An investigation of some key physico-chemical water quality parameters of an Integrated Multi-Trophic Aquaculture (IMTA) system operating recirculation methodology in the Western Cape of South Africa

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DECLARATION

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JOSEPH ANTHONY DE PRISCO, September 2019

Abstract

Over the last few decades, Integrated Multi-Trophic Aquaculture (IMTA) in South Africa has developed from early experimental designs to large scale, commercially operating farms. This was in response to uncertainty regarding food availability for stock (primarily kelp in the case of abalone farms) and a desire to recirculate water whilst reducing the environmental footprint of the abalone farms. The growing prevalence of IMTA as a commercially viable activity has brought about a need for an expansion of the knowledge pool regarding the physico-chemical processes at work in such systems. Of particular interest to researchers are mechanisms and dynamics of nutrient transfer between components of the system and how these could be manipulated to increase efficiency and reduce running cost of farms. This work was conducted to try and quantify some of the changes in some physical and chemical characteristics of the water stream on a large-scale IMTA farm cultivating seaweed of the genus *Ulva* (*Ulva rigida*) and the locally named perlemoen abalone (*Haliotis midae*) on the south west coast of South Africa (Viking Abalone Farm at Buffeljagsbaai, Western Cape, South Africa) (34.7550° S, 19.6154° E).

Experiment one was a three-day experiment taking place in December of 2018, there was no particular reason for the choice of month, analyses of this nature are potentially useful on any given day of any given month as although the literature contains plenty of gaps, there is no single identifiable data gap sufficient to encourage the use of particular timeframes. The sampling regime involved single sample point testing of three modular clusters each operating a different rate of water recirculation (50%, 75% and 100%) with 50% recirculation being standard farm operation, 75% and 100% tested to gauge effect of increasing recirculation, 75% tested as a potential standard farm operation to reduce load on pumps and reduce volumes of water pumped in, 100% tested in case of emergency situation which requires farm to be isolated from the inbound water stream arriving from the immediate coastal water, ambient conditions were also tested for reference and comparison. Parameters tested were those which the farmers already tested periodically to gauge changes in water quality which may effect the abalone or seaweed, though slightly different methods were used for the testing of ammonia. On the farm the standard method is the Nesler photometric test (Lovibond photometer), whereas this research was conducted using a calibrated indophenol blue spectrophotometric technique (Modified Grasshoff, 1976). Results showed no statistically significant differences (Mood's Median Test, $p>0.05$) between the 50% and 75% recirculation cluster for temperature, pH, Total Ammonia Nitrogen (TAN) or Free Ammonia Nitrogen NH_3 (FAN). At 100% recirculation, statistically significant differences (Mood's Median Test, $p<0.05$) occurred between 100% and 50% recirculation, 100% and 75% recirculation and 100% recirculation and ambient conditions for pH, TAN and FAN whilst no statistically significant differences (Mood's Median Test, $p>0.05$) occurred for temperature. At 100% recirculation, TAN and FAN increased rapidly, though the commensurate rapid and considerable decrease in pH meant the FAN increase was not as high in magnitude as it would be at

a normal seawater pH of around 8.2. Abalone suffered no mortalities at 100% recirculation for three days and later reports from the farmers suggested no noticeable drop in growth rate that could be attributed to this test in the months following the experiment. From the regulatory perspective, the TAN levels breached WWF guideline maximum effluent concentrations for abalone aquaculture (600µM/l) only in the 100% recirculation cluster, and only then during three of the thirteen sampling runs. The TAN concentrations in 50% and 75% recirculation treatments were far below the WWF guideline maximum effluent concentration with maximum concentrations of 7.15 µM/l in 50% and 13.46 µM/l at 75%, the increase in maximum concentration was large but not egregious and resulted from a more pronounced build-up of ammonia as residence time of water in the cluster increases at 75% recirculation.

Experiment two was an intensive 24-hour sampling run; the primary aim was to test the effectiveness of the seaweed biofilter in an Integrated Multi-Trophic Aquaculture (IMTA) farm culturing perlemoen abalone and a green macroalga. Parameters tested were temperature, pH, dissolved oxygen, salinity, TAN, nitrate and nitrite as these are relevant parameters for the farmer and the necessary equipment to test them was available. Samples were stored in a freezer for this experiment due to intensity of sampling regime, and spiked standards were prepared to check shifts in concentration of TAN, nitrate and nitrite that may have resulted from the freezing and thawing processes. Spike recoveries were good in the case of TAN (87%-98%) and nitrite (92%-96%), but random and widely dispersed in the case of nitrate. As such, nitrate and nitrite were removed from the analysis as nitrite values only really held value if taken in conjunction with nitrate values. Minimal and non-useful variation in salinity observations meant that salinity was also discounted from the analysis. Temperatures varied minimally between sampling points during the experiment, though they rose in all sampling points during daytime as would be expected. pH was higher in abalone inbound and *Ulva* effluent water compared to the abalone effluent water. Total ammonia nitrogen percentage removal across the seaweed biofilters ranged from 65%-85% with the mean and median at 73% and 71% respectively. Free ammonia nitrogen percentage removal across the seaweed biofilters ranged from 41%-80% with the mean and median removals at 63% and 66% respectively. A regression analysis demonstrates a strong positive linear relationship between TAN removal and TAN load to the seaweed biofilter (r^2= 0.90). Principal component analysis revealed a strong negative correlation between FAN removal and pH, as pH increased across the seaweed biofilters, the level of FAN removal decreased. This suggests that the perceived benefit of increasing pH in seaweed biofilters during the day-time may have some negative repercussions.

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1. Introduction

1.1 General rationale and overview

Aquaculture has been practiced in some form or other for thousands of years, and the cultivation of aquatic organisms under controlled or semi controlled conditions is nothing new (Stickney, 2013). The Food and Agriculture Organisation (FAO), specialised agency of the United Nations concerned with global food production and exchange, release a biennial report entitled “The State of World Fisheries and Aquaculture” (SOFIA), the most recent of which was released in 2018.The FAO collect data from governments and organisations around the world, which are then compiled and combined to give estimates for gross production and first sale value from the world’s fisheries and aquaculture activities (FAO, 2018). Early, plausible examples of aquaculture given by Parker (2012) involve fisher people keeping excess catch alive for later consumption, possibly in baskets or ponds, and then moving on to feeding and breeding. In a modern context, fisheries and aquaculture are becoming more isolated from each other as aquafarmers can breed, grow and harvest stock without any reliance on wild caught fish, plants or mammals and the report discusses aquaculture’s growing role in providing fish for human consumption. Figures provided in the report, show that aquaculture now provides approximately 50% of the globally produced fish for human consumption and suggests that aquaculture now provides more fish for human consumption than wild fisheries; it’s growth to achieve this milestone has been significant in the last three-four decades with especially rapid expansion occurring during the 1990s (De Silva and Soto, 2009) . In the FAO (2018) report, the word “fish” includes crustaceans, molluscs and other aquatic animals, the name “fish” does not include aquatic mammals, reptiles, seaweeds and aquatic plants.

This work was focussed on the water stream of a South African abalone (*Haliotis midae*) farm, located on the south coast of South Africa (Buffeljags Abalone Farm, 34.7550° S, 19.6154° E). The primary stock is the farmed abalone, with an integrated production of a marine macroalga as a secondary crop (*Ulva rigida*) for abalone feed. Worldwide production of seaweeds in aquaculture is primarily for direct human consumption, though Indonesia has drastically increased output in the last decade or so to produce the colloid carrageenan from the tropical seaweeds *Kappaphycus alvarezii* and *Eucheuma sp.* where it is valuable as an emulsifying agent in food production sectors (Pauly and Zeller, 2017).

In historic terms, China is widely considered the cradle of aquaculture, with Parker (2012) giving a date of around 3500 BC for the freshwater cultivation of common carp on Chinese silkworm farms where pupae and faeces from the silkworms were used as a supplementary feed for the carp; providing a sound example of an ancient aquaculture design which employed a rudimentary recycling methodology where a nuisance waste product becomes a valuable feed. In literature there are other examples of aquaculture occurring in ancient Egypt, Rome and Japan, and in more recent history (19th century) two commercial French fishermen concerned about depletion of trout stocks in streams sought assistance from scientists M. Miline Edwards and M. Coste. The combined observations and experiences of the fishermen and the expertise of the scientists came together to produce one of the first trout hatcheries in 1852, which became widely known and began providing trout eggs for farms across central Europe (Stickney, 2013).

Whatever it's roots, aquaculture in a modern context remains a blend of trial and error, innovation, and cooperation between fishermen, aquafarmers, scientists and other interested parties. Today, the world's biggest aquaculture producer by far is China, with the majority of Chinese aquaculture accounted for by freshwater culturing of various carp species including grass carp (*Ctenopharyngodon idellus*), the silver carp (*Hypophthalmichthys molitrix*) and the common carp (*Cyprinus carpio*). The vast majority of output from Chinese freshwater finfish farms goes to feeding local populations in China or other Asian countries, and Chinese scientists are responsible for some impressive genetic modifications in carp species to yield fast growing, disease resilient hybrids named jian carp (FAO, 2017). Many of the developments in aquaculture in the last few decades have been associated with mitigating and managing the proliferation of pathogens and disease as aquaculture experiences many of the same problems with disease as agriculture, particularly when stocking densities are high and mitigation techniques are poorly implemented (Schryver and Vadstein, 2014). Other significant developments have been centred around increasing ecological sustainability of aquaculture activities, by the improvement of water quality in effluent waste streams by various methods of remediation.

In the world's oceans there are signs of significant stress on ecological systems resulting from anthropogenic pressures (coral bleaching, rapid migration of fish stocks due to changing conditions, increasing trend in temperature and eutrophication arising from concentrated nitrogenous waste). The FAO (2018) reports that fishery captures worldwide increased rapidly in the mid to late 20th century, before plateauing somewhere between 1990 and 1995. This situation is unlikely to undergo any significant changes in the near future as harvesting of fisheries resources has reached or exceeded maximum sustainable levels in as many as half of the world's fisheries (FAO, 2018).

As the importance of aquaculture as a food production sector continues to grow, and the aquaculture industry itself continues to flourish, so too will the need for sustainable and environmentally friendly practices which mitigate the harm done to surrounding ecosystems, whilst providing nourishing and healthy food. Sustainable development goal number 14 of the United Nations Development Programme, concerns "Life below water" and makes a commitment "To conserve and sustainably use the oceans, seas and marine resources". Sustainable and eco-friendly aquaculture will have its role to play in the achievement of this goal, whilst also helping to secure a stable global food supply in years to come.

One of the ways in which aquaculture is becoming more sustainable is by the increased cultivation of marine macroalgae (Bolton *et al.*, 2016). Macroalgae demonstrate many remarkable properties: as well as providing many nutritional benefits to humans, they are resilient and can tolerate a fairly broad range of water conditions, as well as showing impressive conversion rates of dissolved nutrients to biomass (Robertson-Andersson *et al.*, 2008). South African aquaculture is dominated by the locally named Perlemoen Abalone (*Haliotis midae)* but there are impressive innovations in the local industry which employ an integrated co-cultivation methodology (Troell *et al.*, 2006). In most South African examples, the two species co-cultured are Perlemoen Abalone and *Ulva*, and this co-cultivation approach holds many benefits, both financial and environmental. The general idea is that the macroalgae is an unfed crop, the input/feed to the algae comes from the effluent waste stream of the abalone, and thus a nuisance waste stream which was previously being pumped directly out to sea and potentially causing harm to coastal ecosystems by the addition of nutrient/organic loaded water, is now being used as a resource to grow a second crop (Robertson-Andersson *et al.*, 2008). This research project was conducted to discern some of the changes in the physico-chemical characteristics of farm water arising from the integrated cocultivation technique. It is hoped that in building a better understanding of how the cultured species interacts with the water and its contents, that aquafarmers may be able to streamline their processes, increase efficiency by increasing water recirculation and enjoy increased resilience to emergency situations and changing conditions in future.

1.2 Innovations and developments in global aquaculture

Aquaculture and aquafarmers share many similarities with agriculture and farmers, and a constant battle against pathogenic disease is one of these (Parker, 2012). High stocking density culture of any animal (aquatic or terrestrial), in an agriculture or aquaculture operation, is liable to proliferate the spread of diseases. In the case of fish, a wild fish which becomes diseased will likely slow down and become easy prey for predators, thus removing the vector for the disease to spread, but in aquaculture operations (particularly monoculture operations) there are no predators and often high stocking densities so the disease is far more likely to spread through the population. Much of the research associated with aquaculture is concerned quite naturally with the microbiome, and how to control pathogenic microorganisms and stop disease from proliferating. The fundamental importance of microbes in aquaculture, and their role in the recycling of nutrients, the degradation of organic materials and their influence on water quality is discussed by Bentzon-Tilia et al., (2016). Also mentioned is that although microbes do occasionally kill and harm the cultured species, their larvae, or their live feed, they often play a protective/nurturing role for the cultured species and can yield beneficial synergistic effects. Aquaculture stocks are often cultured at both high density and large scale to maximise profit for the aquafarmers, but this becomes somewhat problematic not only in terms of pathogenic proliferation, but also with respect to maintaining acceptable water quality. This is evidenced in the tanks themselves which may be polluted by excess inorganic nutrients, faecal matter and uneaten foodstuffs, but it is also evidenced in surrounding environments where excess nutrient loading can contribute significantly to eutrophication (Chávez-Crooker and Obreque-Contreras. , 2010).

Aquaculture in the 21st century exists in many forms, and aquafarmers exercise varying levels of control in their operations. The level of control exercised can be as minimal as spreading shell on the bed of a bay to encourage oyster settlement, to maximal control involving hatcheries, recirculated water supplies and chemical additives to induce larval settlement (Parker, 2012). Cages and pens in themselves are a form of control, but there is a wide spectrum of control exerted in the aquaculture industry, from high levels of control to minimal levels of control. The more control exerted by the aquafarmer, the greater the opportunity to refine and streamline the process, and thus increase output. More control however, generally means increased deployment of technology, and more technology gives more scope for problems to arise (i.e. mechanical failure, incorrect dosage from automated systems), so high levels of control in aquaculture often require almost constant attention from trained staff to ensure smooth operation of the plant. There are various methods aquafarmers employ in an attempt to maintain agreeable water quality, not only in terms of excess nutrient loading, but also in terms of organic pollution of receiving water but these methods all vary widely between location, practicability, and local regulation (Chávez C. and Obreque C., 2010). Aquafarmers, regulators, and other interested parties are becoming increasingly concerned with water quality and point-source pollution arising from the operation of aquaculture farms, and this has led to a variety of methods to be employed in an attempt at remediating the water stream (Merino *et al.*, 2012). There are fairly standard mechanical methods such as filters (mesh, sand, charcoal etc.) to remove suspended/colloidal particles, and foam fractionators, which serve to remove organic waste from the water (Stickney, 2013). Many artificial feeds which aquafarmers feed to fish are high in protein, which when metabolised by fish generates ammonia. This ammonia can accumulate to toxic levels particularly in recirculating systems, so a variety of bioremediation techniques have been explored in an effort to maintain water quality. Bacterial nitrification is one such method, which proceeds under aerobic conditions to convert ammonia to nitrite and then nitrate, this process consumes significant levels of oxygen as ammonia-oxidising bacteria (AOB) oxidise ammonia to nitrite via hydroxylamine, and then nitrite oxidising bacteria (NOB) oxidise nitrite to nitrate (Chávez C. and Obreque C. , 2010). These bacterial biofilters, whilst proven effective in remediation of water streams, require high levels of maintenance and care as they are sensitive to operating conditions, pH in particular. Another method receiving much attention in global aquaculture is the bioremediation of water streams by macroalgae (Bolton et al., 2016). More information regarding bioremediation is given in section 1.6, however the general idea behind bioremediation is to convert harmful or nuisance compounds in to more innocuous ones, occurring at the molecular level, bioremediation does not simply transfer the waste somewhere else. The ultimate goal of bioremediation is the complete mineralisation of unwanted contaminants in to CO_2, H_2O, N_2, HCl etc. (Rhodes and Rhodes, 2015) but for seaweeds as biofilters, the objective in South African abalone farms is the conversion of organic and dissolved nitrogenous waste (ammonia, nitrate, nitrite) to seaweed biomass (Robertson-Andersson *et al.*, 2008).

1.3 Aquaculture in South Africa

The report by the FAO (2018) suggests that aquaculture in South Africa is under-developed when it's full potential is considered, and gives a number of constraints which are currently disrupting development. These constraints include access to water, suitable sites and technology, prohibitive transaction costs, ambiguous and erratic governmental oversight and regulation, and barriers to effective marketing. It is also mentioned that these constraints are receiving significant attention from the relevant authorities, primarily the Department for Agriculture, Forestry and Fisheries (DAFF) with a view to improving the situation. DAFF is now the lead governmental department with a mandate to develop and nurture the aquaculture sector of South Africa. Historically, rainbow trout culture is the oldest form of freshwater aquaculture in South Africa, with development occurring from the late 19th century onwards. In terms of mariculture, the oldest subsector of aquaculture in South Africa is oyster farming in embayment systems, which apparently began in the mid to late 17th century, although the modern industry is based on imported pacific oysters. Freshwater fish of interest to South African aquafarmers include: trout, tilapia, catfish, carp and crayfish. In the mariculture sector, the species of interest include: abalone, mussels, prawns, oysters, macroalgae and some finfish. The most important of these is the abalone (*Haliotis midae)* sector, which according to an estimate by (Cook, 2014) contributed around 1450 metric tonnes (mt) to the total aquaculture output of South Africa in 2015. Current farm gate prices for abalone are around $35 (USD) per kilogram. DAFF statistics released in 2016 reported 18 operational abalone farms around the coast of South Africa, and of the six naturally occurring *Haliotis* species found in South African waters, only *Haliotis midae* retains any commercial interest, assumedly because it is the largest of them . The availability of feed is of primary concern to aquafarmers, and for this reason many of the aquaculture farms occur close to natural kelp forests which are harvested at sustainable levels in line with guidelines provided by the South African Department for Agriculture, Forestry and Fisheries (Troell *et al.*, 2006)

1.4 *Haliotis midae*

Haliotis midae is endemic to South Africa, and the geographic distribution is wide but patchy and concentrated primarily on the south/south western coast, with abalone found on coastlines from Port St. Johns in the Eastern Cape, to Saldanha Bay on the west coast (31.62 ° S, 29.54 ° E- 33.02° S, 18.19° E) (Vosloo *et al.*, 2013). Abalone farms are concentrated on the south west coast, where there is plentiful kelp for farmers to harvest and feed to the stock, depending on regulation and accessibility (Robertson-Andersson *et al.*, 2008).

The full taxonomic classification for *Haliotis midae* is as follows; Phylum- Mollusca, Rank- Species, Kingdom- Animalia, and it is a marine, herbivorous, univalve, gastropod mollusc. There is currently a small legally regulated abalone fishery in South Africa, with the 2018/2019 season total allowable catch set at 96.5 tonnes by the South African Minister for Agriculture, Senzeni Zokwana (M. Cyrus. Personal communication, June 2019). There is, however, a booming illegal abalone trade in South Africa; by the trade's very nature, accurate values for the size of the market are difficult to estimate (Cook, 2014). The primary market for South African abalone (illegal and farmed) is China and other South East Asian countries, where abalone is considered a delicacy, and is often consumed to celebrate/mark special occasions. China itself is a large producer of *Haliotis* species for consumption, with shrinking production of lower value species such as the *Haliotis diversicolor supertexta*, which commonly sells for around $20 (USD) per kilogram and ever-increasing levels of production of higher value *Haliotis* species such as *Haliotis discus hannai* and *Haliotis discus discus*, or hybrids thereof (Cook, 2014). The reason that South African abalone continues to attract high demand and a correspondingly good price from Chinese consumers, is that it is considered to be of premium quality when compared with much of the Chinese aquaculture output (Troell *et al.*, 2006). Investigations done by (Cook, 2014), found that a first sale value (farm gate) for abalone in South Africa is somewhere in the region of $30-40 (USD) per kilogram, but the value added chain is such that by the time it reaches a consumer, it can fetch $300-400 (USD) per kilogram. Prices fluctuate widely with shift in local supply, consumer attitudes and as a result of incidents such as human virus/pandemic scares.

