, *Scenedesmus*, *Pediastrum*, and *Spirogyra* and these species accounted for 66 % of the gut content. The algae reduced in algal ponds eventually ends up in fish guts, where its nutrients are then assimilated; further evidence that *O. mossambicus* do remove microalgae from brewery effluent ponds.

3.5.2.2 Water Quality Parameters

- pH

Fish presence/absence does not affect the brewery wastewater pH. Carbon dioxide sparging is an effective method of reducing water pH (Gomà *et al*., 2010). There was a significant difference in pH between pH manipulated and non-manipulated tanks. pH manipulated tanks were almost neutral whereas the non-manipulated were alkaline.

pH is credited with being "one of the key drivers of biological responses" (Wu *et al*., 2017). In *O. niloticus* for instance at pH six the average haemoglobin concentration in the blood decreases (El-Sherif and El-Feky, 2009). This response could also ultimately lead to impaired fish feeding. Both treatments followed a similar trend in that, from hour zero both treatments showed a decline in pH up until hour five at which time pH peaked again. pH is inversely proportional with temperature in water i.e. when the temperature increases the pH decreases (Baron *et al*., 2006). From the time the experiment started the temperatures steadily increased only to decrease later on hence the lower pH values when temperatures were the highest.

- Carbon dioxide

Carbon dioxide is the environmental parameter that decreased brewery wastewater pH levels (Brennan, 2017). However, CO_2 concentration was not influenced by fish presence/absence. When sparging was introduced, CO_2 increased from a mean of approximately one milligram per litre (hour zero) to lower than ninety five milligrams per litre (second to the fifth hour of the experiment). Therefore a significant difference in CO_2 was observed between pH manipulated and non pH manipulated tanks. Carbon dioxide sparging produced the desired effect, which was a reduction in pH.

- Dissolved oxygen

The absence or presence of fish does not significantly affect brewery wastewater dissolved oxygen; whereas carbon dioxide sparging reduces dissolved oxygen concentration within a water column. During the first hour of the experiment pH was similar between both treatments. In pH manipulated tanks dissolved oxygen concentration steadily decreased from the first to the fifth hour of the experiment whereas this gas gradually increased during the same time frame in tanks in which CO_2 was not bubbled in. Under similar atmospheric pressure, carbon dioxide diffuses 0.86 times faster into water compared to oxygen due to the former being highly soluble in water (Schmidt-Nielsen, 1997). Both carbon dioxide and oxygen were sparged into pH manipulated tanks, creating similar pressures for both gases. Carbon dioxide increasing whilst dissolved oxygen decreased could only be explained by the former displacing the latter which was in continuous use by the fish for respiration (Mallya, 2007).

- Temperature

Carbon dioxide bubbling slightly lowers water temperature. A significant difference was observed between treatments that included CO_2 bubbling (lower temperatures) and treatments without CO_2 input (higher temperatures). According to Turker *et al*. (2003), temperature of the water environment affects the filter feeding rates of *O. niloticus*. In a study conducted by Turker *et al*. (2000), a temperature increase from 23.8 to 31.2 ^{o}C caused *O. niloticus* algae filtration rates to increase considerably. *Oreochromis niloticus* is related to *O. mossambicus* and consequently these fish are similar in certain aspects; for instance since they are both tropical fish and their feeding rates are directly proportional to water temperature (Popma and Masser, 1999; FAO, 2018). Since the temperature recorded in the current study had a relatively narrow range (24.317±0.240 to 26.9±0.466 °C) not much variation in filtration rates would be expected to be caused by this water parameter. Water temperatures were merely

recorded and not manipulated in this experiment. The differences in temperature (over time) could be attributed to the differing times of the day and CO_2 (amongst treatments) sparging (Brennan, 2017). Carbon dioxide lowering the water temperature was probably just a by-product of lowering the pH through sparging.

- Ammonia and temperature in relation to the external environment

The influence of the greenhouse environment caused certain water quality parameters to have a narrow range which was independent of the external environment. In the present study, fish-containing tanks had an ammonia range of 0.068±0.027 to 0.345±0.293 mg/l. García-Trejo (2016) reported an ammonia range of 0.00 to 0.90 mg/l which is a little bit different due to several factors. *Oreochromis niloticus* were used instead of *O. mossambicus*, the fish were fed a commercial diet instead of being on strict microalgae diet and data collection was done over a 60 day period rather than a six hour period. Nevertheless, García-Trejo (2016) conducted the study in a greenhouse and on a related fish species. Ammonia nitrogen in the aquatic environment can range from <0.2 mg/l to >10 mg/l depending on water contamination (DAFF, 1996). In fish-containing-tanks there was a temperature range of 24.317±0.240 to 26.9±0.466 °C, over six hours in the present study. In a study by García-Trejo, (2016) water temperatures ranged from 26.0 to 32.3 °C, over a 60 day sampling period. External air temperatures ranged from 22 to 27 °C during the day of sampling (2nd of February 2016) (Timeanddate, 2018). Water quality parameters (ammonia and temperature) in the greenhouse were different to those observed in the external environment.

