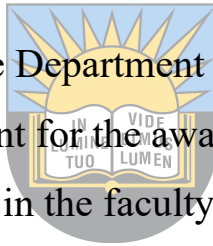


**The effect of water-off events on the growth,
survival, and biochemical stress indicators of the
South African abalone, *Haliotis midae***

Bongi Langa Mngqushu

A dissertation submitted to the Department of Zoology and Entomology in
fulfillment of the requirement for the award of the degree of Master of
Science (MSc) in Zoology in the faculty of Science and Agriculture



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Supervisor:

Prof. Niall Vine

DECLARATION

I, Bongi Langa Mngqushu, (201822765) hereby certify that this dissertation is my original work and has not been submitted in this or any other form to any University other than the University of Fort Hare, Alice, South Africa. Where use has been made of the research of others, it has been duly acknowledged in this text.

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ABSTRACT

South African abalone farms are dependent on seawater from the surrounding environment which occasionally may experience the occurrence of harmful algal blooms (HABs). To avoid HABs entering the abalone tanks, the farm may implement water-off events, resulting in ammonia accumulation which potentially stresses the abalone, influencing the metabolism, growth, and survival. This study aimed to better understand how long the water-off interruptions might be implemented before they have a negative impact on the survival, growth, and health of the abalone, *Haliotis midae*.

The study involved placing baskets of abalone in different raceway tanks (control and experimental) where the water was switched off for 24, 48, and 96 hours. Prior to, during and after the exposure to the different water-off periods, haemolymph and muscle tissue were taken from abalone and were used to assess haemolymph glucose, muscle glycogen, dopamine, superoxide dismutase, and total haemolymph protein content, as indicators of abalone health. There were differences in the water quality parameters over the sampling period period. Four months after the start of the experiment there was a 100% survival for the abalone across all the treatments with no differences in their growth. There was no difference in the concentration of glycogen, glucose, dopamine, and superoxide dismutase over time in any of the treatments. Total protein concentration in the treatment tanks showed differences between 6h and 84h fluctuations, however, it did not show any differences between the treatments.

Despite fluctuations in the water quality parameters over the 96-hour time period, the water quality parameters remained within a safe range for *H. midae*. The results of this study therefore show that it is safe for farm managers to turn the incoming seawater off for up to 96-hours without any negative effect on the health, growth, and survival of *H. midae*.

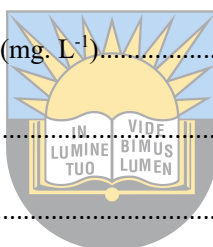
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CHAPTER 1: INTRODUCTION

1.1. Literature review



Figure 1.1: The South African abalone, *Haliotis midae* (Two Oceans Aquarium, 2020).

Abalone, the common name given to the *Haliotis* (sea ear) genus, includes about 70 species distributed around the world (FAO/UNDP, 1990; Daume *et al.*, 1999). South Africa is home to five endemic species of abalone which are *Haliotis midae*, *Haliotis parva*, *Haliotis queketti*, *Haliotis spadicea*, and *Haliotis speciosa* (Evans *et al.*, 2004). Of these five species, *H. midae* (Figure 1.1) is the biggest and is colloquially known as perlemoen (Visser-Roux, 2011).

This abalone species grows to become 230 mm in standard length at the age of 30 years (Newman, 1968; Wood, 1993). *Haliotis midae* has a distribution range that spans from along the coast of Port St. Johns in the Eastern Cape Province through to Saldanha Bay in the Western Cape Province of South Africa (Visser-Roux, 2011; Rhode, 2014).

Typically, a resident of shallow coastal waters, *H. midae* is mostly found around rocky areas (van der Merwe, 2009), on substrata of granite and limestone (Bryan & Qian, 1998). However, there have been sightings at depths of 36m (Newman, 1969; Griffith & Grobler, 2017). Juvenile abalone have been identified to prefer associating themselves with sea urchins (*Parechinus angulosus*), from which the abalone seem to be receiving protection from predators (Day & Branch, 2002).

Haliotis midae has a high market value in the Far East because its unique flavour, size, and texture (Wagner & Engel, 2021), which therefore makes it one of the country's most economically valuable invertebrates (Kain & Brown, 1999). In 2019 South Africa was the fourth largest exporter of farmed abalone in the world with an estimated production of 436 metric tonnes exported (Wagner & Engel, 2021). In 2021 South Africa was the largest producer of abalone outside of Asia (Cook, 2023). The cultivation of abalone was driven by over-exploitation of wild abalone stocks due to poaching as well as the high demand in Asia and associated market prices (Troell *et al.*, 2006).

The commercial value of *H. midae* in South Africa became more apparent around the 1980s (Franchini *et al.*, 2011). Being able to obtain relatively affordable labour, as well as coastal water with favourable quality and infrastructure, also assisted in the fast growth of the South African abalone industry. The high market demand of the South African abalone resulted in it becoming highly valued (Visser-Roux, 2011). *Haliotis midae* is currently the most exported species in the South African aquaculture sector (Britz & Venter, 2016).

The South African government regards aquaculture as a sector requiring further expansion, as its historical focus was on species of high value which include abalone, mussels, and oysters (Wagner & Engel, 2021).

Because South Africa has a high energy coastline which makes ocean farming difficult, local abalone farms are all on land and generally in (or close to) coastal towns. Most farms use a flow through water system that pumps water through the tanks and back to the ocean while some farms have the option of partially recirculating their water through macroalgae ponds (DAFF, 2018)

and seawater is pumped into specially built abalone tanks with fresh sea water being provided 24-hours a day (Aqunio, 2019).