Haliotis midae reproduce by releasing egg and sperm cells in to the water column; the fertilised eggs then become free swimming larvae which obtain energy from a yolk sac (Sales and Britz, 2001). Metamorphosed adult abalone are unipedal, and they move by either hopping or crawling (Genade *et al.*, 1988). *Haliotis midae* spend their lives in 2 primary stages; the first being a free swimming larval stage (2-5 days), which locates favourable substrate and conditions in which to settle, before settling and undergoing an irreversible metamorphosis process, which results in a crawling benthic organism (Genade *et al.*, 1988). Much research has been done to ascertain what conditions and substrates abalone find favourable, with some diatoms and other microalgae seeming to encourage more settlement than other substrates (Sales and Britz, 2001); settlement however is no guarantee that a larval *Haliotis midae* will survive, and there are other factors for abalone farmers to consider when selecting substrates to offer for settlement, such as success of metamorphosis and survival rates post-metamorphosis.

Abalone are more susceptible to deleterious changes in water quality when they are in their larval and juvenile stages of growth, with resiliency building with size (age). *Haliotis midae* occur naturally in rocky crevices and on reefs, but reach maximum densities in kelp forests, which they graze on in a way that minimises the exposure of their soft vulnerable underbelly by allowing tidal action to carry grazing to them (Hahn, 1989; Vosloo *et al.*, 2013). There have been studies conducted on the toxicity to *Haliotis midae* of unionised ammonia (NH_3) which show that levels of unionised ammonia, often referred to as free ammonia nitrogen (FAN) effect *Haliotis* midae differently during different life stages with levels of resilience increasing with age (Reddy-Lopata et al., 2006). Free ammonia nitrogen refers to the fraction of total ammonia nitrogen accounted for by NH_3 which is a neutral, lipid soluble toxic substance, of particular concern to aquarists and aquafarmers. One of the measures used is LC_{50} (lethal concentration in 50% of the population) at a temperature of 15°C and pH of 7.8. For juvenile abalone (1.0-2.5 cm shell diameter) the LC_{50} reported is 9.8 $\mu g\ L^{-1}\ NH_3$, for cocktail size abalone (5-8cm shell diameter) the LC_{50} reported is 12.9 $\mu g\ L^{-1}\ NH_3$, and for broodstock size abalone (10.0-15.0 cm shell diameter) the LC_{50} reported is 16.4 $\mu g\ L^{-1}\ NH_3$. Further to increasing resilience with size (age), (Reddy-Lopata *et al.*, 2006) mention the acclimatisation factor as playing a role, whereby abalone conditioned to higher levels of ammonia for prolonged periods, are more resilient to mortality arising from elevated concentrations of unionised ammonia. Further to this, at lower concentrations of unionised ammonia there is still the potential for growth inhibition, so monitoring of ammonia concentrations must be carried out regularly on farms to ensure healthy stock and good growth rates. Abalone farmers must also closely monitor temperature, pH and to a lesser extent salinity, as these parameters not only influence the abalone directly, but they also influence the ionisation of ammonia in seawater (see equation three below), with higher pH and temperature yielding higher proportions of free unionised ammonia in the water stream (Bower and Bidwell, 1978).

1.5 *Ulva*

The genus *Ulva* is cosmopolitan in shallow seas and the majority of the species in the genus may be found in near-shore marine and estuarine waters, upper to mid-intertidal regions and the subtidal zone (Hoek *et al.*, 1996). *Ulva* species are opportunistic pioneer species which will rapidly colonise bare substrata (Vergara *et al.*, 1998). Highest *Ulva* densities are often found in the intertidal zone in dynamic environments where there are frequent changes in cover due to shifting sand, but they also achieve high densities in estuarine habitats or more protected embayment systems; in these kind of environments they are found to create a dense, continuous green 'blanket' on rocks, boulders or even sediments in salt marshes (South and Whittick, 1987). *Ulva* are also found to adapt well to reefs where they receive protection from high wave shear and plentiful nutrient supply (Lee, E, 1999). The proliferation of *Ulva* also occurs in environments where there are high levels of organic enrichment arising from pollution.

Ulva species are found naturally all over the world and although most *Ulva* species attach to a substrate, some are capable of floating freely, primarily in sheltered estuaries and embayment systems. The ability of *Ulva* to grow in a free floating state is one of the reasons it has garnered so much attention as a potential crop in land-based aquaculture (Bolton *et al.*, 2016). In Asian countries *Ulva* is often grown for human sustenance, but in Africa and Europe, *Ulva* is grown as a feed supplement, although it's popularity as a foodstuff is growing. There is significant ambiguity in the taxonomy and identification of different *Ulva* species, and Bolton *et al.*, (2016) discuss the lack of coherency between more traditional morphologically based taxonomic names, and molecular clades. A personal communication by Charles J. O'Kelly, referenced by Bolton *et al.*, (2016) states that *Ulva* "perhaps counter-intuitively, is a recent taxon of green algae, currently undergoing rapid and rampant speciation, and we don't yet know where the barriers to gene flow are. Consequently, species designations, at present, are no better than arbitrary". There is much literature concerned with specific identification and speciation of *Ulva* but Bolton *et al.* (2016) state that if the global *Ulva* industry expands rapidly as predicted, then there will be a requirement for detailed molecular population genetic studies to identify, and subsequently name successful species/strains. Commonly known as sea lettuce, *Ulva* species are grown for direct human consumption in eastern countries such as Japan and China (Bolton *et al.*, 2009). It is also reported by Bolton *et al.* (2009) that *Ulva* species have been authorised for human consumption by the French government since 1990.

Aquaculture operations cultivating aquatic plants are dominated by brown algae (primarily *Saccharina* and *Undaria*), and to a lesser extent red algae (mostly *Pyropia*) and some *Eucheuma/Kappaphycus* and *Gracilaria* for phycocolloids (Bolton *et al.*, 2016). The share of green macroalgae production in global aquaculture is currently so minor as to be almost negligible when compared with production levels of brown and red macroalgae, and it is noted by Bolton *et al.* (2016) that production figures for "green nori" such as *Ulva* and *Monostroma* only began appearing in globally compiled statistics (FAO) regarding marine plant production in 2003. As mentioned previously, other than feed crop production, there are several reasons why "green nori" make attractive candidates for large scale commercial production including the ease with which they can be cultivated at high densities and their ability to remain in vegetative states for long periods, their ability to efficiently convert dissolved nutrients in to biomass, and their ability to grow unattached, whilst also providing numerous health benefits to consumers (Bolton *et al.*, 2016). The harvest of macroalgae such as *Ulva* is also easier to achieve than microalgae, as the system may be flushed and the macroalgae can be caught in a net or basket. Of particular significance to this research project, the proven ability of *Ulva* to bioremediate effluent water (Troell *et al.*, 2006) by the removal of excess dissolved nitrogenous nutrients will be investigated.

1.6 Integrated Multi-Trophic Aquaculture (IMTA)

Integrated multi-trophic aquaculture methodologies in aquaculture have been shown to be effective in increasing farm production/profitability in certain South African examples, as well as mitigating environmental impacts and increasing sustainability (Nobre *et al.*, 2010). Integration involves a cocultivation technique where the waste from one organism, often fish, prawns or molluscs, is made useful by another organism that is farmed concurrently, often algae, or bivalve filter feeders. In the South African example, integrating macroalgae with abalone may provide the abalone with a source of high protein nutrition, whilst also facilitating the recirculation of water due to its bioremediation effect. The integrated approach produces a variety of features which increase the ecological and economic sustainability of the system (Nobre *et al.*, 2010). Utilising the bioremediator macroalgae as feed for the abalone reduces the farm's dependence on alternative feedstuffs, such as expensive pellet feeds or locally harvested kelp. If the cultured *Ulva* is used as a supplementary diet to the kelp (*Ecklonia maxima*), research suggests that growth rates of abalone are improved when they are fed a mixed algal diet as opposed to a single algal species diet, but whether this applies to *Ulva* is not specified by the authors (Naidoo *et al.*, 2006). The seaweed is not only used as a feed, it also takes on the role of a biofilter of abalone effluent water, allowing recycling of the water itself, and the nutrients excreted by the abalone. This recycling reduces pumping costs significantly and reduces the need for any 'artificial' fertilisation of the *Ulva* whilst also reducing the ecological impact of the farm's operation. Another potential benefit of recirculating water may arise in the case of harmful algal blooms (HABs), which are not uncommon occurrences on the southern and western coastlines of South Africa (Probyn *et al.*, 2011). A local example provided by Grant Pitcher (Personal

Communication, 2018) details the drastic effect of a HAB in 2017 which consisted of yessotoxins from a mixed *Gonyaulax*/*Lingulodinium* bloom, which caused the loss of 270 tonnes of abalone across three farms in the Hermanus area (south west coast of South Africa). In the case of a harmful algal bloom, then a farm operating recirculating and remediating methodology may be able to close the primary inlet to the farm and move to a 100% recirculation situation whilst the harmful microalgae (often dinoflagellate assemblages) disperse (N. Loubser, Pers. Comm. 2018). Decreases in pH, increases in temperature and rapid build-up of ammonia are likely to create significant problems, but the option is certainly preferable to the alternative of having to allow in the high concentrations of toxic algae species.

Seaweeds suitability as bioremediators is evidenced by their high capacity for the uptake of dissolved nutrients, with work by Neveux *et al.* (2017) suggesting that up to 90% of dissolved ammonia nitrogen can be removed from aquaculture effluent by macroalgae (depending on nutrient loading and system design), which may then contain significantly more protein (true and crude; true being the total content of proteomic amino acids) than macroalgae that have grown in the wild (Robertson-Andersson et al., 2011). This in turn increases growth rate of any organisms which consume the high protein seaweed compared to wild grown lower protein content seaweed. In South Africa, there are numerous aquaculture farms that cultivate marine macroalgae for feed, primarily *Ulva* and *Gracilaria* but of these, not all operate recirculating integrated methodology due to concerns about biosecurity and the proliferation of disease, they instead run on a flow through system where water is continuously pumped through the system with no recirculation (Neveux et al., 2017). The threat of disease proliferation has been mitigated in commercial setups where modular clusters are employed which exist in isolation from each other on the same farm. See methods section for a general schematic but each modular cluster contains rows of abalone baskets connected to an *Ulva* raceway, in this way a disease outbreak in one cluster will not necessarily spread to other modular clusters but if the pathogen is entering with fresh seawater then all clusters will be affected. A recirculating methodology has been employed on one farm in the Gansbaai area for at least six years, with no adverse effects on the abalone stock (N. Loubser, personal communication).

The considerable kelp resources available to aquafarmers on the west and southwest coasts of South Africa have been harvested for aquaculture feed, but some farmers on the south west coast in the Gansbaai area, have been subjected to restrictions on kelp harvesting in recent times (Nick Loubser, personal communication) furthermore, there are sections of the coastline particularly on the eastern and southern coasts of South Africa, which do not possess any significant kelp resources which could be harvested for aquaculture purposes (Troell *et al.*, 2006). Although all farms use formulated feeds, this diet can be supplemented by locally harvested kelp plants if possible, these formulated feeds are often high protein feed pellets which are widely utilised in South African abalone culture. Abfeed (Abfeed®, Marifeed Pty Ltd, Hermanus, South Africa) is often used, and according to a report by The

International Trade Administration Commission of South Africa (2006) consists mainly of fishmeal and starch based products. Currently in global aquaculture the feed for stocks often consists of fishmeal, and it is interesting to note that fishmeal is almost always made from wild caught fish; there are losses in protein as the wild caught fish goes from fishmeal to cultured fish feed to human feed, compared to direct fish consumption by humans where loss of protein is less (Merino *et al.*, 2012). There are many companies worldwide which sell fish food pellets that owe their primary protein content to fishmeal, a fair proportion of which comes from the highly productive boundary currents of the eastern Pacific Ocean. Aquafarmers who cannot access feed in surrounding ecosystems for whatever reason, can either purchase feed from elsewhere (formulated feed pellets) or try and cultivate their own feed depending on the needs of their stock. In the case of South African abalone farmers, the herbivorous nature of *Haliotis midae* allow aquatic plants to be grown with abalone from the nutrient loaded abalone effluent, which can then be fed back to the abalone. *Ulva* grown in this fashion possess significantly more protein than wild grown *Ulva* (Abreu *et al.*, 2011). For the majority of land-based aquafarmers, where pumps are used to introduce water to the farm, the two primary costs are likely to be feed and electricity. Through the integration of a primary crop with a secondary bioremediator crop which occurs on a lower trophic level, feed and electricity costs can be reduced by recirculating water (reduced pumping costs) and feeding *Ulva* back to the abalone (reduced feed costs); coupled with these two benefits are the reduced nutrient concentrations in the water which flows back out to the surrounding environs which may drive eutrophication, this eutrophication is instead directed to useful and desirable organisms (Bolton *et al.*, 2009).

1.7 Water quality and chemical analysis

The oceans are complex, and their physical and chemical properties/characteristics arise through the myriad and complex interactions of the planet's biosphere, atmosphere, lithosphere, pedosphere and cryosphere. In an oceanographic context, it is useful to consider the oceans as a two-part system, with one part consisting of chains of water molecules (approximately 96.5%) and the other part consisting of dissolved constituents (approximately 3.5%) (Morel and Hering, 2013). The 3.5% dissolved component is then grouped in to two categories, major/conservative components and trace components. The average salinity of seawater is around 35 g/l, or 3.5%. Na^+ and Cl^- are the two primary constituents The conservative components undergo only minor changes arising from biological processes, and it is often the case that changes due to chemical and geochemical processes occur over long time scales (Grasshoff *et al.*, 1999); the distribution of these conservative components are regulated primarily by physical processes such as advection, convection, diffusion, turbulent mixing and more. The trace constituents then, as well as being altered by the same physical processes mentioned previously, are also highly and sensitively influenced by biological processes such as uptake, excretion and biodegradation (Grasshoff *et al.*, 1999). To add to the complexity, there are chemical reactions taking place due to the pressure and temperature dependent readjustments of equilibria taking place according to Le Chatelier's principle, where equilibria move to counteract changes in systems. There are also physico-chemical exchanges and reactions occurring at various surfaces and boundaries (sea-air, water-sediment, suspended particle-water to name a few). As such it is necessary for small research projects to attempt to design experiments which yield better understanding of the investigated system without adding layers of complexity which confound the original objective through information overload. The physical parameter of temperature is easy to measure to a reasonable degree of accuracy and has influence over all water quality parameters to a greater or lesser extent. Temperature is fundamentally a measure of movement, and its level governs the patterns of life in all forms, whether in the oceans or on land. The relationship between pH and temperature is an inverse one, but that is not to say that a cooler solution is more alkaline than a warmer one, the whole pH scale shifts based on temperature. The pH probe used in this study is equipped with a temperature probe which automatically compensates for temperature, so lengthy calculations to correct for temperature, or equilibration of samples to some set temperature is not necessary. The pH scale in a simplistic form is a measure of the amount of H^+ ions in a given solution, with a lower pH denoting higher levels of H^+ ions, and a higher pH denoting lower levels of H^+ ions. Small fractions of pure water (H_2O) dissociate spontaneously according to the equation below, known as the autoionisation of water.

$$H_2O\ (l) \rightleftharpoons H^+\ (aq.) + OH^-\ (aq.)$$

Equation 1- Autoionisation of H_2O

The dissociation of pure water produces equivalent numbers of H^+ (hydrogen) ions and OH^- (hydroxyl) ions, the hydroxyl ions can float freely in solution whilst the H^+ ions bond immediately to another water molecule to form H_3O^+ ions (hydronium). When H^+ ions are denoted in water chemistry equations, they truly represent hydronium ions but the conventional shorthand of H^+ is still commonly used. In pure water at 25 °C the concentration of hydrogen ions (H^+) is 1×10^{-7} moles per litre ($mol\ l^{-1}$) and the concentration of hydroxyl ions is equivalent 1×10^{-7} moles per litre ($mol\ l^{-1}$), in the conventional notation of square parentheses to denote concentration

$[H^+] = [1 \times 10^{-7}]$

$[OH^-] = [1 \times 10^{-7}]$

Acidic solutions possess higher concentrations of H^+ ions than pure H_2O ($[H^+] > [1 \times 10^{-7}]$) and basic solutions possess lower concentrations of H^+ ions than pure H_2O ($[H^+] < [1 \times 10^{-7}]$). The pH of a solution then is a measure of it's hydrogen ion (H^+) concentration compared with that of pure water, and is a logarithmic scale of the form:

$pH = -\log_{10} [H^+]$. If a hydrogen ion concentration of $[1 \times 10^{-7}]$ is plugged in to this formula thus:

$pH = -\log_{10} [1 \times 10^{-7}]$

$pH = 7$

pH 7 is often referred to as neutral pH and is the central point about which the pH scale moves. In aqueous solutions, the $[H^+]$ moves away from this neutral central point when either an acid or a base is added to it. This basic description is an over simplification of the system, acid and base theory is more complicated but when describing biological systems and processes this simplification is a useful one. The situation in seawater, and therefore mariculture (marine aquaculture) water streams is extremely complicated, as the input of acids or bases to the system will not immediately cause changes in H^+ or OH^- ion concentration. This is due to the buffering capacity of seawater, which arises primarily as a result of the carbonate system.

$$\begin{tikzcd}
CO_2\,(aq.) + H_2O\,(l) \arrow[r, shift left, "k_1"] & H_2CO_3\,(aq.) \arrow[l, shift left] \arrow[r, shift left, "k_2"] & H^+\,(aq.) + HCO_3\text{-}\,(aq.) \arrow[l, shift left] \arrow[d, shift left, "k_3"] \\
 & & 2H^+\,(aq.) + CO_32\text{-}\,(aq.) \arrow[u, shift left]
\end{tikzcd}$$

Equation 2- Simplified version of the oceanic carbonate system

Equation 2 shows a simplified form of the ocean carbonate system, which accounts for the ocean's high capacity for buffering changes in pH. K_1 is kinetically slow, in the order of seconds, whereas k_2 and k_3 occur almost instantaneously (Smith, 1988). The position of these dynamic equilibria is driven by pH, and at common seawater pH of around pH 8, the predominant form of dissolved inorganic carbon is bicarbonate (HCO_3^-). In an integrated mariculture system like the one in which this work will be conducted, the abalone change the carbonate system by excreting CO_2 during respiration, and the macroalgae change this carbonate system through photosynthesis and respiration. The abalone excrete CO_2 during respiration, which combines with water molecules to form carbonic acid as per k_1, the carbonic acid formed rapidly dissociates in to HCO_3^- and H^+, the generation of H^+ then decreases the pH. In biological systems, the interconversion between CO_2 and HCO_3^-/H^+ is often catalysed by carbonic anhydrase, which is a large, zinc containing enzyme with a monomeric relative molecular mass of around 30 000, it is ubiquitous in biological systems having been identified in bacteria, algae, higher plants, vertebrates and invertebrates (Henry, 1984). Many isozymes of this enzyme exist in nature, with each of them possessing their own kinetic properties; With a few exceptions however, all the enzymes in this group are characterised by exceptionally rapid turnover times.