3.5.2.3 Relationship between algae concentration and various physico-chemical parameters

Turbidity was inversely proportional to pH. A decrease in pH was accompanied by an increase in water turbidity. A similar result to that observed in the *C. gariepinus* experiment. The National Statistical Coordination Board (1999) arrived at a similar conclusion even though data collection was done over a period of twelve years, from 1980 to 1992; sampling was done on a lake (a fresh water body). However according to Mandal (2014), wastewater pH and turbidity have no direct significant relationship; Mandal (2014) sampled seven stations along a river using a nephelometer, therefore sampling on a different kind of study site and use of different equipment may have contributed to the difference in results.

The relationship observed between turbidity and pH may have been due to a third party, namely CO_2. Carbon dioxide is required for photosynthesis which leads to more algae production, increasing water turbidity and in the present study CO_2 was in constant supply; furthermore this gas reduced water pH through a series of proton releasing reactions resulting in the formation of carbonate (Wurts and Durborow, 1992; Brennan, 2017). This indicates that pH does have a relationship with algae concentration.

Turbidity had a positive relationship with CO_2 concentration. High CO_2 levels were mirrored by high turbidity values. The relation may be due to the carbon dioxide being a fundamental component for algae production (Pfannschmidt, 2003). Carbon dioxide influences turbidity. The CO_2 did not only lower the pH, it probably also acted as a carbon source for algal production.

Algae concentration, whether based on dry total suspended solids or turbidity readings did not have a significant relationship with ammonia ($N\text{-}NH_3$); temperature; turbidity and chemical oxygen demand. Extreme conditions have been reported by several researchers to contribute to increased cortisol levels, and a stressed fish cannot assume normal functioning including

feeding. One possible explanation for the lack of a clear relationship between dry TSS (particle size >8.0 µm) and the water quality parameters could be that these parameters ranged within a narrow spectrum, so that the fish adapted and resumed normal function throughout the period of experimentation.

3.6 Conclusion

pH was not similar between CO_2 sparged and non CO_2 sparged tanks. Carbon dioxide sparged tanks had a lower water pH than tanks where there was no CO_2 sparging. There was no significant difference in water pH between light and dark tanks. Therefore what this experiment demonstrated is that CO_2 sparging does reduce water pH whereas the absence or presence of light has little to no effect at all as far as water pH is concerned.

Oreochromis mossambicus filter feed significantly on brewery effluent algae. Algae concentration was not similar between fish-containing tanks and fish-absent tanks. Furthermore, although fish gut weights were similar in fish-containing tanks algae were present in all the fish guts providing evidence that the fish were consuming algae.

In the pH moderated tanks, CO_2 produced an undesired effect of lowering the oxygen content of the water. This might have been responsible for the result that, there was no difference in algae concentration between pH moderated/oxygen deficient tanks and un-moderated/alkaline tanks.

Algae concentration is related to two water quality parameters (pH and CO_2), therefore algae concentration either influences or is influenced by pH and CO_2.

Chapter 4: Overall Conclusion and Recommendations

There are not many studies which have approached this research from a similar area of focus, hence the reason for the present study. The objectives of the present study were met in that, fish age and the width between the gill rakers were measured in section 2.2.1, leading to the conclusion that *Clarias gariepinus* age was directly proportional to the width between the gill rakers. This paved the way for the realisation of the following objectives:

Section 2.2.2: (1) to assess whether *C. gariepinus* remove algae from algal tanks, and it was concluded that under the employed experimental design, algae removal by *C. gariepinus* was not significant; to (2) determine if flocculation using chitosan improves alga removal, and this led to the discovery that for chitosan flocculation to be successful in improving algae removal, a low pH was mandatory; to (3) monitor water quality parameters, and these occurred within ranges devoid of extremes which would have warranted termination of the study; and to (4) determine whether a relationship exists between algae concentration and the various water quality parameters, and a relationship between algae concentration and (chemical oxygen demand and pH, ammonia and phosphate) was revealed, indicating factors which may affect algae removal by *C. gariepinus*.