The sea water on an abalone farm regularly undergoes environmental change in variables such as carbon dioxide (CO₂), nitrogen, oxygen, pH, and temperature, while incoming sea water may introduce harmful algal bloom (Morash & Alter, 2016).

Daily monitoring of the water by the farms is vital as there is a real risk that if harmful algal bloom species enter the farm, widespread damage can occur. The term “Red Tide” which may at times be referred to as harmful algal bloom originated after the colour change that occurs from the dense concentrations of phytoplankton in both the south and west coasts of the cape provinces in the late summer and autumn seasons (National Oceanic and Atmospheric Administration, 2024). Harmful algal blooms take place as a result of coastal upwelling which brings nutrient enrichment to the surface which both the non-toxic and toxic algae then utilize to rapidly grow. During this growth period, each cell can produce up to 1 million daughter cells. The red coloured cells can cause the colour of water to change and is referred to as a red tide (Guy, 2014). Red tides of a certain nature can cause toxic contaminations and even mortalities in abalone and mussels (Attwood, 2000; Guy, 2014).

The decrease of algal blooms and harmful algal blooms occurs when the supplied nutrients decrease. This phenomenon then leads to oxygen depletion in the water. Which, in turn, has caused large-scale mortalities in fish and shellfish populations which can have a serious impact on fishing industries (Sanseverino *et al.*, 2016). The algal blooms then subside as the concentration of nutrients in the upper mixed layer decrease to levels that limit growth.

Even though harmful algal blooms occur due to natural causes, there are some factors, including the input of nutrients due to activities related to untreated sewage and industrialization by humans can promote their spread (Davies *et al.*, 2015). An increased amount of nutrient run off resulting from the use of terrestrial fertilizers has seen an increase in the incidence of harmful algal blooms and the subsequent presence of paralytic shellfish poisoning (PSP) in the Goukou estuary (CSIR, 2011).

Paralytic shellfish poisoning is a lethal syndrome caused by eating molluscs contaminated by saxitoxins or STX. The concentration of PSP toxins in shell fish is caused by elongated exposure period during filtration of toxic algae produced by different dinoflagellates (e.g. *Alexandrium* and *Gymnodinium*) (Grattan *et al.*, 2016).

Some harmful algal bloom species (i.e., the dinoflagellates *Heterocapsa circularisquama* and *Karenia mikimotoi*) have a toxic effect on *Haliotis discus hannai* (Matsuyama *et al.*, 1998), while there have been cases of paralytic shellfish poisoning (PSP) toxicity in *H. midae* (Pitcher *et al.*, 2001). The latter case of the occurrence of HABs resulted in the paralysis of some abalone, including brood stock, animals dropping off the sides of holding tanks and mortalities (Pitcher *et al.*, 2001).

Although at times there needs to be an implementation of water-off events as to avoid or minimize the introduction of HABs on abalone farms, abalone farms generally have back-up systems to prevent air or water supply interruptions, as there are occasions when such interruptions are unavoidable (e.g., during installations or maintenance, loadshedding (planned power outages)) or occur because of system failure.

During such periods of interruptions, there may be oxygen depletion, carbon dioxide (CO₂) build-up as well as ammonia accumulation. Changes in these parameters cause stress to the abalone influencing their survival, growth, and metabolism (Harris *et al.*, 1997, 1998a, 1999; Basuyaux & Mathieu, 1999; Reddy-Lopata *et al.*, 2006). If the impact of these parameters are known, managers can use this information to inform decision making regarding priorities when these interruptions occur or due to the presence of *Gonyaulax spinifera* (Mouton, 2017).

During the 2017 “red tide” event in South Africa, the dinoflagellate *G. spinifera* emerged as a front runner with densities of 1.4 million cells per litre (Mouton, 2017). *G. spinifera* vary in toxicity, with different strains producing varying analogues of yessotoxin (Rhodes *et al.*, 2006). Yessotoxin is a toxin produced by dinoflagellates of the *Gonyaulacaceae* family. This toxin harms *H. midae* by compromising gill integrity and by causing apoptosis by the activation of caspase proteins (Rhodes *et al.*, 2006).

1.1.1. Water Quality

To gain access to large quantities of seawater, South African abalone farms mainly cultivate abalone onshore on land based tank systems (Wagner & Engel, 2021). These land-based abalone farms make use of flow through systems (Siikavuopio *et al.*, 2004; Colt, 2006). Intensive flow-through land-based aquaculture systems can be classified by the degree of control on the environment of the species being cultured. These systems may involve the transfer of large water volumes through single usage or serially re-used water raceways, tanks, or ponds (Snow *et al.*, 2012). It is therefore important that

species-specific water quality limitations of the flow through systems are assessed and understood (Siikavuopio *et al.*, 2004; Colt, 2006).

To maintain the water quality within safe levels, it is important to understand processes that may affect it which result in the inhibition of growth and survival of the cultured animal (Datta, 2012). Poor water quality e.g., low oxygen levels, very high or low temperature, very high or low pH levels, high ammonia, nitrogen, and hydrogen sulphide levels in an aquaculture system may also affect the feed intake of the animals (Datta, 2012).