Smith (1988) discusses that although there are similar levels of CO_2 in air and water, CO_2 diffuses approximately 1000 times slower in water than in air. For this reason, photoautotrophs such as the *Ulva rigida* in this mariculture system, may face shortages of CO_2 for use in photosynthesis. Over millennia, some marine macroalgae have evolved to utilise bicarbonate (HCO_3^-) in conjunction with carbon dioxide, particularly when there are low levels of carbon dioxide available, the use of HCO_3^- is unsurprising given that the dominant form of dissolved inorganic carbon in the oceans at present is bicarbonate (Beer and Koch, 1996). Membranal diffusion of bicarbonate is minimal according to Gutknecht (2004), so other transport mechanisms must be at work. It has been hypothesised that the bicarbonate is converted to CO_2 at the surface of the thalli according to its equilibrium constant, temperature and pH level in the immediate surroundings. This CO_2 is then transported across the membrane and utilised for photosynthesis after various exchanges and transport mechanisms which concentrate the carbon, the catalyst here is hypothesised to be an isozyme of Carbonic Anhydrase (Smith, 1988; Beer and Koch, 1996). In the water stream in integrated aquaculture systems like the one which will be studied here, the expected reductions in pH caused by input of CO_2 arising from the respiration process of the abalone should be counteracted to some degree by the integrated *Ulva* during periods of photosynthesis.

Ammonia is of importance to this project as ammonia arising from excretory processes may accumulate in recirculating aquaculture systems if mitigation techniques are not well implemented (Neori *et al.*, 1998). Of particular concern is the fraction of ammonia present in the water stream accounted for by unionised ammonia (NH_3). The ionisation of ammonia in fresh water is driven by temperature and pH; in seawater systems such as the one being studied there is also a salinity effect which must be accounted for. Work done on the ionisation of ammonia in seawater by Bower and Bidwell (1978) shows that increasing temperature and pH yield a higher proportion of unionised ammonia, but it is also noted that increased salinities reduce unionised ammonia fractions, at salinities of 32-40% anticipated in this project. Bower and Bidwell (1978) state also that the fraction of unionised ammonia is approximately 20% less in seawater than in freshwater at the same temperature and pH. When measuring ammonia in aquaculture farms then, it is vital to measure temperature, pH and salinity also so that the fraction of unionised ammonia which demonstrates high toxicity to fish and molluscs (thought to be due in part to it's high solubility in lipids (Bower and Bidwell, 1978)) can be deduced. South African Department for Agriculture, Forestry and Fisheries guidelines for aquaculture facilities state that levels of Total Ammonia Nitrogen in effluent waters from aquaculture facilities should not exceed 43 µM/l.

$$NH_4+(aq.) \quad + \quad H_2O\,(l.) \quad \rightleftharpoons \quad NH_3(l.) \quad + \quad H_3O^+(aq.)$$

Equation 3- Ionisation of ammonia in water

Testing for Ammonia will be carried out by a spectrophotometric method, which is explained in some detail in the methods section. As such, the basic principle of spectrophotometry will be briefly discussed here with reference to the Beer-Lambert law on which spectrophotometry is predicated. The Beer-Lambert law takes the form:

$$A = \epsilon l c$$

Where A is the measured absorbance (no units), ε represents the molar extinction coefficient ($L \cdot mol^{-1} \cdot cm^{-1}$), l represents the path length (cm) which is very often 1cm in spectrophotometry, as a 1cm sample cuvette is standard for most determinations, and c represents the concentration of the analyte (mol/L). The absorbance of a compound/molecule/analyte of interest at a given wavelength, can be determined by the amount of light which enters the sample and the amount of light which leaves the sample. The wavelength of light used in spectrophotometric determinations is the wavelength at which the compound/analyte of interest demonstrates peak absorption. The indophenol blue dye generated by the Berthelot reaction which this TAN methods uses, demonstrates maximum absorption between 600-670nm wavelengths of light, and the optimum wavelength is 630nm. This is the wavelength of monochromatic light which the spectrophotometer is set to in the Grasshoff ammonia determination method which will be employed in this study. The absorbance can be calculated using the following equation where I_0 is the light intensity before the light passes through the sample cuvette and I_t is the intensity of the light after it has passed through the sample:

$$\text{Absorbance (A)} = -\log \frac{I_t}{I_0}$$

During determination, the sample is irradiated with light of wavelength 630nm and intensity (I_0) following the addition of the necessary testing reagents (discussed in methods section) and allowing sufficient time to elapse to allow for full colour development of the dye. The spectrophotometer then detects the intensity of the light that has passed through the sample (I_t) and outputs a unitless absorbance value. This absorbance value is related linearly to the concentration of the indophenol dye according to the beer-lambert law, and the indophenol dye concentration is equivalent to the ammonia concentration, as they react in 1:1 molar ratio quantities (figure 1.1 below).

$$NH_4^+ + OH^- \rightarrow NH_3 + H_2O$$

$$NH_3 + OCl^- \rightarrow NH_2Cl + OH^-$$

$$NH_2Cl + \text{Phenol} \rightarrow \text{Indophenol (intensely blue)}$$

Figure 1.1- Simplified reaction scheme for Berthelot reaction yielding indophenol blue dye measured spectrophotometrically in Grashoff Total Ammonia Nitrogen (TAN) method

The last reaction which forms the intensely blue indophenol dye is catalysed by Nitroprusside ions (octahedral Iron^{3+} centred metal-ligand complex commonly found in the dihydrate form) in the Grasshoff ammonia determination method which will be used in this work. This allows for high sensitivity, and the Beer law is obeyed in this method to an absorbance of 0.800, above which the response is no longer linear and a new slope must be fitted (T. Probyn, Pers. Comm. December, 2018).

Dissolved Oxygen is also of fundamental importance to aquatic organisms, and in combination with temperature it governs most natural biological processes. The health and fitness of populations is often diminished at temperature and oxygen extremes (Vosloo *et al.*, 2013). In the wild, *Haliotis midae* may experience periods of oxygen extremes driven by events such as the decay of algal blooms and dissolved oxygen levels are naturally variable in near shore marine systems where abalone occur naturally. (Pörtner and Knust, 2007) have hypothesised that the tolerance of aquatic organisms for changes in temperature is driven by oxygen delivery to tissues, meaning that oxygen extremes will be one of the key drivers of extinctions or relocations in warming oceans. For *Haliotis midae*, juveniles seem better able to acclimatise to changing levels of dissolved oxygen than adults (Vosloo *et al.*, 2013), which is hypothesised to result from their exposure to highly variable oxygen levels when in the diatom biofilm. This is in contrast to *Haliotis midae*'s tolerance for ammonia, which increases as the specimen ages (Reddy-Lopata *et al.*, 2006). Minor hypoxia and hyperoxia can negatively affect *Haliotis midae* in terms of growth rate, as energy is required to acclimatise, and high levels of either could yield mortality of stocks and although dissolved oxygen concentrations are only likely to approach dangerous levels (low or high) in extreme situations, it must be monitored closely by farmers in case of such events.

1.8 Aims

1. Gauge changes in water quality (temperature, pH, ammonia) arising from increasing recirculation percentage
2. Gauge effectiveness of the seaweed biofilter in terms of removal of dissolved nutrients (particularly ammonia), and gauge how changing environmental conditions effect the unionised ammonia fraction
3. Identify patterns in pH, temperature change and dissolved oxygen concentration in the water as it moves through integrated modular clusters

2. Materials and Methods

2.1 System description

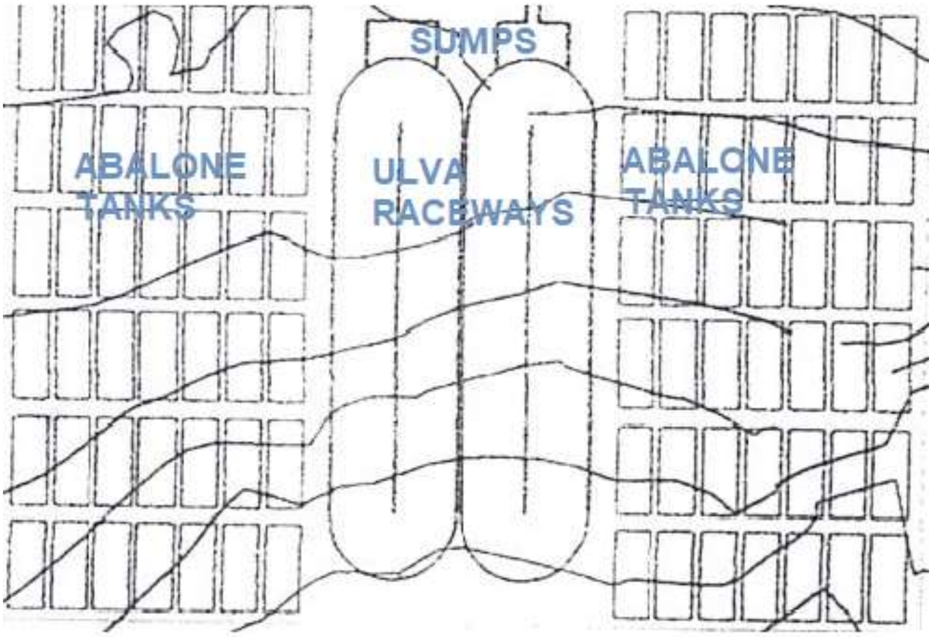

Figure 2.1- Basic schematic of two modular clusters on the farm (curved lines show contours)

Figure 2.1 shows the general layout of two modular clusters. Although positioned side by side, the clusters are isolated from each other; each has their own sump attached to the *Ulva* raceway which receives half it’s water from the raceway, and half of the volume is pumped directly in from the sea.

Volume of the *Ulva* raceways is 300 000 litres, volume of each abalone tank is 8000 litres, and sump volumes are approximately 25 000 litres. Water flow from the sump to the abalone tanks is spread across 84 taps, with two taps feeding each tank (42 tanks in each cluster) and each individual tap outputting a volume of 18.1 litres per minute. Each row of abalone tanks has seven tanks, each with two taps for 14 taps per row

$$14\ (taps) \times 18.1(l\ per\ minute)\ = 253.4\ l\ per\ minute$$

Each row has a single effluent pipe, which discharges 253.4 litres per minute in to the *Ulva* raceway to maintain approximately constant volume in each abalone tank.

$$6\ (effluent\ pipes)\ \times 253.4\ l\ per\ minute\ = 1520.4\ l\ per\ minute$$

The *Ulva* raceway then receives 1520.4 litres per minute of water from all of the abalone rows combined and discharges approximately half of this volume as effluent to the sea, and half of this volume flows by gravity back to the sump for recirculation.

2.2 Experiment One- Variable Recirculation

Sampling regime

Experiment one was conducted as a pilot investigation to attempt to quantify changes in temperature, pH and TAN (Total Ammonia Nitrogen) concentration in modular clusters operating variable rates of recirculation where recirculation percentage was switched one hour before commencement of sampling so that the systems were recirculating variable amounts of water by the time sampling commenced. Three modular clusters (isolated from each other) were sampled in the sump tank attached to the *Ulva* raceway as per figure 2.1 (10cm below surface) which receives recirculated water from the *Ulva* raceways and fresh seawater pumped in directly without filtration. In the sump there is a foam fractionator preventing build-up of organic matter, and the mixing this provides combined with input from the *Ulva* raceway and fresh seawater means the water is well mixed in the sump and can be assumed to be homogenous, negating the need to take multiple samples at depth. The sump was chosen as the sample point for the variable recirculation experiment as the sump water is directly pumped to the abalone baskets. *Ulva* was weighed and redistributed to give as close to practically possible biomasses between clusters, 24 hours before the experiment started to allow for equilibration of conditions. Biomass estimates were obtained from the abalone farmers. As can be seen in figure 2.2 below, one modular cluster was operating at a 50% recirculation for the duration of the experiment (72 hours, sampling every 6 hours, beginning at midnight on the 5th December 2018 and ending at midnight on the 8th of December 2018), one modular cluster was operating at 75% recirculation for the duration, and one cluster was operating at 100% recirculation for the duration. Recirculation percentage was adjusted one hour prior to commencement of sampling run one. Ambient conditions were measured in the main inlet to the farm.

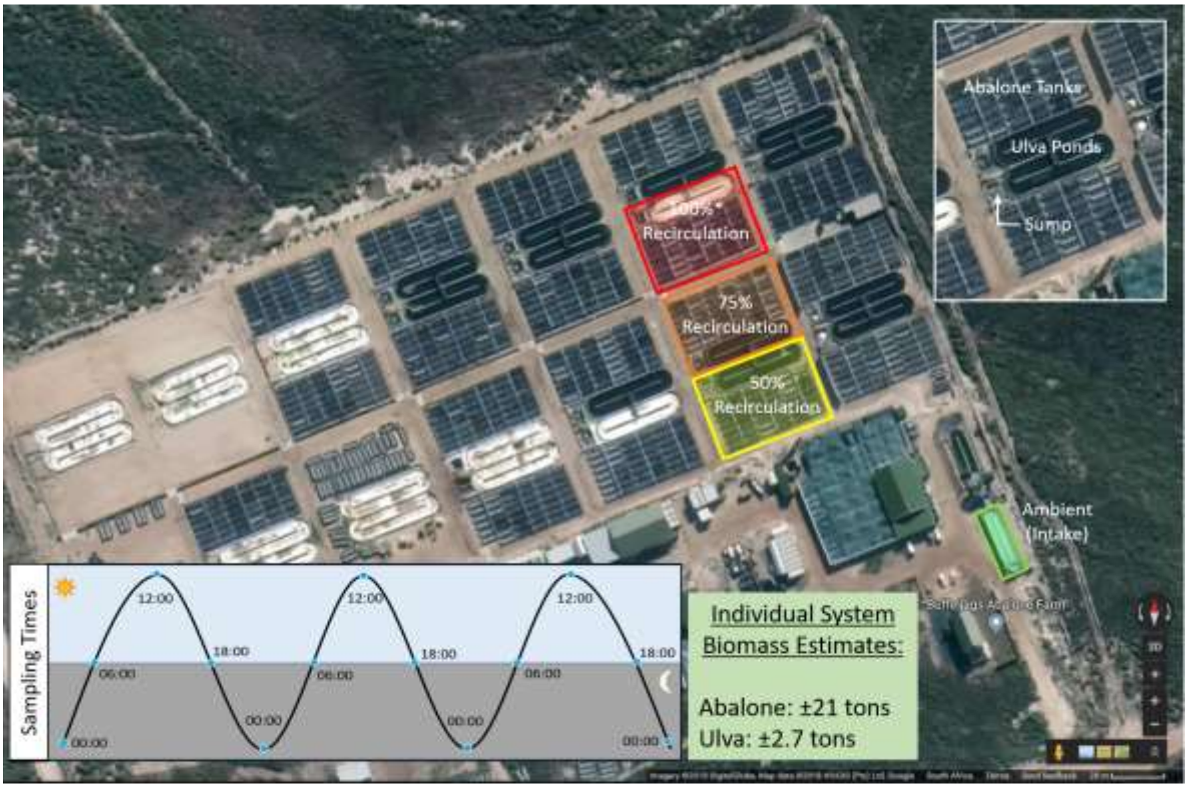

Figure 2.2- Satellite image showing clusters with sumps (sampling points) and ambient collection point, with zoomed image top right (Google Earth, November 2018)

Testing regime- varied recirculation experiment

Four samples were collected immediately at the start of each sampling run, one from each sump and one from the farm inlet, and tested for temperature, pH and conductivity using a DTK2017-SD (Lovibond Tintometer GmbH.) multi meter with automatic temperature compensation set to a working temperature of 25°C. Probes were calibrated at the beginning of each sampling run (3 point calibration at pH 4, 7 and 10 for pH probe, one point calibration for conductivity probe at 12.88 mS/cm) and operated according to manufacturer's instructions. Total ammonia nitrogen was measured in duplicate and absorbances were adjusted for turbidity arising from reagent addition to sample water by the addition of the citrate and phenol reagents only, to the standard solutions. A blank correction was made for the spectrophotometric absorbances using low nutrient seawater which had been treated with the same reagents as the samples, to attempt to account for the matrix effect and scattering of light caused by the cuvette. TAN was measured using a modified version of the spectrophotometric Grasshoff (Grasshoff *et al.*, 1999) method employed by Department for Agriculture, Forestry and Fisheries analytical chemists, where the modification involves lower sample and reagent volumes but maintains mass ratios of approximately 25:1 phenol to available chlorine (chlorine available for reaction at all oxidation levels), recommended in the Grasshoff method. The general premise of the method is that ammonia reacts in moderately alkaline solution with excess hypochlorite to yield monochloramine which then reacts with two molecules of phenol (Aminot *et al.*, 1997), high sensitivity is achieved by adding catalytic quantities of nitroprusside ions, to produce an indophenol blue dye (Berthelot reaction), the absorbance of which is proportional to the sum of NH_4^+ and NH_3 (TAN) when irradiated with a monochromatic light source at wavelength 630nm (frequency= 4.76 x 10 E14 Hz, energy= 3.16 E-19 Joule). The pH sensitivity of colour development, which is influenced by salinity, is accounted for by the addition of NaOH in the trione reagent to buffer the pH of the solution to a level which ensures monochloramine generation sufficient for dye formation. The method provided in the handbook (Grasshoff *et al.*, 1999) involves 50ml of sample volume, with 2ml of each of the three addition reagents. The modified version involves 5ml of sample water, to which is added 0.2ml of trisodium citrate dihydrogen reagent which prevents precipitation of divalent cation salts (primarily Calcium and Magnesium oxides and carbonates) by complexation, 0.2ml of Phenol reagent which contains catalytic quantities of Disodium nitroprusside dihydrate, and 0.2ml of Trione reagent (Trione DTT) which acts as the hypochlorite donor in this reaction, and also contains sufficient sodium hydroxide solution to buffer the pH of the whole solution to a pH which will facilitate monochloramine generation for reaction with Phenol. Final colour development at these reagent concentrations takes a maximum of 6 hours, so each batch of processed samples was covered with foil and left in a dark place for 6 hours before spectrophotometric determination occurred, final absorbance is independent of temperature but strongly dependent on pH, above pH 10 in seawater, magnesium and calcium salt precipitation becomes problematic, hence the addition of NaOH to the trione reagent to achieve pH 9.8.

A calibration curve method was not utilised in this instance, rather a single accurately formulated calibration standard in the mid-range of expected concentrations (12uM) was produced and another nutrient free seawater sample tested to give a 0 uM to give a reference, these were then measured spectrophotometrically following reagent addition as previously described, giving an absorbance value which was then used as a ‘factor’ to calculate concentrations of unknown samples (see results section for calculations). This method is commonly employed by D.A.F.F. research scientists and is considered sufficient for most aquaculture research (Probyn *et al.*, 2017).

TAN stands for Total Ammonia Nitrogen, and the test gives the sum of NH_4^+ and NH_3. The equilibrium dynamics of the hydrolysis of ammonia in seawater are discussed in some detail in section 1.7, and the following free ammonia nitrogen (FAN) values were calculated according to the method described in the paper by (Bower and Bidwell, 1978). The percentage of TAN accounted for by NH_3 is determined by pH, temperature and salinity, with pH exerting the most influence over the position of the dynamic equilibrium. Values are given in the table as in table 3.5 for TAN measurements, with means and medians shown with standard deviation and absolute deviation respectively. Uncertainty in FAN measurements is complicated by the fact that the FAN concentrations are derived directly from the TAN measurement based on environmental conditions. As such, the standard deviation between replicates for the TAN readings was calculated as a percentage of the concentration, and this percentage has been applied to the FAN readings.