Chapter 3's objectives were 3.2.1 to ascertain the most efficient method to reduce water pH, and of the methods tested carbon dioxide sparging proved most efficient; 3.2.2 (1) to assess whether *Oreochromis mossambicus* remove algae from algal tanks, and it was reaffirmed that this fish was an efficient filter feeder; (2) assess the fish gut weights at the end of the experiment, and describe the fish gut contents. (3) to ascertain the role of pH moderation in algae removal, and it was concluded that the method used was not ideal, not because it was ineffective but because it had negative effects on dissolved oxygen concentration; (4) to monitor water quality parameters and these occurred within acceptable ranges (5) to determine

whether a relationship exists between algae concentration and the various water quality parameters including pH modification, relationships were observed between algae concentration and pH (negative)/carbon dioxide (positive); All of these objectives were met meaning the study achieved its purpose.

This body of work did not address all of the questions adequately in order to paint more of a complete picture as far as the research aim and conclusions are concerned. For instance: fish health was not monitored in terms of conducting histology of both fish gills and liver. This would have answered the question of whether the fish remained healthy at the end of the experiment. A vital question considering the potential of the application of algae in aquaculture as a protein substitute in fish diets.

Future work should focus on investigating the necessary *Clarias gariepinus/Oreochromis mossambicus* stocking density and sampling duration for algae concentration to be reduced significantly.

Thirdly, an alternative pH reduction method to carbon dioxide as utilised in the present study is advisable for future research, simply because CO_2 compromised algae reduction by *Oreochromis mossambicus* by displacing dissolved oxygen within the water column.

Lastly, the algae community structure was not analysed, this would have made possible the determination of what kind of algae was mostly consumed. These are aspects that future research may properly address.

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pH, CO_2 and dissolved oxygen non-significant variances

Appendix 1: The mean (± standard error) pH, CO_2, and dissolved oxygen of post-high-rate-algal-pond effluent subject to the presence or absence of light and which included carbon dioxide or no carbon dioxide (Multifactor repeated measures ANOVA: $p>0.05$).

		Time (h)					Interaction		CO_2/none		Light/Dark		
	0	1	2	3	4	5	F	p	F	p	F	p	
pH													
Light	No CO_2	11.34±0.09	10.22±0.09	10.08±0.12	9.99±0.12	11.19±0.06	11.29±0.02	1.86	0. 33	21050.00	0.00	8.93	0.05
Light	CO_2	11.23±0.09	7.42±0.09	7.36±0.12	7.56±0.12	8.49±0.06	8.41±0.02						
Dark	No CO_2	11.39±0.09	10.00±0.09	9.94±0.12	9.92±0.12	11.24±0.06	11.25±0.02						
Dark	CO_2	11.38±0.09	7.31±0.09	7.41±0.12	7.89±0.12	8.31±0.06	8.33±0.02						
CO_2 (mg/l)													
Light	No CO_2	2.67±0.41	3.00±0.67	2.33±0.41	3.33±0.62	3.67±0.60	4.00±0.80	2.88	0.21	7993.90	0.00	12.98	0.03
Light	CO_2	1.67±0.41	91.00±0.67	93.67±0.41	91.00±0.62	90.67±0.60	92.00±0.80						
Dark	No CO_2	3.00±0.41	7.00±0.67	5.33±0.41	6.00±0.62	4.67±0.60	4.67±0.80						
Dark	CO_2	1.67±0.41	91.33±0.67	95.00±0.41	93.67±0.62	91.33±0.60	92.33±0.80						
DO (mg/l)													
Light	No CO_2	3.77±0.72	4.53±0.46	5.20±0.28	6.67±0.33	7.80±0.54	8.43±0.43	8.21	0.06	5.75	0.09	44.13	0.01
Light	CO_2	5.47±0.72	3.63±0.46	3.73±0.28	3.17±0.33	3.17±0.54	3.27±0.43						
Dark	No CO_2	3.97±0.72	2.30±0.46	1.67±0.28	1.33±0.33	1.33±0.54	1.60±0.43						
Dark	CO_2	4.50±0.72	2.67±0.46	2.30±0.28	1.83±0.33	2.23±0.54	2.33±0.43						

Turbidity and temperature non-significant variances

Appendix 2: The mean (± standard error) turbidity and temperature of post-high-rate-algal-pond effluent subject to the presence or absence of light and which included carbon dioxide or no carbon dioxide (Multifactor repeated measures ANOVA: $p>0.05$).