Water Temperature is an environmental determining factor for the metabolism of poikilotherms (Fry, 1971), and has positive correlation to the growth of abalone (*H. midae*) at the 12 - 20°C range where the metabolic rate of the abalone increases with the increase in temperature (Britz *et al.*, 1997). When water temperature increases, the biochemical activity of the microbiota and the rate of respiration increases due to an increase in the demand of oxygen (Singh *et al.*, 2022). Increased water temperature also results in the rise of ammonia levels in water and lower oxygen solubility (Singh *et al.*, 2022). Due to flow through system design of most abalone aquaculture facilities, farmed abalone are exposed to environmental water temperatures (Kang *et al.*, 2019).

The amount of dissolved oxygen available for animals in aquaculture systems could be the most important water quality variable that is routinely monitored (Sticknky, 2001). Even a small decrease in the amount of DO below the minimum desirable amount could result in a growth rate reduction (Sticknky, 2001).

Oxygen is important for maintaining life in aquaculture systems. Oxygen diffusion into water can be increased by increasing the water surface area that interacts with air to allow more diffusion to take place (Sticknky, 2001). In aquaculture systems the surface area can be increased by creating a strong turbulence that results in wave action or aeration in the water, and if this is not achieved oxygen diffusion into water occurs at a very slow rate (Sticknky, 2001; Datta, 2012).

In addition to very little air diffusing into the water, other factors that may contribute to having a decrease in DO levels are high temperatures that decrease the solubility of oxygen, and the crash in phytoplankton blooms which can lead to an increased biochemical oxygen demand (BOD) as the cells decompose (Sticknky, 2001). To help increase DO, aquaculture farm managers often make use of mechanical aeration systems (Sticknky, 2001). Examples of mechanical aeration that are used in

aquaculture farms include agitators, air diffusers, airlift pumps, impellers, liquid oxygen injection, venturia pumps, and vertical sprayers (Datta, 2012).

Another important water quality parameter is pH. The pH of a liquid is an indication of whether the liquid is acidic or basic (Wedemeyer, 2001). Acidic and basic substances liberate and accept H^+ respectively and are influenced significantly by the diffusion of carbon dioxide into (and out of) the water (Wedemeyer, 2001; Datta, 2012). High levels of carbon dioxide in water undergo hydrolysis in the absence of sunlight producing carbonic acid $CO_2 + H_2O \rightleftharpoons H_2CO_3$ (Wedemeyer, 2001; Datta, 2012). In the presence of sunlight, the same reaction also produces glucose and oxygen (photosynthesis), thereby decreasing the pH and alkalinity of water (Wedemeyer 2001, Datta 2012).

Because the intensity of sunlight is typically strongest around noon (11 – 1 PM), the rate of photosynthesis is also highest during this period resulting in oxygen levels being at their highest while carbon dioxide levels are at their lowest (Datta, 2012). It is therefore important that when dissolved oxygen, free carbon dioxide, pH, acidity, and alkalinity are being assessed, the period (dawn or noon) at which the sampling takes place should be considered (Datta, 2012).

Ammonia is the main end-product of the catabolism of proteins and can account for about 40-90% of nitrogen excretion for crustaceans (Parry, 1960). Total ammonia nitrogen (TAN) exists as a sum of ionized (NH_4^+) and unionized (NH_3) forms of ammonia. High levels of ammonia can occur as a result of overfeeding protein rich feed (which decays) all of which liberate ammonia (Datta, 2012).

Under certain conditions, the amount of excreted ammonia can accumulate to levels that are dangerously high for farmed animals. Fortunately, nitrifying bacteria can partially buffer this increase in ammonia levels by converting it to the less toxic nitrate (NO_3^+) (Datta, 2012).

Fish excrete more amounts of ammonia than urea as waste into the water. The aerobic bacteria *Nitrosomonas* and *Nitrobacter* convert ammonia and ammonium nitrogen into nitrite (toxic) and nitrate (nontoxic) respectively (Datta, 2012). The concentration of gaseous ammonia gradually increases in aquatic systems when the levels of O_2 are low (Datta, 2012).

At pH levels below 8.0, the fraction of unionized (toxic) ammonia (NH_3) and ionised (nontoxic) ammonium (NH_4^+) changes. Ammonia is the major end product in the catabolic reaction of protein and can be responsible for about 40-90% of nitrogen release in crustaceans (Parry, 1960). As the concentration of Free Ammonia Nitrogen (FAN) in water increases the ammonia excreted by aquatic

organisms decreases, and the ammonia levels in the bloodstream and other animal tissues increases (Datta, 2012). High ammonia levels can harm animals by elevating oxygen uptake by tissues, thus damaging the gills and reducing the ability of the animal to transport oxygen (Datta, 2012). Mechanistically the increase ammonia increases oxygen levels by causing a disruption in the intracellular energy production. Ammonia tends to interfere with key steps in cellular respiration, by inhibiting enzymes such as α – ketoglutarate dehydrogenase in the Krebs cycle (TCA cycle) The disruption results in the reduction in the production of important energy molecules such as NADH and FADH₂, resulting in less efficiency in the electron transport chain, which is responsible for using oxygen to generate adenosine triphosphate. As a compensatory measure for the reduction in energy production, the metabolic activity in cells increases and an increase in metabolic activity requires oxygen as a fuel.

Under low oxygen conditions, microbial metabolism of nitrous compounds can also produce ammonia in water. The unionized form of ammonia is said to be more toxic than the ionized form due to its ability to readily diffuse across a cell membrane (Emerson *et al.*, 1975; Fromm & Gillette, 1968). The effect of FAN on the susceptibility to *Vibrio parahaemolyticus* and immune response of the Taiwan abalone *Haliotis diversicolor supertexta* inhibited the abalone growth (Cheng *et al.*, 2004). Similarly, the growth inhibition occurred when juvenile blacklip abalone (*Haliotis rubra*) were exposed to metabolic waste (i.e., ammonia) increase (Huchette *et al.*, 2003a).