After determining that data were non-normally distributed by histogram plots, and that homogeneity of variance did not exist between categories (ambient, 50%, 75% and 100% recirculation) determined by creating a linear model of temperature vs category, plotting the residuals and observing heteroscedasticity; a statistical test was conducted which is described in statistical handbooks as being robust against departure from normal distribution, in this case the Mood's Median Test, which was conducted at the 95% confidence level, and pairwise median tests were conducted posthoc to determine where the differences existed.

Results are given as tables showing basic summary statistics, time series plots, and for statistical analysis Mood's Median Test's were performed followed by posthoc pairwise median analysis. The following is a quote from (Liu *et al.*, 2013) concerned with the problems associated with meaningful statistical analysis in aquaculture water testing; "Aquaculture water is an open, nonlinear, dynamic, complex system. Water quality is affected by many factors such as physics, chemistry, hydraulics, biology, meteorology, and human activities, and the water quality parameters are nonlinear, time varying, random and delayed, because of the interactions between them. Thus, it is difficult to describe them quantitatively using accurate mathematical models". The solutions to such problems mentioned in the paper include highly complex statistics such as:

- Genetic algorithms, which borrow ideas and reasoning from natural genetics and the evolutionary principle
- Novel machine learning methods based on artificial neural networks
- Support Vector Regressions, which are alternatives to neural networks based on statistical learning theory and a structural risk minimisation principle

This work was conducted on a large-scale operational aquaculture farm, logistical constraints meant it was only possible to conduct this work in single systems with adjusted recirculation percentages, as such the data are intended to give preliminary indications of changes in water quality which may arise from increasing recirculation percentages

2.3 Experiment Two- comparison of system components

Sampling regime

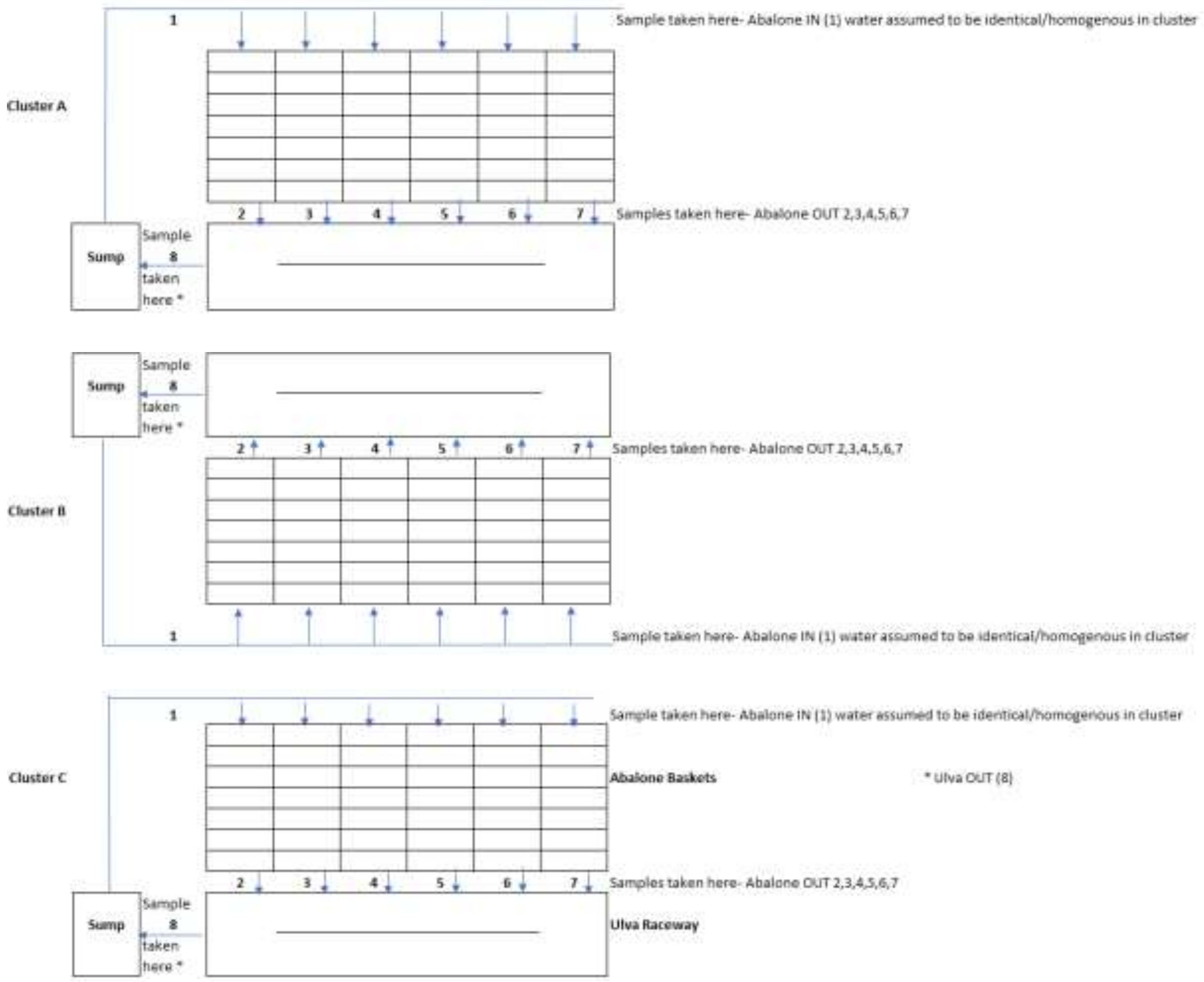

Figure 2.3- Simplified schematic of clusters with sampling points shown

Experiment two was conducted to try and ascertain patterns in the fine scale nutrient cycling of the integrated clusters, particularly with respect to nitrogenous dissolved nutrients (TAN, nitrate and nitrite). Storage of samples for later testing was unavoidable in this experiment as discussed in chapter two, so spiked samples were prepared to attempt to calibrate for the storage process (freezing and thawing) on the concentrations of the nutrients. The spiked samples showed good coherency with fresh standards in the case of TAN and nitrite, but wide and random discrepancy between prepared nitrate spikes and fresh standards. This major discrepancy in spike recovery for nitrate forced the nitrate results to be discounted and removed from the analysis. It is generally accepted that the high concentrations of chloride (Cl^-) ions in seawater (on average approximately 19 000 parts per million) greatly diminish the toxicity of nitrite (NO_2^-) to marine species as the chloride will “outcompete” the nitrite and be preferentially taken up by marine species, literature on the toxicity of nitrite to *Haliotis midae* is scarce to the point of non-existent, but it is generally accepted by South African abalone farmers that nitrite toxicity is of minimal concern (N. Loubser, December 2018); in fresh water with minimal chloride concentrations nitrite toxicity is a far more pressing concern. As such, the nitrite values are useful in this instance if taken in conjunction with the TAN and nitrate values to build a “Total Nitrogen” picture in terms of dissolved nutrients. For these reasons, the nitrate and nitrite values obtained in this experiment, have been excluded from the analysis. The parameters tested were temperature, pH, salinity, dissolved oxygen, TAN, nitrate and nitrite; nitrate and nitrite were discounted so temperature, pH, salinity, dissolved oxygen and TAN are the remaining parameters to be presented, these were selected as aquafarmers consider them to be of particular importance to their stock, and they are the parameters most often encountered in IMTA literature. This experiment was designed to determine the effectiveness of the seaweed biofilter in the modular clusters, and to measure environmental conditions not just as stand-alone parameters, but also as influencers of the ammonia equilibrium.

Experiment two was conducted as a more in-depth analysis of the specific changes in water quality parameters arising from a 50% recirculation (standard farm operation). The volumes and flow rates are as described in section 2.1 and the biomasses in each modular cluster are as follows in table 2.1:

Table 2.1- Biomass estimates for clusters A,B and C

Cluster	Abalone biomass (kg)	*Ulva* biomass (kg)
A	9 837	1 061
B	10 534	1 094
C	13 904	1 105

An intense 24 hour period of sampling was conducted beginning at midnight on the 13th June 2019 and ending at midnight on the 14th of June 2019, again there was no particular reason for choosing this time of year, as there was no single identifiable gap in literature it may have been useful to try and fill. Samples were collected from eight different points in three clusters (A, B and C, figure 2.3) and from the main farm inlet (ambient). Each cluster was sampled consecutively with each separate cluster sampling taking 2 hours, with one sample being taken from the abalone tank inflow water, six samples being taken from each of the six abalone effluent pipes, and the final 8th sample being taken from the exit sluice of each *Ulva* raceway. Samples were collected one after the other in batches of 9 (8 samples from the modular cluster, and one from the ambient seawater).

Testing regime

Upon collection, each individual sample was tested immediately with a DTK-2017SD (Lovibond Tintometer GMbH.) meter (automatic temperature compensation to a working temperature of 25°C) for pH, conductivity/salinity and dissolved oxygen, whilst another subsample was filtered through a glass fibre filter with a nominal pore size of 0.45 uM and frozen immediately for later dissolved nutrient analysis; this was done in an upright position to avoid issues of spatial freezing leading to analyte loss through sample container lid as per standard practice (Grasshoff *et al.*, 1999). Samples were frozen in this instance as the number of samples and intensity of each sampling run was such that immediate analysis would have been impossible. Time from sample collection to probe testing and freezing at $^{-}$18 °C to $^{-}$20 °C for each sample was no more than fifteen minutes. Following sample storage and probe testing, frozen samples were conveyed at the end of the sampling runs back to the DAFF marine research aquarium in Seapoint, Cape Town, where they were thawed in batches to room temperature and analysed spectrophotometrically for TAN, nitrite and nitrate within 4 days of sample collection.

Total Ammonia Nitrogen

The TAN method in this instance was similar to experiment one in terms of reagent addition and analysis, except a single accurately-made standard of 4uM was prepared which was then used as a 'factor' to determine unknown concentrations from absorbances in accordance with the Beer-Lambert Law. A lower concentration calibration standard was used in this instance as recirculation percentage was standard farm operation of 50%, and anticipated concentrations were lower. To try to calibrate for the storage and freezing/thawing process, during the sampling runs of cluster C, the 2nd abalone effluent pipe water from the cluster (C3) was collected in the same way as other samples, and then a subsample was used to prepare 'spiked standards'. To prepare a 4uM standard, 100ml of dilute standard was prepared by the addition of 1ml of concentrated stock standard containing 10uM mL^{-1} N (NH_4Cl) to 99ml of MilliQ water (100uS/cm) which gives a dilute standard concentration of 100uM L^{-1}. 1ml of this dilute stock standard is then made up to a final volume of 25ml using low nutrient seawater to account for matrix effect (T. Probyn, pers. comm., December 2019) to yield a final concentration of 4uM L^{-1}. For the spiked frozen standards, the same dilute stock standard was produced as described (100uM L^{-1}) and 1mL of this dilute stock standard was made up to a volume of 25ml using sample water from C3 (second abalone effluent pipe in cluster C). The absorbance of this 'spiked' standard should then be approximately equal to the absorbance of the freshly made standard plus the absorbance of the sample water. When analysis is carried out, the absorbance of

C3 water is subtracted from the absorbance of the spiked standard, to give an absorbance which can then be compared to that of a freshly made 4uM standard. This allows the analyst to get a good idea of how the freezing/thawing storage process is altering the analyte concentrations by comparing the spiked standard absorbance to the freshly made standard absorbance. If there is a wide variation between the two, then the frozen standard absorbances were used to calculate unknown concentrations.

For the spectrophotometric TAN method used here, uncertainty is minimised during the mechanical and chemical process of sample preparation by the following steps:

- Sample pH is standardised by the addition of NaOH to each discrete replicate, in this case the addition of NaOH to the citrate reagent
- Impact of impurities is minimised by preparing standard solutions in low nutrient seawater and filtering samples across a clean, flushed filter
- The uncertainty caused by reaction between sample and reagents is minimised by the preparation of a 'turbidity blank', in this case the turbidity blank was generated as per the methods section (addition of phenol and trione reagent ONLY to a standard solution) and the absorbance of the turbidity blank is then subtracted from the standard solution absorbances following addition of all three testing reagents
- The error arising from the light scattering caused by the sample cuvette and sample matrix are minimised by the preparation of a low nutrient seawater calibration standard, which should contain zero analyte (ammonia). The absorbance of this low nutrient seawater standard after addition of the method reagents, is subtracted from all sample and standard absorbances
- The volume of reagent can also introduce significant uncertainty, this is minimised in this methodology by a reduction in sample volume. The Grasshoff method in the handbook (Grasshoff *et al.*, 1999) utilises 50ml of sample reagent and 2ml of each of the testing reagents, this modified version utilises the exact same testing procedure but the sample and testing reagent volumes are scaled down by a factor of ten, 5ml of sample reagent, and 0.2ml of each testing reagent were used
- There was no dilution involved in this methodology
- The final absorbances in the Grasshoff method are not influenced by temperature
- Spatial distribution of analyte concentration can cause uncertainties, to try and achieve homogeneity in the sample, samples were mixed thoroughly using an electronic agitator after the addition of the testing reagents

Despite steps taken to minimise uncertainty and error in measurement, there are other sources of error in the measurements, including instrumental uncertainty of the glassware, pipettes and balances used in the production of standard solutions and testing reagents, as well as the calibration uncertainty of the spectrophotometer. A recent report of abalone farm effluents drafted by DAFF analytical chemists (Probyn *et al.*, 2017), used the same method as the one used in this instance, and uncertainties in TAN are given in the report as standard deviation between replicate measurements. For this reason, uncertainty in the TAN measurements will be given as the standard deviation between replicate measurements across the treatment (ambient, 50, 75 and 100% recirculation).

Total ammonia nitrogen readings were analysed with the objective of determining TAN removal from the water stream as it passes through the *Ulva* raceways. As such, the analysis was conducted using the amount of ammonia that passes through the abalone effluent pipes in to the *Ulva* raceway, and the water that leaves the *Ulva* raceway. To calculate the reductions, the per-minute flow rates were determined for each abalone effluent pipe (litres per min), and the per-minute flow rate for water leaving the *Ulva* raceway was determined. The absolute concentration of TAN in the water was then determined on a mg/l basis, and the concentration was multiplied by the volume of water per minute. Example calculations are given below:

Number of abalone effluent pipes in cluster = 6

Number of *Ulva* effluent sluices in cluster = 1

$$Abalone\ effluent\ pipe: Volume\ of\ water\ per\ min. = 253\ litres$$

$$Ulva\ effluent\ sluice: Volume\ of\ water\ per\ min. = 759\ litres$$

$$Rounded\ concentration\ of\ water\ leaving\ abalone\ effluent\ pipes\ @\ time\ of\ testing\ (\frac{mg}{l}):$$

$$Pipe\ 1: 0.09$$

$$Pipe\ 2: 0.10$$

$$Pipe\ 3: 0.13$$

$$Pipe\ 4: 0.19$$

$$Pipe\ 5: 0.13$$

$$Pipe\ 6: 0.09$$

$$TAN\ per \min leaving\ pipe\ 1 = 253l\ \times \frac{0.09mg}{l}$$

$$= 21.71\ mg\ TAN\ per\ min$$

$$TAN\ per \min leaving\ pipe\ 2 = 253l\ \times \frac{0.10mg}{l}$$

$$= 24.62\ mg\ TAN\ per\ min$$

Calculations identical for pipes 3-6, combined total for 6 pipes added together to give total mg TAN per min leaving abalone effluent pipes and entering *Ulva* raceway

$$Total\ TAN\ per \min leaving\ abalone\ effluent\ pipes\ (mg\ TAN\ per\ minute) = 21.71 + 24.62 + 33.33 + 47.55 + 32.87 + 23.70 = 183.78\ mg\ TAN\ per\ min$$

$$Rounded\ concentration\ of\ water\ leaving\ Ulva\ effluent\ sluice\ @\ time\ of\ testing\ \left(\frac{mg}{l}\right) = 0.07$$

$$TAN\ per \min leaving\ sluice = 0.07\frac{mg}{l} \times 759l$$

$$= 56.88\ mg\ TAN\ per\ min$$

$$Reduction\ in\ TAN = Total\ TAN\ per \min leaving\ abalone\ eff.pipes - Total\ TAN\ per \min leaving\ Ulva\ sluice$$

$$183.78 - 56.88 = 126.90\ mg\ TAN$$

Removal then converted to a percentage of total input and worked out identically in all instances for both TAN and FAN.

3. Results and Discussion

3.1 Experiment One results

Experiment one was conducted as a preliminary investigation to gauge how increasing recirculation percentage effects certain water quality characteristics, relative to a standard farm operation recirculation of 50%. Results will be given for separate parameters, and then discussed individually and more broadly as interplaying parameters.

Temperature

Table 3.1 shows minimal difference between means and median between treatments and the global mean/median, both mean and median were calculated but the non-normal spread of data meant that median was more useful in describing the average. The most noticeable differences occur between the maximum value at 100% recirculation and the maximum temperatures in the other treatments where the maximum temperature at 100% recirculation was approximately 1.5°C warmer than the maximum value at 50% and 75% recirculation as well as ambient conditions. Uncertainties in the temperature measurements are primarily limited by the uncertainty provided by the manufacturer for the temperature probe, which is given as ± 0.08°C. The probe was regularly checked against a mercury thermometer and the agreement was good. Table 3.1 shows minimum and maximum values are given as ± 0.08°C; for the mean values, the measurement is given as observed mean ± standard deviation. For the median values, the measurement is given as observed median ± median absolute deviation.

Table 3.1- Basic summary statistics for Temperature in experiment one (52 observations across 4 sampling points, 13 observations in each)

	Minimum (± 0.08°C)	Mean (± s.d.)	Median(± m.a.d.)	Maximum (± 0.08°C)
Temperature (°C) (all sampling points) 52 obs.	18.2	19.2± 0.9	19.0± 0.7	22.4
Temperature (°C) (Ambient) 13 obs.	18.5	19.1± 0.5	19.0± 0.6	19.9

Temperature (°C) (50% Recirc.) 13 obs.	18.3	19.2± 0.9	18.7± 0.6	21.0
Temperature (°C) (75% Recirc.) 13 obs.	18.2	19.1±0.7	18.9±0.6	20.5
Temperature (°C) (100% Recirc.) 13 obs.	18.2	19.5±1.3	19.3±1.2	22.4

Figure 3.1 is a time series plot of temperature for all treatments across the 72-hour sampling period. Temperatures across treatments are similar during night-time when temperatures are lower. During daytime and late afternoon in particular when temperatures are higher; the 50% and 75% recirculation clusters show minor increases compared to ambient, and at 100% recirculation the temperature increase is considerable.