		Time (h)						Interaction		CO_2/none		Light/Dark	
		0	1	2	3	4	5	F	p	F	p	F	p
Turbidity (cm)													
Light	No CO_2	4.00±0.33	3.33±0.58	4.33±0.41	4.67±0.50	3.67±0.25	3.67±0.29	1.90	0. 32	1.96	0.31	9.13	0.05
Light	CO_2	4.00±0.33	3.67±0.58	4.33±0.41	3.67±0.50	3.67±0.25	3.67±0.29						
Dark	No CO_2	4.0 0±0.33	3.33±0.58	4.00±0.41	4.00±0.50	4.17±0.25	4.00±0.29						
Dark	CO_2	4.33±0.33	3.67±0.58	3.33±0.41	3.67±0.50	5.00±0.25	3.67±0.29						
Temperature (˚C)													
Light	No CO_2	23.07±0.35	28.78±0.90	2.33±0.80	28.17±2.16	31.27±1.00	30.93±0.90	0.71	0.67	4.00	0.14	0.90	0.59
Light	CO_2	23.10±0.35	24.47±0.90	24.97±0.80	26.33±2.16	27.07±1.00	27.17±0.90						
Dark	No CO_2	22.60±0.35	26.17±0.90	25.87±0.80	27.23±2.16	28.00±1.00	27.90±0.90						
Dark	CO_2	22.13±0.35	24.13±0.90	24.53±0.80	25.80±2.16	26.43±1.00	26.73±0.90						

pH, carbon dioxide, dissolved oxygen and temperature non-significant variances

Appendix 3: The mean (± standard error) pH, carbon dioxide, dissolved oxygen and temperature which included fish or no fish and in the absence or presence of pH moderation over five hours (Multifactor Repeated Measures ANOVA: $p>0.05$).

	Time (h)							
	0	1	2	3	4	5	F	p
pH								
Fish	11.76±0.02	9.32±0.07	9.06±0.04	9.09±0.02	9.33±0.04	10.09±0.06	0.24	0.94
No fish	11.74±0.02	9.32±0.07	9.07±0.04	9.07±0.02	9.35±0.04	10.03±0.06		
Carbon dioxide (mg/l)								
Fish	1.17±0.20	48.12±0.46	48.12±0.37	47.83±0.29	46.50±0.24	46.12±0.24	0.78	0.57
No fish	1.50±0.20	48.00±0.46	48.00±0.37	48.12±0.29	45.83±0.24	46.00±0.24		
Dissolved oxygen (mg/l)								
Fish	9.70±0.37	11.98±0.59	11.43±0.39	10.17±0.69	12.12±0.59	12.45±0.61	0.26	0.93
No fish	9.68±0.37	12.00±0.59	11.85±0.39	9.37±0.69	11.72±0.59	12.22±0.61		
Temperature (°C)								
Fish	24.32±0.24	26.38±0.77	26.72±0.83	26.65±0.72	26.90±0.47	26.80±0.68	1.02	0.42
No fish	24.63±0.24	27.05±0.77	27.93±0.83	27.55±0.72	26.52±0.47	27.78±0.68		

Ammonia ($N\text{-}NH_3$) and chemical oxygen demand non-significant variances

Appendix 4: The mean (± standard error) ammonia ($N\text{-}NH_3$) and chemical oxygen demand (COD) which included fish or no fish and in the absence or presence of pH moderation over five hours (Multifactor Repeated Measures ANOVA: $p>0.05$).

	Time (h)							
	0	1	2	3	4	5	F	p
$N\text{-}NH_3$ (mg/l)								
pH moderation	0.32±0.02	0.14±0.02	0.52±0.19	0.21±0.03	0.46±0.29	0.08±0.03	0.38	0.86
No pH moderation	0.09±0.02	0.13±0.02	0.18±0.19	0.14±0.03	0.33±0.29	0.07±0.03		
Fish	0.09±0.02	0.18±0.02	0.17±0.19	0.21±0.03	0.35±0.29	0.07±0.03	0.66	0.66
No fish	0.31±0.02	0.10±0.02	0.52±0.19	0.14±0.03	0.45±0.29	0.09±0.03		
COD								
pH moderation	83.33±2.12					82.00±2.26	0.86	0.38
No pH moderation	83.67±2.12					86.12±2.26		
Fish	83.00±2.12					85.33±2.26	0.72	0.42
No fish	84.00±2.12					82.83±2.26		