As the temperature or pH increase, the levels of toxic ammonia increase relative to the level of nontoxic ammonia, and as a result ammonia becomes more toxic to animals (Datta, 2012). Free ammonia (NH₃) levels above 0.05 mg. L⁻¹ of water can be harmful to fishes. It shows that the bottom of aquaculture ponds may become foul as a result of excessive amounts of decomposition of anaerobic nature (Datta, 2012). The following reaction depicts how unionized ammonia (NH₃) exist in an equilibrium state with the ammonium (NH₄⁺) ion in water: $\text{NH}_3 + \text{H}_2\text{O} \rightleftharpoons \text{NH}_3 \cdot \text{H}_2\text{O} \rightleftharpoons \text{NH}_4\text{OH} + \text{H}_2\text{O}$.

Due to the daily cycling of pH, it is difficult to accurately evaluate the toxicity of TAN in aquaculture environment as the concentration of FAN changes continuously. In natural environments, such as ocean, river, and lakes, the FAN may never reach dangerous levels, as a result of fish densities being low, whereas fish farmers who maintain high fish densities run the risk of FAN increasing to toxic levels (Swann, 1997).

Each species has its own species-specific tolerance range of acute exposure to un-ionized ammonia. Values that are lower than 0.02 ppm NH₃ are however generally considered to be safe (Datta, 2012). Waste products such as carbon dioxide and ammonia which exist in ionized and un-ionized forms in water may occur in higher concentrations in high intensity aquaculture systems (Sanni & Forsberg 1996; Huchette *et al.*, 2003b; Piedrahita, 2003; Naylor *et al.*, 2011; Colt *et al.*, 2012; Liu *et al.*, 2022).

Provided that alkalinity concentration and hardness continue being constant, concentrations of dissolved carbon dioxide can be determined by monitoring pH changes that take place as a result hydrogen ions (H⁺) being released when carbon dioxide hydrates in water. The unionized forms of ammonia and carbon dioxide are the ones mostly responsible for exhibiting toxicity (Thurston *et al.*, 1981; Sanni & Forsberg, 1996).

A few studies have been done in trying to understand the water quality at South African abalone farms (Yearsley, 2008; Naylor *et al.*, 2011; Probyn *et al.*, 2017; Eze *et al.*, 2021), however, none of these studies examined the effect of the water-off event on abalone growth, survival, and health. During a water-off event a decrease in dissolved oxygen, increase in carbon dioxide and a rise in ammonia, nitrite, and nitrate are to be expected. These change in water quality parameters could result in physiological and immunological dysfunction, reduced feeding and growth, and an increase in mortalities. Determination of the first limiting water quality variable for the health and growth of *H. midae* during a water-off period is important for refining the best response and management for such a problem.

Stocking density of the farmed species and water flow are two of the main factors that affect water quality in intensive aquaculture (Harris *et al.*, 1998b; Colt *et al.*, 2009). It is thus also important to consider how growth and health of the farmed animals may be affected by the flow index (L.h⁻¹.kg⁻¹) in order to improve the efficiency in production (Fivelstad, 1988; Colt & Orwicz, 1991). Low flow rates or the lack of water flow may cause health and growth problems for abalone (Naylor *et al.*, 2011). If water quality parameters and the switching off of water flow are well understood, managers can use this information to inform decision making regarding priorities when these interruptions occur and when there are HABs.

1.1.2. Stress in animals

There have been different ways in which the word “stress” has been used. The word has been used in describing both the stress response and the stressor (Barton, 1997). According to Parker (2002) as well

as Asres & Amha (2014) conditions that result in an animal not being able to maintain normal physiological state and affect the animal's well-being are considered to be stress. Factors that cause stress in animals can be classified into a number of groups which are chemical, physical, biological, immunological and procedural stressors (Asres & Amha, 2014; Martinez-Miro *et al.*, 2016; Morash & Alter, 2016; Inbaraj *et al.*, 2019).

Chemical stressors include diet composition, poor water quality, pollution, and metabolic wastes. Secondly, physical stressors include dissolved gases, light, sound, and temperature. Thirdly, there are biological stressors which include food competition, genetically modified species, introduced species, stocking density, etc. The fourth stressor which may affect animals are immunological stressors (Martinez-Miro *et al.*, 2016). Immunological stressors are those which cause diseases to animals (Martinez-Miro *et al.*, 2016). Lastly there are procedural stressors, and these include disease treatments, grading, handling, and shipping (Morash & Alter, 2016).

When properly managed and operated, many aquaculture procedures can be effectively operated to minimize how stress the animals are exposed to (Rehman *et al.*, 2017).

Effective management of stress in animals is dependent on which species are chosen, the history of the cultured animals, the chemistry of the water, the flow rate of the water, the water temperature, ambient light cycles and conditions, bottom substrates, physical stimuli including noise, stocking density, how much the animals are being handled and the use of anaesthetics (AFS, 2004).

With increasing stress, more energy is used by the animal, resulting in them growing less than those that are not stressed, and as a consequence, the risk of mortality increases (Reay, 1979; Fallu, 1991). The gills of abalone collapse during periods of aerial exposure and therefore respiration becomes difficult to achieve (Fallu, 1991; Ragg & Taylor, 2006).