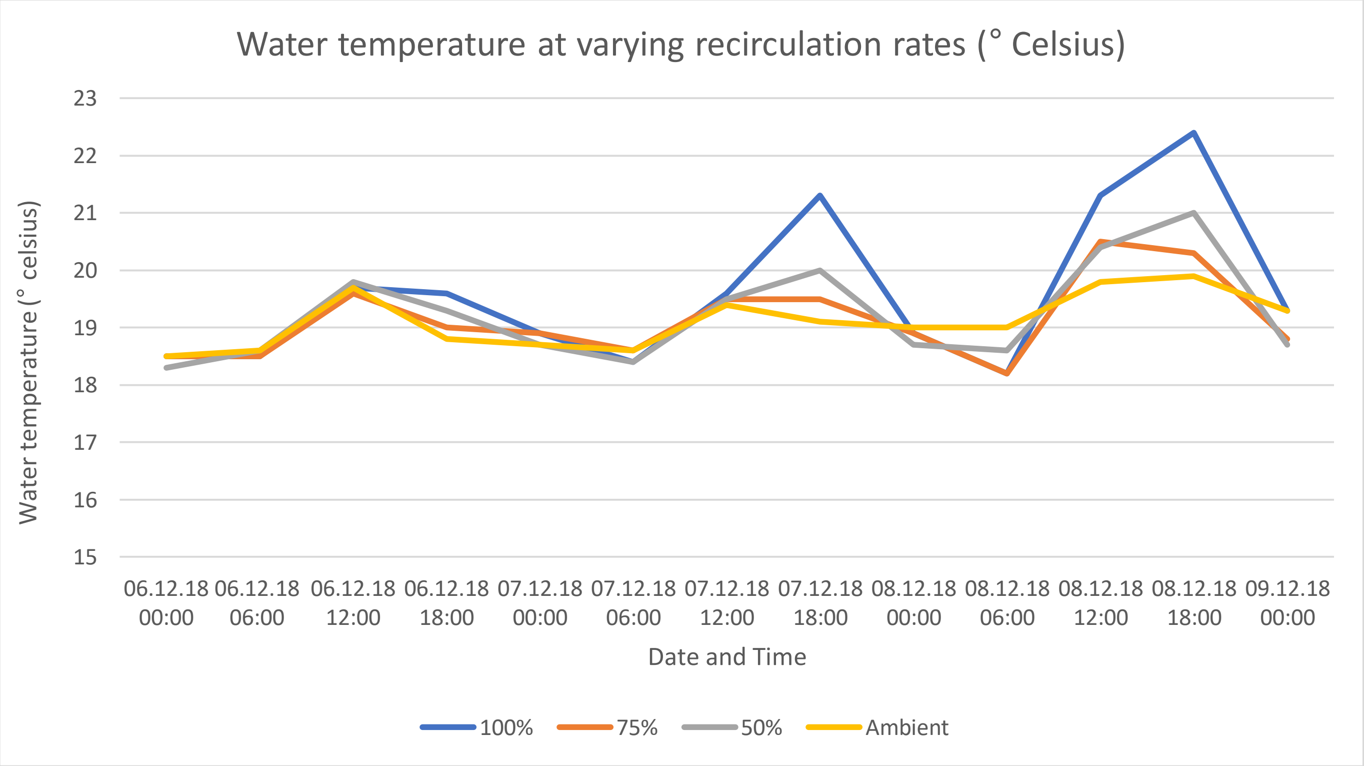

Figure 3.1- Time series of temperature experiment one

Results for the Mood’s Median Test and posthoc pairwise test for temperature (table 3.2), which demonstrate no significant differences among medians; this is unsurprising considering the trends observed in figure 3.2 and medians shown in table 3.1. The differences between medians among the categories are so small that the output from the test shows no significant difference between any of the clusters or the ambient conditions; this is unsurprising as the test looks for significant differences between medians, the main difference in temperature occurs only in the late afternoon when the water has been warming through the day, but the differences in median are small. The 100% recirculation cluster temperature appear to be elevated during daytime sampling runs (12:00, 18:00), and for the abalone farmer the plot is descriptive enough to inform them that temperature increases during the summer would rapidly become a problem at 100% recirculation.

Comparison	p value
Ambient-50% Recirc	1.0000
Ambient- 75% Recirc	0.6971
Ambient- 100% Recirc	0.7005
50%-75% Recirc	1.0000
50%-100% Recirc	0.7005
75%-100% Recirc	0.7005

Table 3.2- Pairwise median test of temperatures (DF=3, α=0.05)

pH

Summary statistics for pH are given in table 3.3, during the sampling runs, the probe was regularly checked against buffer solutions and calibrated to ensure that it was reasonable to use the manufacturers stated instrumental uncertainty. It is clear in the table that pH was consistently lower in the 100% recirculation cluster, with the minimum, maximum, mean and median levels the lowest in any treatment. It can be observed in the table that pH levels decrease going from ambient to 100% recirculation, but the difference is only distinct in the 100% recirculation cluster. Differences between pH in the ambient, 50 and 75% recirculation clusters do exist.

Table 3.3- Basic summary statistics for pH in experiment one (52 observations across 4 sampling points, 13 observations in each)

	Minimum (±0.02pH units)	Mean(±s.d.)	Median(±m.a.d.)	Maximum (±0.02pH units)
pH (pH units) all sampling points (52 obs.)	7.02	8.00±0.32	8.07±0.26	8.49
pH (pH units) Ambient (13 obs.)	7.74	8.15±0.17	8.23±0.07	8.39
pH (pH units) 50% recirc. (13 obs.)	7.51	8.11±0.26	8.13±0.30	8.49
pH (pH units) 75% recirc. (13 obs.)	7.50	8.09±0.24	8.05±0.12	8.41
pH (pH units) 100% recirc. (13 obs.)	7.02	7.64±0.31	7.55±0.27	8.14

Figure 3.2 below is a time series plot for pH readings across all treatments for the duration of the experiment. The pH is consistently lower (approximately 0.5 pH units) in the 100% recirculation cluster compared to the other clusters, and differences in pH between ambient and 50 and 75% recirculation are negligible. The ambient line is smoother than the three recirculation treatments, the peaks and troughs are less pronounced although a similar pattern is observed. Peaks in pH are observed at midday during photosynthesis, with lower pH at night-time and in the evening, when the abalone and seaweed are respiring.

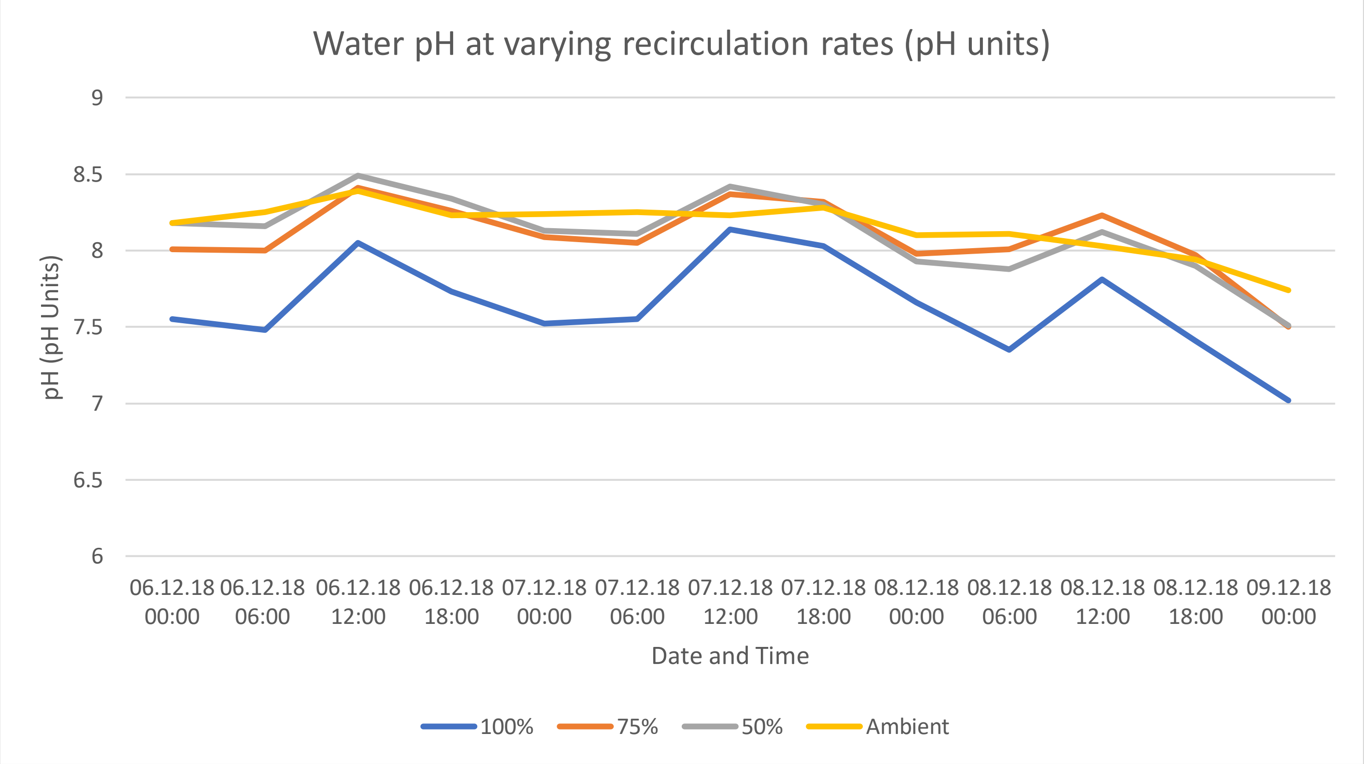

Figure 3.2- Time series of pH across all 12 sampling runs

The results of a Mood’s Median Test and posthoc pairwise median test are displayed in table 3.4 below (* denotes significant differences). The significant differences occur between the ambient conditions and the 100% recirculation cluster, the 50% recirculation cluster and the 100% recirculation cluster, and the 75% recirculation cluster and 100% recirculation cluster. The lack of statistically significant differences between ambient conditions/50% recirculation cluster, ambient conditions/75% recirculation cluster and 50%/75% recirculation clusters is coherent with trends observed in figure 3.2 and the values observed in table 3.3.

Comparison	p value
Ambient-50% Recirc	0.2486
Ambient- 75% Recirc	0.2486
Ambient- 100% Recirc	<0.001 *
50%-75% Recirc	0.2486
50%-100% Recirc	<0.001 *
75%-100% Recirc	<0.001 *

Table 3.4- Pairwise median test of pH (DF=3, α=0.05)

Ammonia

In table 3.5 below, the means are given as mean ± standard deviation, medians as median ± median absolute deviation, and the minimum and maximum values are given as the observed measurement± standard deviation between replicate measurements.

The trends in the table include:

- Concentrations in 100% recirculation cluster are markedly higher than concentrations in all other treatments
- Concentrations in the 75% recirculation cluster are higher than the 50% recirculation cluster
- Ambient concentrations and 50% show minimal variation in median, means show wider variation (approximately three times higher in 50%) suggesting that outliers and extreme results are exaggerating the variation in mean, reasons for these outliers may be fluctuations in ammonia uptake by the *Ulva* or increased waste production by the abalone

Table 3.5- Basic summary statistics for TAN in experiment one (52 observations across 4 sampling points, 13 observations in each)

	Minimum	Mean(± s.d.)	Median(± m.a.d.)	Maximum
TAN (µM/l) Ambient (13 obs.)	0.15± 0.031	0.45± 0.36	0.31± 0.23	1.38± 0.031
TAN (µM/l) 50% recirc. (13 obs.)	0.15± 0.078	1.50± 2.05	0.46± 0.34	7.15± 0.078
TAN (µM/l) 75% recirc. (13 obs.)	0.15± 0.089	2.96± 4.39	0.84± 0.80	13.46± 0.089
TAN (µM/l) 100% recirc. (13 obs.)	19.08± 0.141	33.75± 13.37	30.69± 13.12	59.85± 0.141

Figure 3.3 below is a time series plot of TAN concentration with a unit conversion from µM/l to mg/l for the duration of the experiment, unit converted as mg/l used in abalone dialogue standards. Trends in table 3.5 and figure 3.3 show that ammonia builds up rapidly at 100% recirculation. As the 100%

recirculation line extends the y axis so noticeably, figure 3.4 is plotted below so that the trends in the ambient, 50 and 75% recirculation cluster are more identifiable.

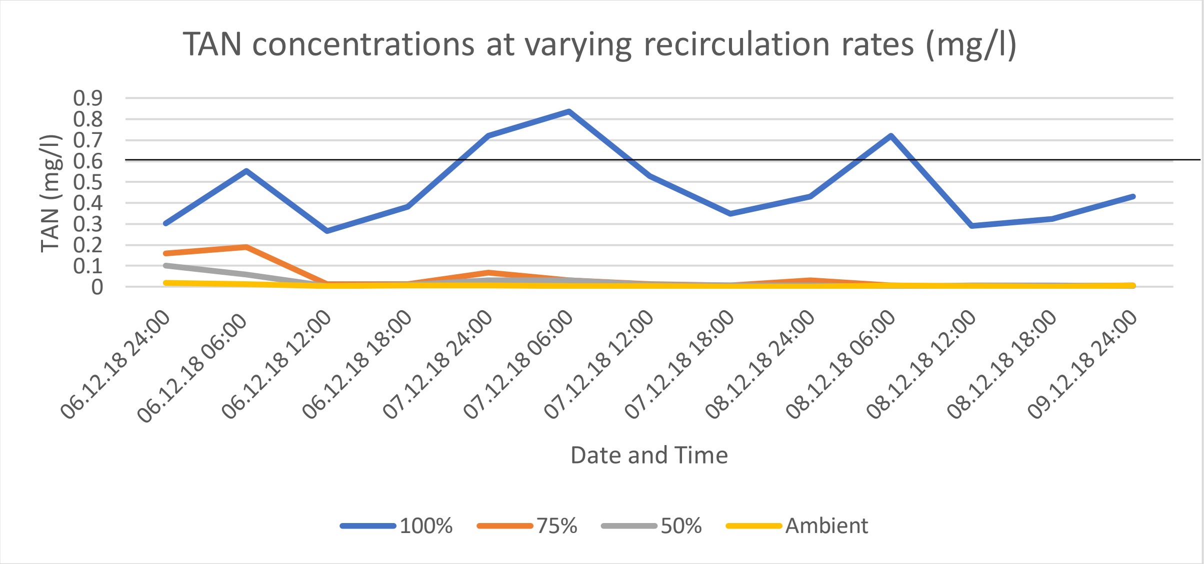

Figure 3.3- Total ammonia Nitrogen (TAN) concentrations over time showing the WWF abalone dialogue limit (standard deviations between measurement replicates below 0.0028mg/l so error bars excluded)

Figure 3.4 is identical to figure 3.3 except for the removal of the 100% recirculation sampling run. This was done so that the trends in the other recirculation clusters and ambient conditions could be more readily observed. The interesting outcome of the removal of the 100% recirculation series, is that the comparatively high TAN concentrations in the first two sampling runs become evident. Interestingly, in the ambient conditions and 50% recirculation cluster the higher of the two readings occurs at midnight, whereas in the 75% recirculation cluster the highest reading is at 06:00. Later in the time series the 75% recirculation peaks in ammonia occur at midnight. After the first two sampling runs, the readings converge again between 0-2µM/l, before another peak in concentration is observed at midnight in the 50% and the 75% recirculation clusters. By midday, the lines have converged again between 0-2µM/l before there is another spike at midnight in the 75% recirculation cluster only. This would appear to demonstrate ammonia levels do increase in a 75% recirculation compared to a 50% recirculation, but consideration of the unionised ammonia fraction is of crucial importance.

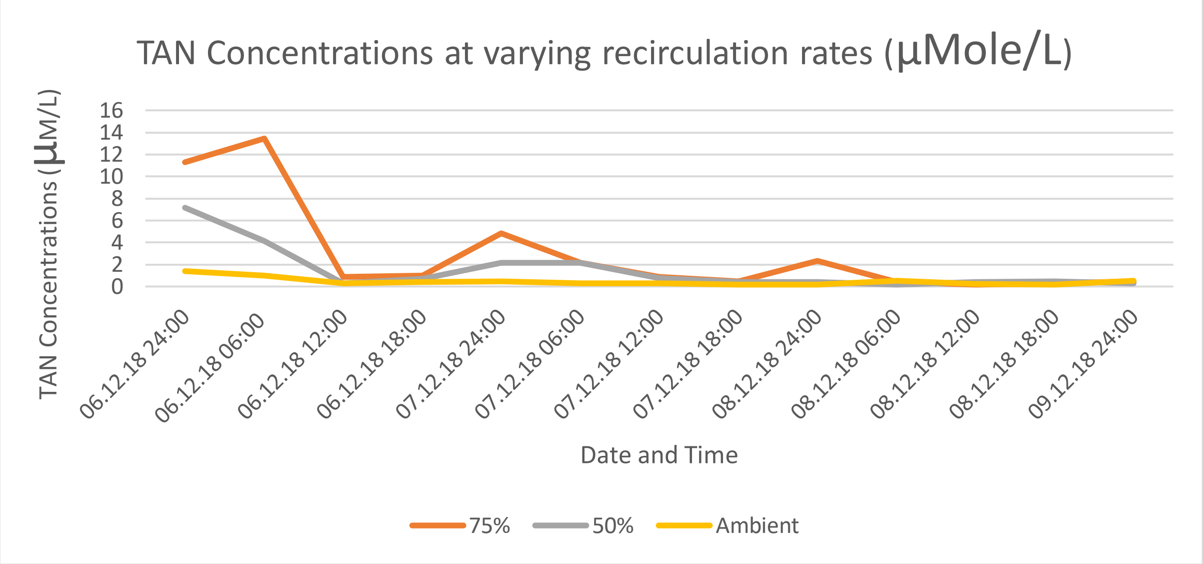

Figure 3.4- Time series of TAN across all 12 sampling runs with 100% recirculation excluded (standard deviation between measurement replicates all below 0.2μM/l so error bars excluded)

Table 3.6 below shows the results for the statistical tests performed on the TAN observations. Statistically significant differences occurred between ambient-100% recirculation, 50-100% recirculation and 75-100% recirculation. No statistically significant difference between ambient conditions and the 50/75% recirculation cluster, and no statistically significant difference between the 50 and 75% recirculation clusters.

Table 3.6- Pairwise median test, TAN (DF=3, α= 0.05)

Comparison	p value
Ambient-50% Recirc	0.4404
Ambient- 75% Recirc	0.1228
Ambient- 100% Recirc	<0.001 *
50%-75% Recirc	0.2486
50%-100% Recirc	<0.001 *
75%-100% Recirc	<0.001 *

Table 3.7 below shows basic summary statistics for free ammonia nitrogen results. These numbers were derived from the measurements as described in methods section.

Table 3.7- Basic summary statistics for FAN in experiment one (52 observations across 4 sampling points, 13 observations in each)

	Minimum	Mean (± s.d.)	Median (± m.a.d.)	Maximum
FAN (µg/L) Ambient (13 obs.)	0.05± 0.010	0.27± 0.25	0.23± 0.17	0.84± 0.010
FAN (µg/L) 50% recirc. (13 obs.)	0.04± 0.021	0.85± 1.16	0.38± 0.49	4.03± 0.021
FAN (µg/L) 75% recirc. (13 obs.)	0.06± 0.036	1.32± 1.67	0.85± 1.05	5.22± 0.036
FAN (µg/L) 100% recirc. (13 obs.)	3.38± 0.025	7.17± 4.42	5.93± 1.51	19.72± 0.025

Figure 3.5 below shows FAN concentrations across the three-day sampling period, at 100% recirculation the peaks occur at midday, in contrast to the TAN reading peaks which occur at 0600. Trends in 50,75% recirculation clusters and ambient conditions are again difficult to discern; so figure 3.6 below shows the same plot with the 100% recirculation line removed.

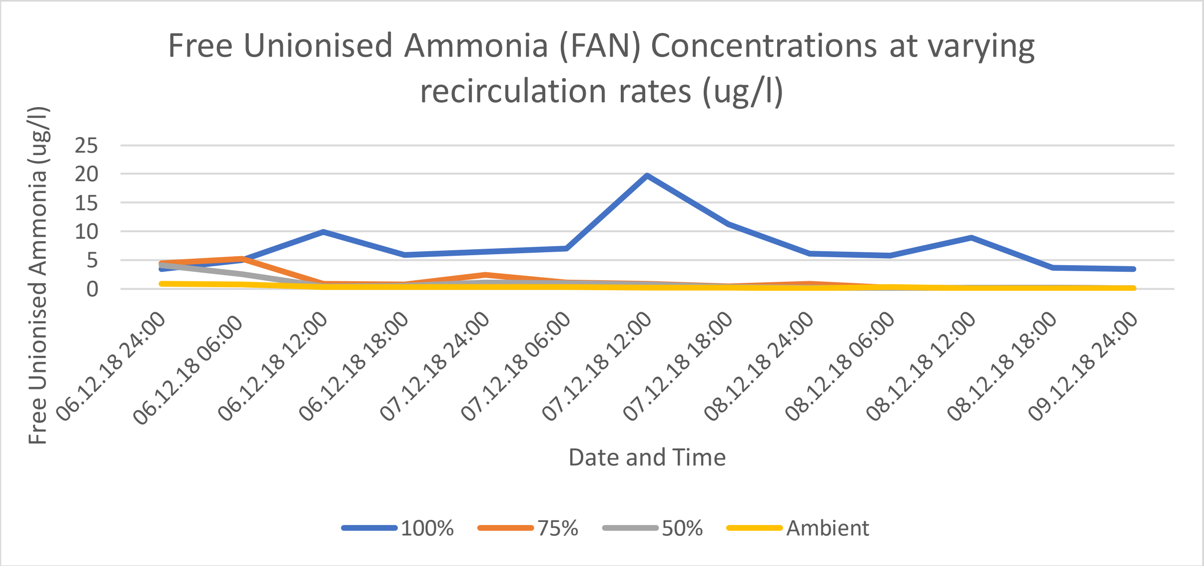

Figure 3.5- Time series of FAN across all 12 sampling runs (standard deviations between measurement replications all below 0.1µg/l so error bars excluded)

Figure 3.6 below shows peaks in FAN concentration in the 75% recirculation cluster coinciding with peaks in TAN concentration in the 75% recirculation cluster. Ambient FAN concentrations are low (below 1µg/l), 50% recirculation FAN concentrations show peaks coinciding with the peaks in TAN concentration show in figure 3.4.

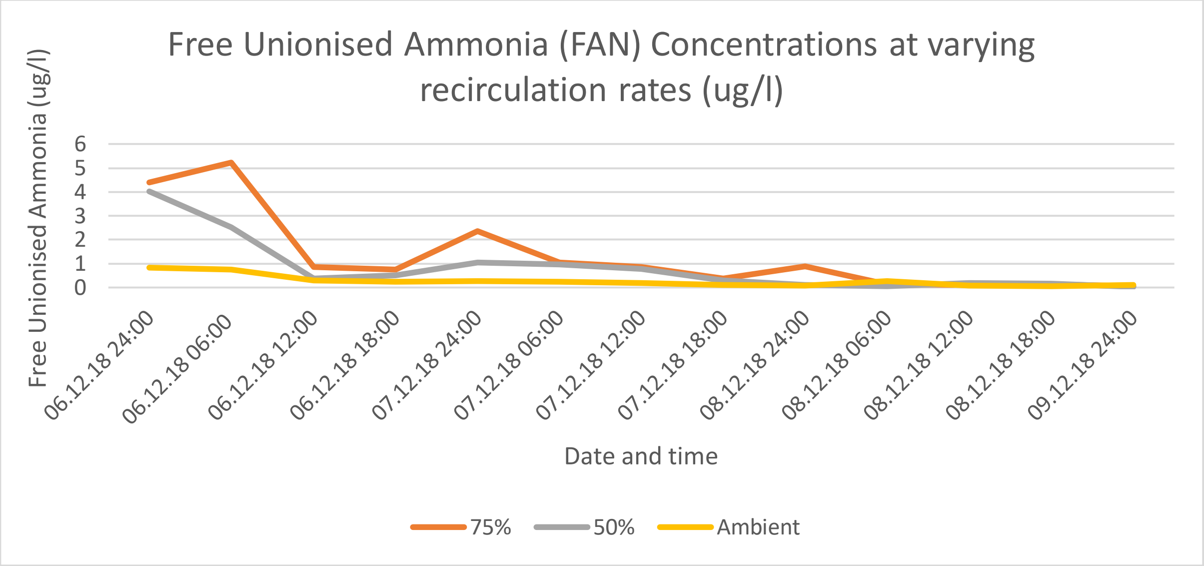

Figure 3.6- Time series of FAN across all 12 sampling runs with 100% recirculation excluded (standard deviations between measurement replications all below 0.1µg/l so error bars not included)

Table 3.8 below shows the results of the pairwise median test for FAN concentrations. Significant differences between ambient/50/75% recirculation clusters and the 100% recirculation cluster. This picture is coherent with the TAN median test results, though the difference between ambient and 75% recirculation is very nearly significant.

Comparison	p value
Ambient-50% Recirc	0.2486
Ambient- 75% Recirc	0.05447
Ambient- 100% Recirc	<0.001 *
50%-75% Recirc	0.2486
50%-100% Recirc	<0.001 *
75%-100% Recirc	<0.001 *

Table 3.8- Pairwise median test, FAN (DF=3, α= 0.05)

3.2 Experiment Two Results

Temperature

Table 3.9 shows water temperatures at various sampling points within the modular clusters over the course of the 24-hour experiment. The variation between sampling points (abalone in, abalone out and *Ulva* out) is minimal. As would be expected, temperatures increase during the day (inside the black box) compared to the night-time observations. Table 3.10 shows no statistically significant differences between sampling points over the whole 24-hour sampling period which is unsurprising when the very minor variations in figure 3.8 are considered.

Table 3.9- Basic summary statistics for temperature in experiment two (36 observations across three sampling points, 12 observations in each)

	Minimum (± 0.08°C)	Mean (± s.d.)	Median(± m.a.d.)	Maximum (± 0.08°C)
Temperature (°C) (Abalone IN) 12 obs.	14.1	14.9± 0.6	14.7± 0.5	16.1
Temperature (°C) (Abalone OUT) 12 obs.	13.9	14.7± 0.9	14.4± 0.7	16.2
Temperature (°C) (*Ulva* OUT) 12 obs.	13.6	14.6± 1.0	14.3± 0.6	16.4

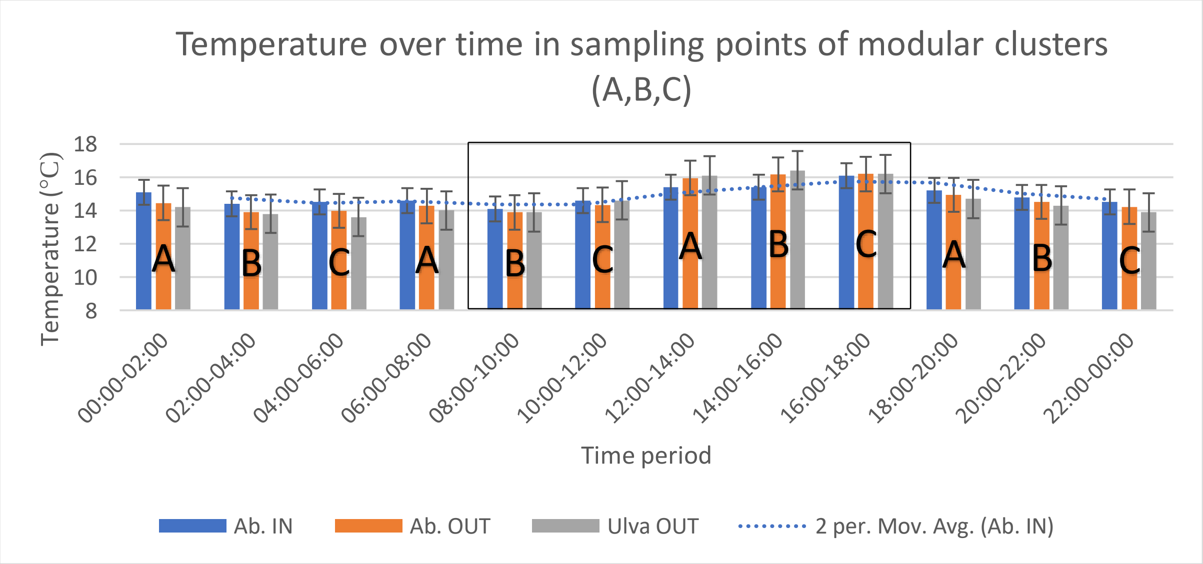

Figure 3.8- Temperature over time across three modular clusters (A,B,C) with black box showing sampling during day light hours (distributions abnormal so error bars show interquartile ranges)

Table 3.10- Pairwise median test for temperature (DF=2, α.= 0.05)

Comparison	p value
Abalone IN-Abalone OUT	0.1099
Abalone IN- *Ulva* OUT	0.4176
Abalone OUT-*Ulva* OUT	0.4241

pH

Table 3.11 shows water pH as it moves through the system from the abalone inlet to the *Ulva* outlet, means and medians of the observations show the lowest water pH is to be found in the abalone out, where the narrowest range was also found. Figure 3.9 below suggest diurnal patterns in the water pH, between the sampling points at night time and the last sampling time of the day (16:00-18:00) the abalone inbound water has the highest pH, with the abalone outbound and *Ulva* outbound water showing approximately coherent and lower pH than the abalone in water. From 08:00-16:00 the *Ulva* outbound water has the highest pH, with abalone inbound water second and unsurprisingly abalone outbound water the third and lowest pH. The pH appears to increase during daytime as would be expected. Table 3.12 shows statistically significant differences in median between the abalone inbound and abalone outbound water, and the abalone inbound and *Ulva* outbound water. This makes sense when the graph is considered as the abalone out and *Ulva* out water are very similar in pH during night-time and the first few hours of day-time.

Table 3.11- Basic summary statistics for pH in experiment two (36 observations across three sampling points, 12 observations in each)

	Minimum (± 0.02pH units)	Mean (± s.d.)	Median(± m.a.d.)	Maximum (± 0.02pH units)
pH (pH units) (Abalone IN) 12 obs.	7.71	7.84± 0.09	7.82± 0.06	8.03
pH (pH units) (Abalone OUT) 12 obs.	7.57	7.64± 0.06	7.63± 0.06	7.79
pH (pH units) (*Ulva* OUT) 12 obs.	7.52	7.73± 0.22	7.64± 0.11	8.15

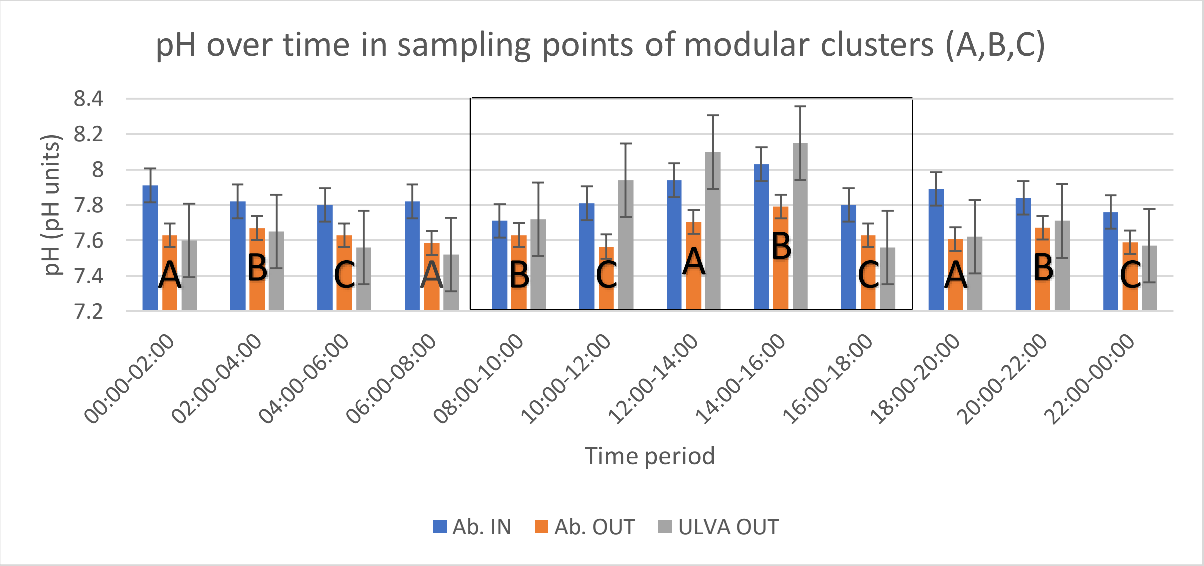

Figure 3.9- pH over time across three modular clusters (A,B,C) with black box showing sampling during daylight hours (distributions abnormal so error bars show interquartile ranges)

Table 3.12- Pairwise median test for pH (DF=2, α =95%)

Comparison	p value
Abalone IN-Abalone OUT	6.428 e-05 *
Abalone IN- *Ulva* OUT	0.04494 *
Abalone OUT-*Ulva* OUT	0.6884

Dissolved Oxygen

Table 3.13- Basic summary statistics for Dissolved Oxygen in experiment two (36 observations across three sampling points, 12 observations in each)

	Minimum (± 0.4 mg/l)	Mean (± s.d.)	Median (± m.a.d.)	Maximum (±0.4 mg/l)
Dissolved Oxygen (mg/l) Abalone IN 12 obs.	9.2	10.1± 0.4	10.2± 0.4	10.5
Dissolved Oxygen (mg/l) Abalone OUT 12 obs.	8.5	9.1± 0.5	9.1± 0.7	9.8
Dissolved Oxygen (mg/l) *Ulva* OUT 12 obs.	8.5	10.1± 1.1	9.8± 1.0	11.6

Table 3.13 shows that the dissolved oxygen in the abalone out water was the lowest of the three sampling points, and once again the graph in figure 3.10 is needed to discern that the dissolved oxygen appears highest in the abalone inbound water at night time, and the *Ulva* outbound water during the day time. Dissolved oxygen levels apparently increase during the day in the *Ulva* outbound water, decrease in the abalone outbound water and stay approximately constant in the abalone inbound water.

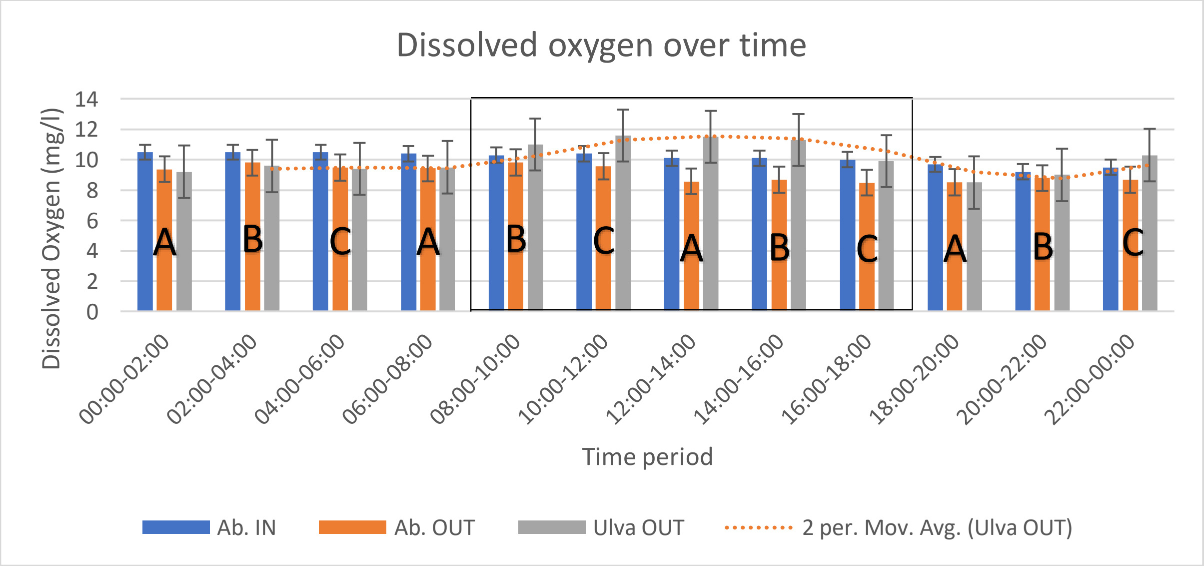

Figure 3.10- Dissolved oxygen over time across three modular clusters (A,B,C) (distributions abnormal so error bars show interquartile ranges)

Table 3.14- Pairwise median test for dissolved oxygen (DF=2, α= 0.05)

Comparison	p value
Abalone IN-Abalone OUT	<0.001 *
Abalone IN- *Ulva* OUT	0.6884
Abalone OUT-*Ulva* OUT	0.1099

TAN and FAN

Four spiked standards for TAN, nitrite and nitrate were prepared to try and gauge how the freezing and thawing process was altering the concentrations of each nutrient. The percentage recovery shows how close the spiked standard was to the freshly made standard, in the case of TAN and nitrite the spike recoveries were within 10%, whereas nitrate was up to 70% above or below the fresh standard. The minimum and maximum values in table 3.15, have been given as ±100-spike recovery; to be conservative the spike recovery furthest from 100% has been used for TAN and FAN, which is a derivative of FAN.

Table 3.15- Basic summary statistics for flow weighted ammonia removal (% removal from *Ulva* IN to *Ulva* OUT) in experiment two (12 observations in each)

	Minimum (± 13%)	Mean (± s.d.)	Median (± m.a.d.)	Maximum (± 13%)
TAN (flow weighted % removal) 12 obs.	64.55	73.40± 6.95	71.02± 8.34	84.84
FAN (flow weighted % removal) 12 obs.	40.84	62.96± 11.22	65.62± 10.72	80.29

Testing Parameter	Spike Recoveries (%)
TAN	90, 98, 87, 89
Nitrite	96, 93, 97, 95

Figures 3.11 and 3.12 show flow weighted ammonia reductions over time as the water moves from the abalone effluent pipe to the *Ulva* raceway effluent sluice, with the reductions being calculated using concentrations in the water at the pipe outflow and the sluice outflow, combined with the flow rates as discussed previously. Figure 3.11 appears to show higher percentage removals of TAN during day time (in the black box) compared with night time, whereas figure 3.12 seems to demonstrate higher percentage removals of FAN during night time (outside the black box). Table 3.14 shows statistically significant differences between the percentage of TAN removal and the percentage

of FAN removal, the FAN removal is a function of TAN removal, but is primarily driven by the pH of the water. Total ammonia nitrogen removal overall would seem to be influenced by TAN load and temperature, however it is more likely that photosynthetically active radiation (PAR) increases TAN reductions, but as PAR was not measured, temperature has here been used a proxy.