Abalone are however generally able to survive short periods of time exposed to air as they have the ability to undergo anaerobic respiration (Gade, 1988; Baldwin *et al.*, 1992; Behrens *et al.*, 2002; Leonart *et al.*, 2003). Live juvenile abalone can survive for up to 56 h when exposed to air at 16°C (Sales & Britz, 2001).

Being exposed to air during grading (for example) is a common stress causing factor in farmed abalone, mostly due to their need to shift to using anaerobic respiration (Olley & Thrower, 1977; Gade, 1988). Anaerobic respiration is more energetically expensive as glucose molecules only get split into

pyruvate molecules which then gives rise to 2 Adenosine Triphosphate molecules, whereas aerobic respiration produces carbon dioxide which produces 38 ATP molecules and more energy (Granchi *et al.*, 2010).

1.1.3. Abalone biochemical stress indicators and their assessment

Being a member of the gastropods (class Gastropoda, phylum Mollusca) also means that abalone are also susceptible to a few diseases (O'Brien & Pellett, 2022). Some of the diseases that may be found in abalone could be caused by different classes of pathogens which include viruses (herpesvirus-1 (HaHV-1), bacterium (*Clostridium sp.*), fungi (*Atkinsiella awabi sp.*), and endoparasites (Lauckner, 1980; Dixon *et al.*, 1991; Newton & Smolowitz, 2018; O'Brien & Pellett, 2022). The pathogens may affect the animals by compromising the immunity of the animals by causing stress which may lead to mortalities (Lauckner, 1980).

The ciliated, moist and mucus producing epithelial layer of gastropods such as the abalone forms the first layer or physical barrier to prevent the colonization by pathogens, thus preventing pathogen related stress from occurring (Loker, 2010). Isolation of achacin from the mucus covering the body of the *Achatina fulica* (giant African land snail) has suggested that mucus forms a chemical barrier (Ehara *et al.*, 2002). The achacin is a dimeric flavoprotein that generates peroxide (H_2O_2) from the oxidative deamination of other amino acids and possesses antibacterial properties (Ehara *et al.*, 2002).

In addition to the mucus acting as a defence against foreign bodies, the snails produce hemocytes and rhogocytes in their open circulatory system (Loker, 2010). The hemocytes are defence cells that are responsible for the production of reactive oxygens species (ROS) and antimicrobial peptides (AMPs) which work with hemocytes in getting rid of pathogens, while the rhogocytes are involved in ingesting small foreign particles (Loker, 2010). Not a lot is known about the immune response of gastropods to pathogens, however different tests can be used as an assessment to different pathogens, and how the responses might be altered in snails from natural habitats, including those experiencing rapid environmental changes that may cause stress (Loker, 2010). The metabolism of marine species such as abalone may be affected by environmental factors (Widdows, 1985).

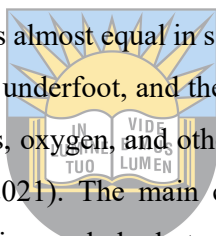
An example of this was depicted by a study that showed a long-term increase in the water temperatures off Korea (Lim & Chang, 2016). Such increases decrease the immunity and change the blood

parameters of aquatic animals (Perez-Casanova *et al.*, 2008). In addition, *H. discus hannai* has a reputation of being sensitive to low levels of salinity, and they have been reported to lower both the metabolism and growth of the abalone (Kong *et al.*, 2017; Boamah *et al.*, 2020). Under stressful conditions, the metabolism of carbohydrates increases for marine life (Paterson & Spanoghe, 1997).

It is for the above- mentioned reasons that growth and biochemical reactions can be used as stress indicators in abalone.

Biochemical reactions take place in a fluid called haemolymph (Report, 2006; Dolashka *et al.*, 2011). Haemolymph is an animal body fluid in arthropods, insects, and molluscs, and is composed of various biochemically and pharmacologically active substances is found in an open circulatory system (Findley & Stickle, 1978; Report, 2006; Dolashka *et al.*, 2011, 2015). This fluid acts as a hydrostatic skeleton and is found in the foot of the abalone (Kilburn & Ripper, 1982; Payne & Crawford, 1989; Mgaya & Mercer, 1994; Hickman *et al.*, 2006; Venter *et al.*, 2016).

The foot of abalone is large and flat and is almost equal in size to the shell of the abalone. It is divided into several parts, the epipodium and the underfoot, and the underfoot is discoid (Di *et al.*, 2012). Its major function is to transport metabolites, oxygen, and other components to the animal's tissues and organs (Machałowski & Jesionowski, 2021). The main compositions of molluscs include water, inorganic salts, and organic compounds (i.e., carbohydrates, enzymes, lipids, and proteins) hormones and hemocytes (Machałowski & Jesionowski, 2021).



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1.1.3.1. Abalone growth

Growth in animals can be defined as food that being ingested later being converted to body tissue (Barnabé, 1994). To ensure successful commercial production, sufficient, and viable, it is a fundamental management step to maximize the growth rate of the abalone (Keesing & Wells, 1989; Capinpin *et al.*, 1999; Lee, 2004; Setyono, 2005; Naidoo *et al.*, 2006). When abalone growth is suppressed, productivity is reduced, and as a result profitability of the commercial abalone farm is also reduced (Lyon, 1995).