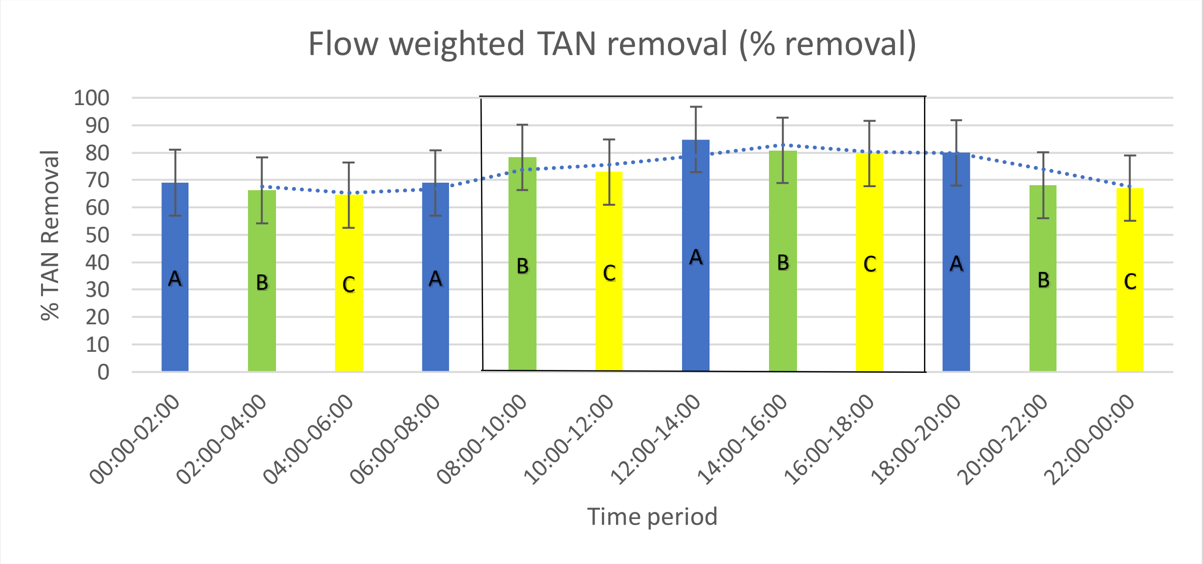

Figure 3.11- Flow weighted total ammonia nitrogen (TAN) removal, % removal from *Ulva* IN water to *Ulva* OUT water. Black box shows daytime sampling, letters A, B and C represent modular clusters, dotted blue line is a two period moving average line (error bars show interquartile ranges)

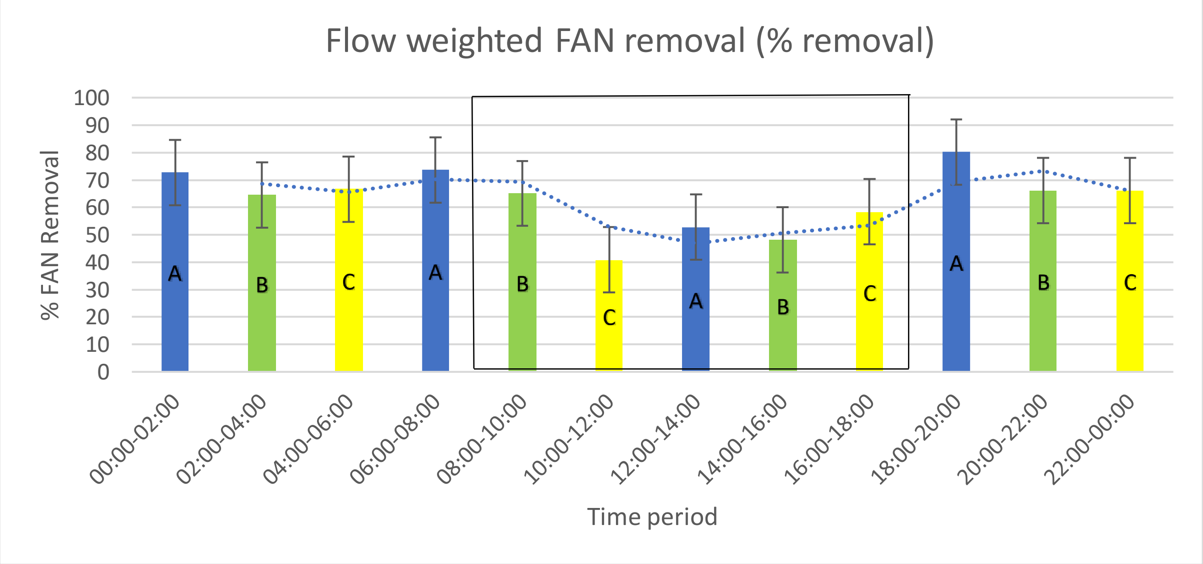

Figure 3.12- Flow weighted free ammonia (FAN) removal, % removal from *Ulva* IN water to *Ulva* OUT water. Black box shows daytime sampling, letters A, B and C represent modular clusters, dotted blue line is a two period moving average line (error bars show interquartile ranges)

Table 3.14- Pairwise median test for TAN and FAN (DF=1, α= 0.05)

Comparison	p value
TAN % removal- FAN % reduction	0.01649 *

The TAN removal appears to increase during the day, and the FAN removal decreases during the day as temperatures and pH increase. The principal component analysis in figure 3.14 implied a strong correlation between TAN load and TAN removal, and the scatterplot following (figure 3.15) shows a strong positive relationship (R^2= 0.8983) between TAN load and TAN removal. The principal component analysis in figure 3.13 implies a strong negative correlation between FAN removal and pH, which would be expected as increasing pH increases the fraction of TAN accounted for by FAN.

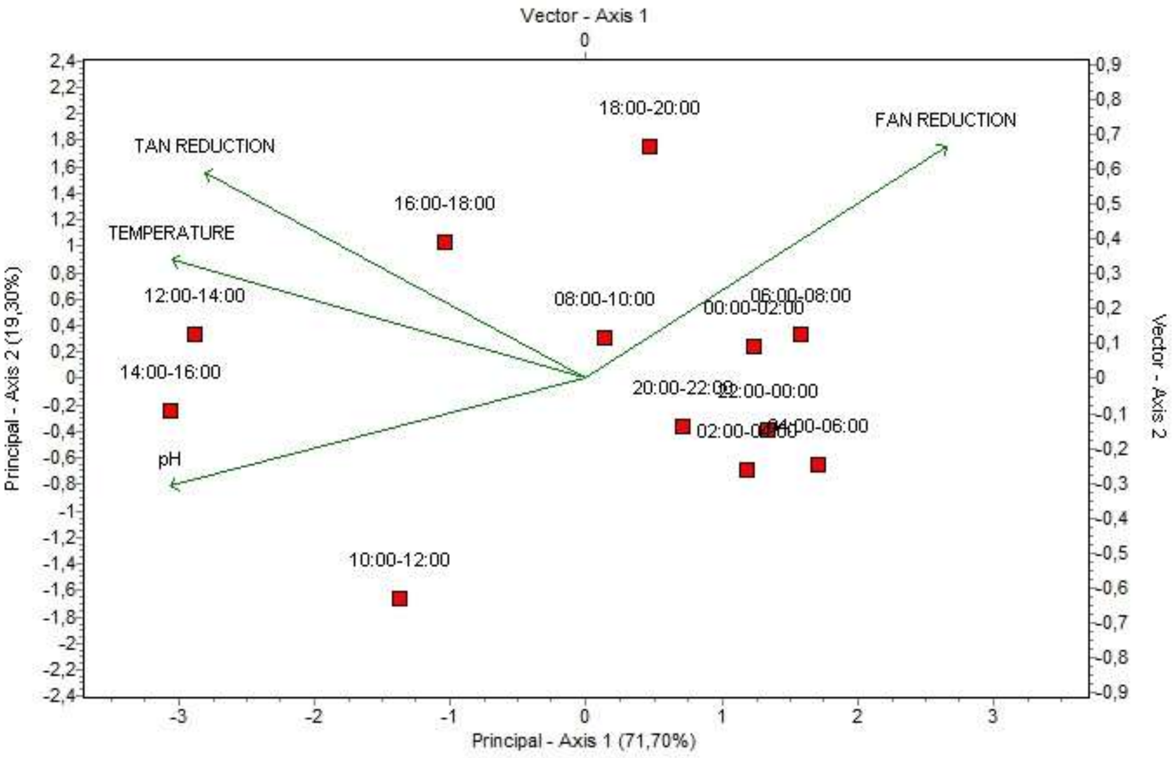

Figure 3.13- Principal component analysis biplot showing relationship between pH, temperature, TAN removal and FAN removal

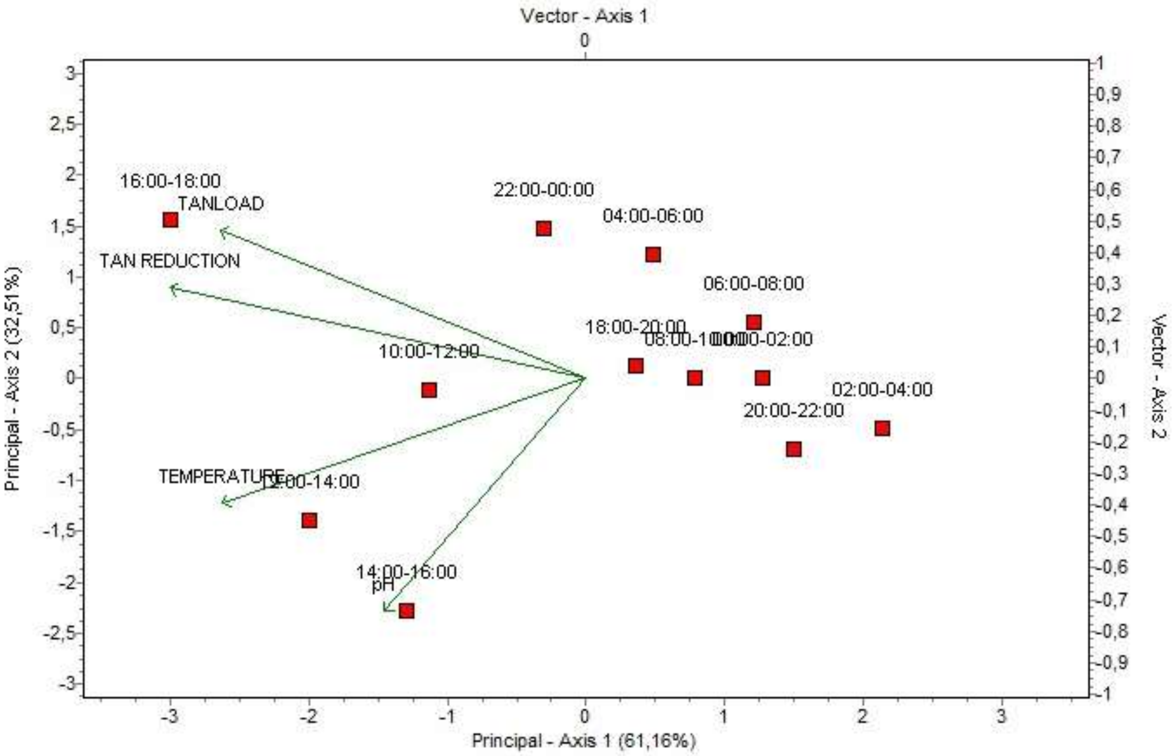

Figure 3.14- Principal component analysis biplot showing relationship between TAN load, TAN removal, temperature and pH

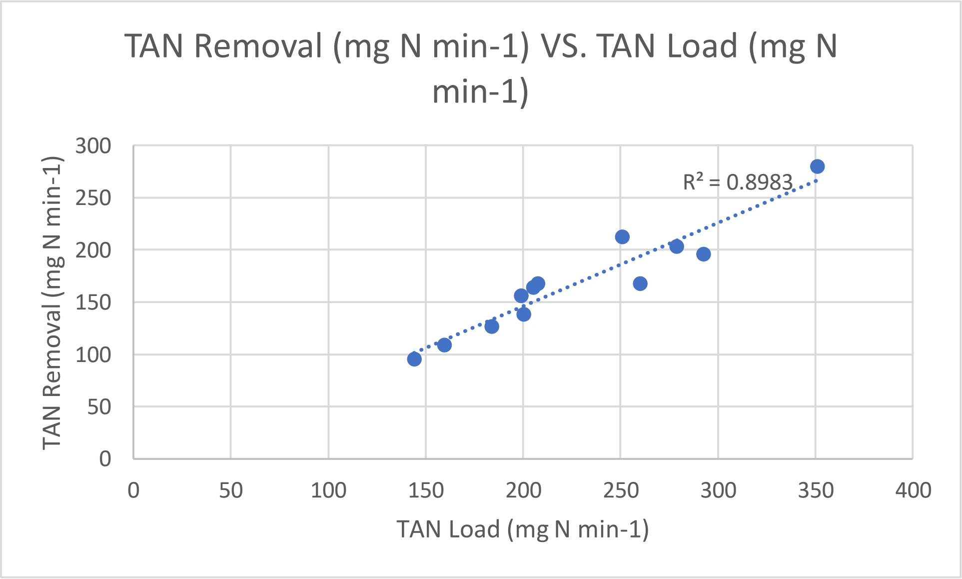

Figure 3.15- Scatterplot showing relationship between TAN load to *Ulva* raceway and TAN removal by *Ulva* raceway (R^2 = 0.90, DF = 3)

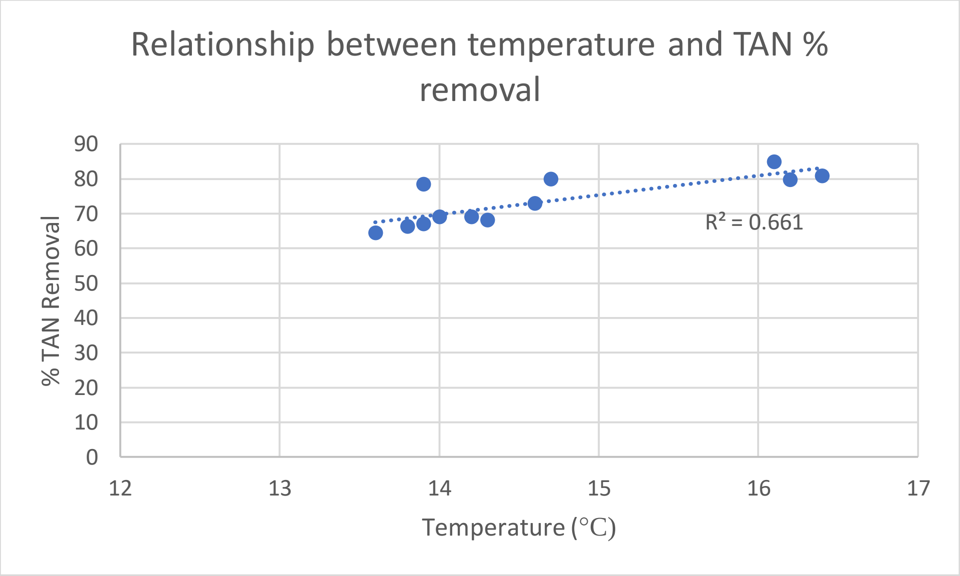

Figure 3.16- Scatterplot showing relationship between temperature (°C) and TAN removal (%) (R^2 = 0.67, DF = 3)

3.3 Experiment One Discussion

Temperature

This experiment was conducted in early December (southern hemisphere summertime), as such the temperatures observed in the course of this experiment are higher than would be expected during spring, autumn and winter. The warmest months (air temperatures) in the western cape of South Africa are January and February (South African Weather Service, 2019), though average temperatures are only slightly higher compared to December. A paper by Towner *et al.* (2013) suggests that monthly mean seawater temperatures in the Gansbaai area where this work was conducted stay fairly constant between 15-17°C throughout the year though upwelling events during summer can occasionally reduce temperatures, and other warm water events may increase them also. Harmful algal blooms tend to occur during the summer months (Probyn *et al.*, 2011) so the temperatures observed in this experiment are likely to be similar during a HAB event, such an event being one of the theoretical reasons why a 100% recirculation would be necessary.

Temperatures show similar trends across all four sampling points, with pronounced temperature increases at 12:00 and 18:00; this would be expected as the sun is actively warming the water at these times. The 50% and 75% recirculation clusters follow very similar patterns, and although they do differ from the ambient conditions, the difference is not large and is unlikely to cause any deleterious effects in the abalone based on optimal temperature values for abalone provided by

Vosloo and Vosloo (2010). The temperatures in the 100% recirculation cluster spike at 18:00, in line with the trend in the ambient conditions and 50% and 75% recirculation clusters. The temperature increase is most pronounced in the final 18:00 sampling run. The exact temperature in this run at 100% recirculation was 22.4 °Celsius, which, according to Vosloo and Vosloo (2010) is above that which *Haliotis midae* may encounter in the wild (temperatures between 12-21°C is the range provided in the paper). Work done by Britz *et al.* (1997) found that feed consumption and growth rate decline sharply in *Haliotis midae* between 20-24°C, and the 50% critical thermal maximum is given as 27.9 °C. The 22.4 °C temperature measured in the final 18:00 sampling run would already be of concern to abalone farmers. If during hot summer months/days, the 100% recirculation continued for another 3 days or more, as may be necessary in the case of a harmful algal bloom (which may last for a few weeks but only come inshore and cause problems for a few days), then temperatures may reach critical levels sufficient to cause mass mortalities among stock. Farmers would need to find a way to stall this temperature increase to avoid such a scenario, potential mitigation techniques may be shading of the clusters, or introducing fresh seawater of a lower temperature. A far more complicated though theoretically possible system to cool the water would be a concentric pipe heat exchange style mechanism such as those commonly found in chemical engineering laboratories; whereby cool fresh water in pipes is run through the water in the cluster, this way some of the heat stored in the water molecules of the cluster stream water may be transferred to the comparatively cooler fresh water in the pipes. 100% recirculation would only be considered in emergencies, and such measures would only be used in extreme circumstances as they would be extremely complicated and costly to execute, particularly when the volume of cool fresh water required is considered.

pH

pH is a fundamental chemical characteristic of water, it is influenced by physical parameters such as temperature as discussed in section 1.7 and by pressure, whereby the pressures and partial pressures of gases in the water and the atmosphere adjust the equilibria positions of the carbonate system, which is the primary controlling influence of pH in seawater. Discussion of pH in experiment one with reference to the abalone and the *Ulva* cultured in the system, is primarily a discussion of the carbonate system and the way that each biotic component of the system influences it. To be considered also is the influence of cations such as Ca^{2+} and Mg^{2+} and the other ionic constituents of seawater which contribute to the ‘total alkalinity’ of seawater. Neither magnesium, calcium or total alkalinity were measured in this experiment however, so the discussion will centre around water pH and the carbonate system as it is effected by the biota in the system. The first trend to be discussed is the comparison of the ambient, 50 and 75% recirculation clusters; during the sampling runs at midnight and 6am (darkness) the pH in the ambient conditions is consistently higher than the 50 and 75% recirculation clusters, during the midday and 6pm (daylight) sampling runs however, the pH is consistently higher in the 50 and 75% recirculation clusters compared to ambient conditions. Across all recirculation clusters and ambient conditions, the seawater will contain primary producers including microalgae (dinoflagellates, diatoms etc.) and cyanobacteria and in the recirculating clusters there is also the cultured macroalga and the cultured abalone. The means by which microalgae and macroalgae take up carbon during photosynthesis are becoming better known and are discussed in section 1.7; the carbon concentrating mechanisms that exist in them are complex and will not be discussed here, but the general idea is that microalgae and macroalgae have evolved complex carbon concentrating mechanisms for dissolved inorganic carbon, whereby they can utilise photosynthetic energy to uptake bicarbonate and/or CO_2 to saturate certain crucial carboxylating enzymes inside the carboxysome or pyrenoids (Singh *et al.*, 2016). The end result of the uptake of either CO_2 or HCO_3^- by microalgae or macroalgae is to affect the carbonate system by decreasing the concentration of H^+ ions and thereby increase pH. Microalgae and macroalgae respire throughout the diurnal cycle, but photosynthesis occurs only during daytime in the presence of photosynthetically active radiation. Photosynthetic processes in the algae take up CO_2 and HCO_3^-, and respiration processes excrete CO_2, the photosynthetic uptake of carbon is faster than the respiratory excretion of CO_2 so during daylight there is a net decrease in carbon dioxide which drives increasing pH. The slightly elevated increase in pH observed in the 50 and 75% recirculation clusters compared to the ambient conditions during daytime is a result of the extra uptake of HCO_3^- and CO_2 by the *Ulva* in the clusters, driving changes in the carbonate system that increase pH. At night time, the lower pH in the clusters compared to the ambient conditions is a result of respiratory activities by the abalone and *Ulva*, which will be excreting CO_2 and driving changes in the carbonate system which decrease the pH (Sales and Britz, 2001).