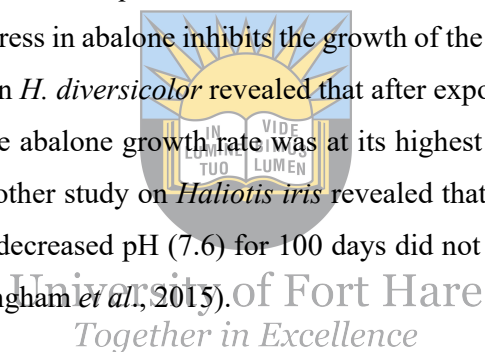
Abalone growth takes place at a slow rate and therefore several studies have focused on abalone growth in an effort to improve production (Day & Fleming, 1992; Ebert, 1991; Forster, 1967; Hooker &

Morse, 1985; Leighton, 1974; Leighton & Boolootian, 1963; Li *et al.*, 2004; Mgaya & Mercer, 1995; Newman, 1968; Poore, 1972b, 1972a; Preece & Mladenov, 1999; Reay, 1979; Shepherd & Hearn, 1983).

The growth rate of abalone has been previously used as a measure of their chronic stress. An increase of metabolic waste such as ammonia as well as a stocking density increase have resulted in decrease in abalone growth (Capinpin *et al.*, 1999; Huchette *et al.*, 2003; Naylor *et al.*, 2011).

Growth parameters can be estimated by measuring each mark between known time periods. The advantage of this measuring method is being able to measure growth over a number of years without a lengthy and expensive field programme (Day & Fleming, 1992).

A study that looked at how ammonia affects the immune response of *H. diversicolor supertexta* and the susceptibility of the abalone to *V. parahaemolyticus*, as well as the one which investigated juvenile blacklip abalone (*H. rubra*) growth in aquaculture tanks revealed that an increase in metabolic wastes (i.e., ammonia) which cause stress in abalone inhibits the growth of the abalone (Huchette *et al.*, 2003; Cheng *et al.*, 2004). A study on *H. diversicolor* revealed that after exposure to different salinity levels (20, 25, 31, 35 and 40 ppt) the abalone growth rate was at its highest at 31 ppt and lowest at 20 ppt (Creencia & Noro, 2018). Another study on *Haliotis iris* revealed that exposure to elevated levels of carbon dioxide which in turn decreased pH (7.6) for 100 days did not increase mortality but reduced the growth of abalone (Cunningham *et al.*, 2015).



1.1.3.2. Biochemical indicators of abalone health or stress

1.1.3.2.1. GLUCOSE

Haemolymph glucose is the main energy provider and regulator in the metabolism of glycogen in molluscs (Borges *et al.* 2004), and it is mainly derived from conversion of glycogen (Principe *et al.*, 2019). During periods of fasting, glucose levels are maintained by breaking down glycogen (glycogenolysis) or protein reserves (gluconeogenesis) (Chourpiliadis & Mohiuddin, 2023). Therefore, glycogen reserve conditions when for survival of an animal during periods of fasting, including under stressful conditions, when energy demands are relatively high. Under normal conditions, the

circulating haemolymph glucose concentration is tightly maintained within a narrow range (Verri *et al.*, 2001).

However, haemolymph glucose levels can spike during stressful conditions (Baeck *et al.*, 2014), and is one of the best well-known indicators of stress in most animals (Carefoot, 1994; Haldane, 2002; Hooper *et al.*, 2007). Glucose can be measured using several methods, including the copper reduction method (modified from Folin Wu) (Services, 1984), the o-toluidine method (Dobowski, 1962), the hexokinase/glucose-6-phosphate dehydrogenase (G6PD) method (Passey *et al.*, 1974), a glucose oxidase assay, and the glucose oxidase and peroxidase (GOD-POD) method. Of these methods, the hexokinase assay, is largely accepted as the standard reference method for glucose estimation (Ambade *et al.*, 1998).

An evaluation of these methods against the hexokinase method was done by Ambade *et al.*, (1998). The GOD-POD method gave good precision and accuracy (CV = 0.7 % -1.4 %), whereas the Modified Folin Wu and O-Toluidine procedures were less precise, with a substantial upward deviation with a coefficient of variation of up to 6 % (Ambade *et al.*, 1998).

The other advantages of the GOD-POD method are that it is both highly specific for glucose and very sensitive. Compared to the hexokinase method, the GOD-POD method has benefits of being simple, rapid, and economical. Methods, which are used to measure animal tissue glycogen can be divided into two general categories: (i) the glycogen can be determined in its original state or (ii) after hydrolysing tissue glycogen to form simple sugars followed by enzymatic measurement of glucose (Passonneau & Lauderdale, 1974).

A study on *Aplysia dactylomela* revealed that after exposure to tidepool stranding there was a rise in the blood – glucose concentration of the sea hare (Carefoot, 1994). Another study which made use of biochemical indicators to measure stress in greenlip abalone (*Haliotis laevis*) indicated that haemolymph glucose levels of *H. laevis* rise in response to an increase in temperature (Haldane, 2002).

1.1.3.2.2. GLYCOGEN

Glycogen, stored in the foot muscle of abalone is a polymer of D glucose that is two branched and it can form fuel that is readily available for the metabolism of carbohydrates (Schmidt-Nielsen, 1998).

By having a metabolism that is carbon-based, glycogen can provide a major form of energy storage for the abalone (Baldwin *et al.*, 1992; Picos-García *et al.*, 2000; Braid *et al.*, 2005). Glycogen is important to the abalone as it provides energy storage for when the abalone needs to be sustained, this includes crucial periods such as those of fasting, dealing severe disease or stress, as well as during periods of low feeding. It can therefore be said that the glycogen levels of abalone may be used as a method of monitoring abalone health and tissue deterioration.

In a study where the basic biochemical constituents in the body tissues of South African abalone was being explored it was found that the muscle glycogen concentration was $81.80 \pm 2.37 \text{ g kg}^{-1}$ ($n = 192$) in older animals (Laas & Vosloo, 2010). Another study that investigated if the end products of anaerobic metabolism could be utilized as indicators of stress during the transportation of *H. midae* revealed that the foot glycogen of abalone at rest is $60 \text{ } \mu\text{moles glycosyl g wet weight}^{-1}$ (Omolo *et al.*, 2003).

1.1.3.2.3. DOPAMINE

Basic knowledge of the biochemical properties of abalone in culture conditions is essential as a management tool of aquaculture systems (Vosloo & Laas, 2010). Of these biochemical properties dopamine is found extensively throughout the animal kingdom. It has a common function in the motor control sensory gating, and a neurotransmitter (Montague *et al.*, 1996; Sawin *et al.*, 2000; Schultz, 2010; Strausfeld & Hirth, 2013). Dopamine is also proposed to act as a proprioceptive signal in the feeding systems of gastropods (Miller, 2020).

A study conducted on *Haliotis tuberculata* revealed that after being exposed to a stressor, the dopamine and noradrenaline of the abalone undergo a rapid rise to $2 - 2.5 \text{ ng/ml}$ (Malham *et al.*, 2003) and then decrease back to normal of $0.5 - 1 \text{ ng/ml}$ levels 15 – 100 minutes after the stress has stopped (Malham *et al.*, 2003).

Another study on *Crossostrea gigas* revealed that after exposure to air exposure at different temperatures (15 and $27.5 \text{ }^{\circ}\text{C}$) revealed that the dopamine levels rise significantly before returning to basal level (Qu *et al.*, 2009). Similarly, a study on *H. discus hannai* revealed that after exposure to blue light stress, the dopamine levels increased (Zhang *et al.*, 2023).

1.1.3.2.4. SUPEROXIDE DISMUTASE

Increased antioxidant responses are elicited by marine organisms when they are exposed to aquatic environments that are complex. During aerobic cellular metabolism, reactive oxygen species (ROS), oxygen free radicals are produced, and these include hydrogen peroxide, hydroxyl radicals, single oxygen, and superoxide anions (Genestra, 2007). Superoxide dismutase is an acidic metalloenzyme that is ubiquitous in microorganisms, animals, and plants.

The role of superoxide includes being a detoxification agent that scavenges on free radicals that are products of exogenous and endogenous substances, to prevent lipid peroxidation, and to delay senescence for the production of the organism (Fukai & Ushio-Fukai, 2011; Che *et al.*, 2016). Superoxide dismutase is commonly utilized as a potential marker for assessing water pollution and environmental stress, due to its ability to improve viral resistance and phagocytosis in cells (Achuba, 2002; Penicaud *et al.*, 2017; Ren *et al.*, 2017).

Two types of superoxide dismutase are found in abalone, and they include: copper and zinc SOD (Cu/Zn-SOD), and manganese SOD (MnSOD) (Smith *et al.*, 2003; Manduzio, 2005; Wu *et al.*, 2008., Lin *et al.*, 2008). The Cu/Zn-SOD can further be classified into extracellular and intracellular Cu/Zn-SOD where the former has an N-terminal signal sequence, and the latter does not have an N-terminal signal sequence (Sheraz *et al.*, 2023).

Cu/Zn-SOD can be found in the cytoplasm, whereas Mn-SOD can be found in the mitochondria and forms part of the of the first line of defence against superoxide radicals that are products of oxidative phosphorylation (Ekanayake *et al.*, 2006; de Zoysa *et al.*, 2009). More data exists about the functioning of the Cu/Zn-SODs than on the other types (Hong-Duk *et al.*, 1996; Kim *et al.*, 2011). Superoxide dismutase is a scavenger of free radical species that are products of endogenous and exogenous substances (Qiao *et al.*, 2020).

Previous studies such as the one which looked at how sub-optimal or reduction in salinity and temperature decrease antioxidant activity and cellularity in the haemolymph of the Pacific abalone (*H. discus hannai*) revealed that the Superoxide dismutase activity was significantly higher $5.5 - 6 \text{ U mL}^{-1}$ at the temperatures $4-6^{\circ}\text{C}$ and salinity level of 26 psu for 7 days than in the control ($2 - 3 \text{ U mL}^{-1}$) at 4°C at 38 psu, which suggests that when stressed, the Pacific abalone has more SOD activity when under stressful conditions (Chadès *et al.*, 2012; Yang & Min, 2019). A study on *Charybdis japonica* revealed that after exposure to different amounts of a xenoestrogen (bisphenol A) for 15 days the SOD

activity of the crabs increased at the initial exposure and decreased afterwards (Peng *et al.*, 2018). Another study on revealed that after exposure to different temperatures the levels of SOD increased (Yasa *et al.*, 2019).

1.1.3.2.5. TOTAL HAEMOLYMPH PROTEINS

Proteins are macromolecules that consist of a single or more polypeptide chains with each of these chains having characteristic sequence with which the polypeptide bonds link the amino acids. A study on *Crassostrea virginica* revealed that after exposure to different seasonal environmental variables (food availability, temperature, and salinity), the haemolymph protein of the oyster showed seasonal fluctuations (Chu & La Peyre, 1989).