The 100% recirculation cluster follows a similar trend to the rest of the clusters and the ambient conditions, yet it is considerably lower in all instances. Beer and Koch (1996) state that photosynthetic pigments are primarily responsible for the majority of light absorption in *Ulva* thalli, and light reflection from the surfaces of the *Ulva* thallus is low, particularly if the incident angle of radiation is close to perpendicular to the surface. At midday and in the afternoon when the sun is high overhead, the angle of incident radiation would likely be close to perpendicular, meaning that minimal light will be reflected off the thalli surfaces. Minimal reflection from the surface would suggest that the photosynthetic pigments in *Ulva* are best able to utilise photosynthetically active radiation (PAR) provided by the sun. Considering that the recirculation was switched only an hour before the experiment began, the reduction in pH is both rapid and considerable in magnitude. It appears that in the 100% recirculation cluster there is still considerable scope for the *Ulva* to utilise light energy for photosynthesis and stabilise pH levels approaching optimal for the abalone. When the *Ulva* and primary producers are not so active however, the pH drops considerably in the cluster. Reasons for this have already been discussed in some detail earlier in the section, but the lack of inbound fresh seawater and the accumulation of inorganic carbon, combine to drop the pH to levels that would certainly concern an abalone farmer. The lowest pH measured was 7.02 in the 100% recirculation cluster, if pH values on this level persisted past acute exposure to chronic (above 24 hours), then consequences could be as severe as mass mortality. There is not much information yet available in scientific literature concerning the specific effects of pH on abalone. Abalone however, like all life forms, have optimal pH values in blood and tissue, which they must expend energy to maintain. If the pH in the surrounding water is such that it forces the abalone to expend high levels energy to regulate, then damage may be incurred to their DNA, metabolic pathways, protein construction, shell formation and more (Naylor *et al.*, 2014). It is worthy of note however, that the abalone in this cluster survived the low pH with no mortalities reported by farmers, so they can feasibly be exposed to the depressed night-time pH levels for at least three nights without mortality, though internal and shell damage was not measured.

Ammonia

TAN

The ammonia levels observed in the 100% recirculation are high, both in comparison to the other sampling points and to seawater ammonia levels more generally. In agreement with a study conducted by Robertson-Andersson (2003), peaks in concentration occur at 6am. There are various guidelines and regulatory frameworks in existence around the world concerned with point source pollution of aquaculture particularly pertaining to ammonia, in Australia and New Zealand the general approach is to provide comprehensive guidelines of trigger values for a host of ecosystem stressors, both physical and chemical; where local ecological and biological data are available, these are preferred in the drafting of these guidelines. If they are not available or not sufficient then reference data are used which are collected from the local ecosystem, or a similar one. A general rule of thumb in these guidelines is that the 80th percentile is used as a broad guideline for stressors that may become problematic at elevated concentrations (Aquaculture Stewardship Council, 2012). Of specific relevance to this research are the ‘Abalone Aquaculture Dialogue Standards’ drafted in 2010 by international cooperating organisations and institutions, facilitated and funded by the World Wildlife Fund. With regards TAN, the upper advisory limit given on annual median concentration in effluent is given as 600μg/l (0.6mg/l). At 100% recirculation this advisory upper limit is breached repeatedly. In the 50 and 75% recirculating clusters, the sampling point in each cluster (sump) is not reflective of effluent conditions, as the sump contains a mixture of fresh seawater and recirculated water arriving from the *Ulva* raceway; in the 100% recirculating cluster however, this distinction ceases to maintain relevance as the water in the system is all recirculating, with no fresh water coming in and only small fractions of water leaving the system. In the 100% recirculating cluster, almost the total volume of the ‘effluent’ the water goes back to the sump, meaning the sump water is a reasonable representation of ‘effluent’ water leaving the system. The black line shows the 0.6mg/l upper limit advised in the abalone dialogue standards, the 100% recirculation cluster breaches repeatedly but is unlikely to cause any serious disquiet among regulators as 100% recirculation would only be necessary in extreme circumstances, and over durations as short as practically possible. As can be seen, the 50 and 75% recirculation clusters fall well short of this limit, although as discussed, the sump water from which a sample is taken is not truly representative of effluent concentration, as there is fresh seawater being continually supplied to the sump. It is a reasonable assumption that effluent concentrations would be higher, but remain well below the 100% recirculation line, therefore unlikely to be in breach of these guidelines, though more detailed analysis would be necessary to state this with certainty. The outliers discussed in the results section which are suggested to have considerably increased the mean values in 50% compared with ambient, are arising during peaks in concentration; these concentration peaks are unlikely to make significant differences to stocks, as levels are still low, and higher levels are short lived.

Regulatory and legal guidelines notwithstanding, these TAN concentrations must be analysed from the perspective of the farmer. The farmer is primarily concerned with abalone and it’s growth, and the way in which TAN may affect it. Total ammonia nitrogen will certainly have an effect, but NH_4^+ is far less toxic to abalone than NH_3, up to 100 times less according to Wurts (2015). The TAN levels shown in at 50 and 75% recirculation, are unlikely to be of concern to either regulators or farmers, as they are not drastically higher than ambient conditions, and on the south coast of South Africa where abalone farms are concentrated, upwelling events are common and a highly energetic surf zone yields high mixing and thorough dilution (Probyn *et al.*, 2017) . Results from this preliminary study indicate that the biofilter (*Ulva*) in the recirculating clusters is doing it’s job, though more detailed analysis would be required to discern specific dynamics of the systems.

FAN

From the regulatory perspective, ammonia regulations tend to be concerned with TAN. This is unsurprising as FAN is derived from TAN, and the abalone dialogue regulations discussed were likely drafted with the general rule of thumb in mind that seawater is at a normal pH of around 8.2, and temperature would be somewhere between 12-20°C as this is often considered an acceptable temperature for the culture of *Haliotis midae* (Britz *et al.*, 1997; Sales and Britz, 2001). At temperatures and pH’s in this range, fractions of TAN accounted for by unionised ammonia will be somewhere between 2-5% (Probyn *et al.*, 2017). The WWF (2010) abalone dialogues maximum suggested TAN level of 600µM/l will likely yield a FAN level somewhere in the range 12-30µM/l NH_3. This is a considerable concentration and consistent effluent concentrations of this level are likely to cause localised problems; this is however, entirely dependent on local conditions and prevailing ecological norms. In the case of the abalone farms on the south coast of South Africa and the results from this experiment, the highest FAN concentration in µM/l found in this experiment was 1.40 µM/l in the 100% recirculation cluster. This is well below the suggested FAN levels which may be encountered close to the 600µM/l limit given by the WWF for abalone effluent, and as discussed, these farms occur on the south coast of south Africa, a region well known for it's upwelling events and highly energetic surf zone yielding high mixing and dilution of coastal contaminants. As such, if 100% recirculation were necessary then it is unlikely to cause major problems in the surrounding ecosystems, as the duration would be as short as possible and the concentrations are not too extreme.

The fact of reducing pH in the 100% recirculation cluster compared to the other clusters, although potentially problematic in and of itself, is advantageous in terms of free ammonia concentrations. pH is the most significant influencer of the dynamic equilibrium of dissolved ammonia nitrogen in seawater (Whitfield, 1974), and lower pH yields lower unionised ammonia fractions. Much of the literature concerned with integrated aquaculture often mentions pH 'rebalancing' as one of the bioremediatory characteristics of seaweed biofilters (Neori *et al.*, 1998; Robertson-Andersson *et al.*, 2008; Shpigel *et al.*, 2019), which is true in that during photosynthesis the pH will increase in seaweed pond effluent water for reasons already thoroughly discussed previously, and this increase in pH towards a normal seawater pH of 8.2 will likely be beneficial to abalone; however it will also increase the fraction of unionised ammonia present in the water. Seaweed biofilters of the form encountered in this work are demonstrably effective in the removal of TAN from seawater (Bolton *et al.*, 2009), but the shifts in environmental conditions that accompany these reductions in TAN, primarily increasing pH and to a lesser extent increasing temperature, combine to drive FAN percentages of TAN higher. Gauging the level of harm which may be inflicted on the abalone by the accumulation of NH_3 under 100% recirculation conditions is difficult, as literature associated with FAN toxicity to *Haliotis midae* is scarce. One paper by Reddy-Lopata *et al.* (2006) suggests a maximum concentration of 7.4 µg/l NH_3 in pond water, and the general rule of thumb for aquarists is that concentrations above 0.02 mg/l NH_3 (20 µg/l NH_3) may be toxic to fish. Figures 3.5 and 3.6 show that concentrations only exceed the 7.4 µg/l NH_3 limit suggested by Reddy-Lopata *et al.* (2006) and the general rule of thumb toxic limit of 20µg/l NH_3 in the 100% recirculation cluster. Over the course of this three day study and follow up consultations with farmers, there were no adverse effects to the abalone in the 100% recirculation cluster noted in this study, there were no reported mortalities or noticeable drops in growth rate reported in the weeks and months following the study when recirculation level had returned to standard farm operation of 50%. Overall, this pilot study suggests that an increase from 50 to 75% recirculation is unlikely to cause any harm to the abalone; further work would be required to evidence this assertion but it seems that 75% recirculation would not be problematic in terms of NH_3 particularly if the farmers switched from 50 to 75% only during the colder winter months, when the weather conditions will drive lower temperatures and slightly lower pH (lower fractions of FAN), as levels of photosynthetically active radiation are likely to be lower. This could potentially save the farmers more money in terms of pumping costs, and it is unlikely to affect the growth rates or quality of the cultured *Ulva*, meaning that the use of the *Ulva* as a feedstuff is unlikely to be affected.

3.4 Experiment Two Discussion

The main findings from experiment two were as follows:

- Mean and median TAN removal across the seaweed biofilters were 73% and 71% respectively, with a maximum value of 85%
- Mean and median FAN removal across the seaweed biofilters were 63% and 66% respectively, with a maximum value of 80%
- Strong positive linear relationship between TAN load and TAN removal, and seemingly strong negative relationship between pH and FAN removal suggesting that increasing pH in seaweed ponds during day-time has the unintended consequence of increasing FAN percentages/concentrations

Studies concerned with TAN removal by bioremediatory seaweeds, particularly *Ulva* species, generally demonstrate that *Ulva* removes anywhere from 10%-90% of the supplied TAN load with averages in the range of 70%-80% being reported (Neori *et al.*, 1998; Robertson-Andersson, 2003; Lidén, 2007; Shpigel *et al.*, 2019). The TAN removal values observed in this experiment were in good agreement with other studies, this study worked out TAN removal by determination of TAN load in the water as it moved through a certain point of the system. Other studies determined TAN load either the same way, or by the areal load of TAN to the system, both methods are used in literature so both are assumed to be valid, this assumption is validated by the coherent results gained from both methods. These results suggest that the *Ulva* biofilters in this IMTA farm are functioning well in terms of the bioremediation of harmful dissolved nitrogenous nutrients. Discussion for experiment one mentioned regulatory limits for abalone aquaculture, and these values are well under the limits discussed; proving that in the case of ammonia, the effluent water from this farm could be considered fairly innocuous to the surrounding coastal waters when the high levels of mixing, dilution and upwelling events are considered.

Work done by Guttmann et al. (2019), Shpigel et al. (2019) and others, demonstrates a strong correlation between TAN load and TAN removal in *Ulva* raceways, and the findings in this experiment were in agreement. In studies where a broad range of TAN loads to *Ulva* biofilters are examined, it has been observed that the relationship between TAN load and TAN removal is linear up to a certain threshold before curving off in what has been described as Michaelis-Menten like dynamics (Guttman *et al.*, 2019). This experiment did not detect any curvature in the trend line which would indicate the presence of Michaelis-Menten like dynamics, but it is highly likely that if increased ammonia input to the *Ulva* biofilter components occurred, then Michaelis-Menten like curves would be observed, however a strong positive relationship was found between TAN load and TAN removal in this experiment.

Many of the studies associated with bioremediation potential of seaweeds in integrated aquaculture, mention the pH rebalancing that occurs in seaweed biofilters (Neori *et al.*, 1998; Robertson-Andersson, 2003; Chávez-Crooker and Obreque-Contreras, 2010). This study found that the seaweed biofilters in this system did indeed rebalance the pH by increasing it towards levels found in the inbound abalone water. This in itself could be viewed as wholly advantageous, until the ionisation of ammonia in seawater is considered; increasing pH drives increasing free unionised ammonia fractions of total ammonia. As such, the fact that seaweed biofilters “rebalance” pH by increasing it during photosynthesis (daytime) could not really be considered wholly advantageous. The pH levels in abalone effluent (*Ulva* inbound) water are depressed due to the respiration and excretory processes of the abalone; this removal in pH actually decreases the free ammonia fraction of the total ammonia; this effect is then reversed by the increase in pH arising from the photosynthetic processes of the *Ulva* which have been discussed previously (CO_2 uptake driving changes in carbonate system which increase pH). The principal component analyses in figures 3.13 and 3.14 demonstrate this contradictory relationship quite neatly, whereby figure 3.12 appears to show decreased FAN reductions during daytime, when the seaweed is photosynthesising and “rebalancing” the pH by driving it upwards. This somewhat negative effect of increasing FAN fractions driven by increasing pH in the biofilter water, is somewhat offset by the suggested increasing dissolved oxygen levels shown in figure 3.10, with work being done by Naylor *et al.* (2014) showing that ammonia toxicity to marine species is increased in oxygen deficient water. The extra oxygen supplied by the *Ulva* would help the abalone to acclimatise and reduce the toxicity of the ammonia.

Literature suggests that ammonia nitrogen removal by seaweed biofilters is dependent on the load and the stocking density of the seaweed (Neori *et al.*, 1998; Guttman *et al.*, 2019; Shpigel *et al.*, 2019), as the stocking densities in this experiment were practically identical, gauging changes in TAN removal at varying stocking densities was not possible. The stocking density dependency is intuitive, as more seaweed would remove more ammonia, though work done by Shpigel *et al.* (2019) suggests that although specific removal increases as stocking density increases, the efficiency of the removal decreases as stocking density increases. Efficiency of removal was not measured here, as it requires the observer to conduct direct tests on the *Ulva* thalli themselves. TAN removal and removal efficiency by seaweed biofilters are TAN load and stocking density dependent; the stocking density would change the penetration of light into the biofilter, even if there is aeration or agitation of the seaweed occurring to mitigate self-shading. The specific removal was measured, and the observed increases during daytime are assumed to be a result of photosynthetically active radiation (PAR) promoting increased uptake compared to night-time. Beer and Koch (1996) state that the angle of incident radiation to the seaweed thalli will effect the photosynthetic processes of the plants. The increases during daytime compared to night time then are most likely a result of the PAR, and the differences in removal between time periods during the day are likely a result of varying TAN load, and varying angles of incident radiation as the sun tracks across the sky during the day. The apparent relationship between temperature and TAN removal is somewhat dubious, as temperature has been used here as a proxy for PAR. Work done by Vergara *et al.* (1998) found significant drops in the photosynthetic efficiency of *Ulva* thalli as the stocking density increased and self-shading became more prevalent. If photosynthetic efficiency of *Ulva* has a direct effect on TAN removal, which would be assumed, then positively identifying the relationship between PAR and its incident angle of radiation versus TAN removal would be necessary; for this an entirely new experiment would need to be conducted whereby the PAR intensity and angle, stocking density of seaweed and TAN removal are measured simultaneously. This experiment however, is coherent with other studies that suggest a strong link between TAN load and TAN removal, and suggests a potential link between PAR intensity and angle, stocking density of seaweed and TAN uptake.

4. Conclusion

The pilot varying recirculation experiment demonstrated that an increase from standard farm operation recirculation percentage (50%) to 75% recirculation may be feasible, with TAN and FAN levels staying below certain critical thresholds and pH and temperature differences between 50% and 75% recirculation being negligible over the course of the three-day experiment. At 100% recirculation, TAN levels increased rapidly, though the commensurate rapid drop in pH that occurred, drove the FAN fraction of the TAN down. As FAN is by far the most toxic form of ammonia to marine species, this drop in pH could almost be described as advantageous, though the drop in pH will cause other problems not related to ammonia nitrogen such as reduced food intake and lower growth rate (Naylor *et al.*, 2014). The experiment proved that 100% recirculation would likely be possible for three days during December under the specific conditions which prevailed when the experiment was conducted, though far more work would be needed to make definitive statements (several experiments during each season of a year). The work also demonstrated that even at 100% recirculation, the TAN levels exceed WWF abalone dialogue limits only during three of the thirteen sampling runs; 50% and 75% recirculation TAN levels were far below the dialogue limits.

The second experiment demonstrated TAN removals by the seaweed biofilters on the farm to be an average of 73% and 75% removal (mean and median) over the course of the 24-hour experiment. Ammonia is by far the most toxic of the dissolved nitrogenous nutrients and removal at these levels demonstrates that the biofilter is functioning effectively. There existed a strong positive linear relationship between the TAN load and the TAN removal, and TAN removal increased during daytime compared to night time. It was found that the increase in pH that occurs most noticeably in the *Ulva* effluent water during daytime increases the fraction of TAN accounted for by FAN (NH_3) and reduces the overall FAN removals compared to TAN removals. TAN levels in *Ulva* effluent water remained well below the abalone dialogue limits laid down by the WWF in 2010 and FAN levels in all sampling points were found to be well below levels which may be considered toxic to the abalone.

Experiment one yielded some useful findings about increasing recirculation percentage in mollusc/seaweed IMTA farms though further work is needed to identify more fully the changes in water quality at 75% recirculation, this would then give farmers a better idea of whether 75% recirculation is a viable option for commercial IMTA farms in the western cape of South Africa. Ideally water quality testing in conjunction with abalone and seaweed performance testing would be conducted monthly over the course of a few years to build a comprehensive picture, though constraints on resources and logistics mean this is unlikely. Continuous and large changes in environmental conditions in the world’s oceans mean that this work is more important, and more complicated than ever. Further research into 100% recirculation would also be advantageous, though the inherent risk associated with 100% recirculation is difficult to manage, running the system empty would of course be useless as only at standard stocking density would the information be useful; small scale experimental designs could be used, which could then be used to model larger designs but there is always uncertainty as to whether larger systems will behave in the same way as smaller systems.

There were also useful findings arising from experiment two, most notably for the scientific body of research around IMTA systems; increasing pH in seaweed biofilters increases FAN percentages. For the farmers, their systems appear to be functioning well with respect to the ammonia removal across the seaweed biofilter, and other readings suggest system is functioning as it should. This work involved testing of the water only, further work should include concomitant testing of the abalone and seaweeds performance so that changes in water quality might be attributed to changes in stock performance.

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