An investigation on the haematological pathology of shell lesions in the New Zealand abalone, *H. iris* (Mollusca: Gastropoda) were able to give an idea of how total haemolymph protein is affected by stress (Nollens *et al.*, 2004). The study showed that at rest, the abalone has a total haemolymph protein reference range of 18.6-21.2 g. L⁻¹ with animals that were unaffected by the lesions having 19.9 ± 0.7 g. L⁻¹ those that were mildly and severely affected had 19.8 ± 0.8 and 21.1±1.0 g. L⁻¹ of total haemolymph protein respectively (Nollens *et al.*, 2004). These results of the above-mentioned study revealed that during stressful conditions, the number of total proteins in *H. iris* increases (Nollens *et al.*, 2004). A study on the hybrid *H. laevigata* and *H. rubra*, revealed that the total haemolymph protein concentration of control abalone was higher than that of the treatment abalone on the first two days of being exposed to heat stress (Hooper *et al.*, 2014).

1.1.3.2.6. HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) form the main part of the chaperone system and are key in the regulation of the process of cellular acclimatization to different stresses and are found almost everywhere in multicellular organisms (Kyeong *et al.*, 2019). There are six major families of heat shock proteins, and they are small HSPs (sHSPs), HSP60, HSP100, HSP90, HSP40, HSP60, and HSP70 (Rosenzweig *et al.* n.d., Ratheesh Kumar *et al.* 2012, Jaspard & Hunault 2016). Heat shock proteins also have a housekeeping role for genes, where they can maintain homeostasis in certain stressful situations by adjusting certain molecules (Kyeong *et al.*, 2019).

These proteins undergo upregulation during different stressful conditions and do not respond only to temperature related stress, as they also function as a common machine for basic cell operations (Kyeong *et al.*, 2019). Heat shock proteins either work individually or work with sHSPs in cell to preform different molecular chaperone tasks (Rosenzweig *et al.*, n.d.). It is worth noting that the levels of HSPs in cells can act as potential indicators to identify the status of thermal stress in abalone (Wan *et al.*, 2012).

Additionally, HSPs and sHSPs become a deciding factor for different housekeeping activities (Rosenzweig *et al.*, n.d.), thus the Pacific abalone proteome is compared to the proteomes of other shellfish species and representatives from various taxa (i.e., plants, animals, bacteria, insects, yeast, and worms) to gain a better understanding to homologs of the HSP family of proteins in *H. discus* (Kyeong *et al.*, 2019). A study was conducted to investigate the response of Hsp70 Gene to the *V. parahaemolyticus* infection and thermal stress and its transcriptional regulation analysis in *H. diversicolor* (Fang *et al.*, 2019). The results of the study revealed a high expression of Hsp70 in hemocytes under both thermal and pathogen stress due to the immune response being activated (Fang *et al.*, 2019). A study on *H. discus* revealed that after exposure to different environmental stimuli there was an overexpression of HdHsp20 which indicates that the gene for HdHsp20 is involved in stress resistance mechanisms and may indicate the stress state of abalone (Wan *et al.*, 2012). A study by Cheng *et al* (2006) where a report on growth and expression of HSP70 expression on *H. discus hannai* inbred and hybrid populations revealed that inbred populations increased expression at low temperatures while hybrid populations increased expression at higher temperatures.

Another study on *H. midae* revealed that after short term (24 hours) exposure to changes in temperature (16, 19, and 22° C) the Hsp70 levels of the abalone high for all temperatures (Vosloo & Vosloo, 2010). The study also examined chronic (1 month) exposure to the same temperatures (16, 19, and 22° C) which revealed Hsp70 were higher at 22° C than at 16 and 19° C (Vosloo & Vosloo 2010).

1.1.4. Rationale and motivation

While all abalone farms have back-up systems to mitigate potential air or water supply interruptions, there are occasions when such interruptions are unavoidable (e.g., during harmful algal blooms, installations, maintenance, or loadshedding) or occur because of system failure.

There is currently no documented information on the immediate and long-term effect of such interruptions on abalone stress and growth response which can inform management on how best to avoid stressing their stock.

Assessing stress and health of abalone is an important monitoring function on abalone farms. Monitoring of these changes allows them to play the role of “early warning signals” which uses biological markers of stress responses (Otitoju & Onwurah, 2007). If these parameters are known, managers can use this information to inform decision making regarding priorities when these interruptions occur.

Many abalone farms are dependent on coastal water inputs which are subject to changes in environmental variables such as CO₂, temperature, oxygen, and salinity (Morash & Alter, 2016). During the periods of air and water interruptions, (which may extend up to 96 hours) oxygen depletion, as well as CO₂ and ammonia accumulation may occur within the tanks. In addition, the current study looks to add to available literature on the effects of water off events on the water quality of abalone farms.

This study therefore set out to investigate the effect of a water-off event on various abalone stress indicators and growth conducted at HIK abalone farm.

The study was conducted during the Covid-19 pandemic when movement and access to facilities was limited. Laboratory analyses were therefore limited to the university’s Alice campus, and as a result, only “kit” type assays were used as they were easier to optimise and use compared to other possibly more recognised methods (i.e., Heat Shock Proteins) which are more technical and equipment specific to implement.

1.2. Research aims and objectives

Aim

To determine the effect of long-term water-off events on the stress indicator parameters (e.g., glucose, glycogen, dopamine, total haemolymph protein and superoxide dismutase), growth, and survival of farmed *H. midae*.

Objectives of the study

The main objectives were to:

- i) Determine the effect of 24, 48, and 96 hours of water-off events on the water quality parameters (e.g., Temperature, Dissolved oxygen, pH, TAN, and FAN) in *H. midae* tanks.
- ii) Determine the effect of 24, 48, and 96 hours of water-off events on the growth and survival *H. midae*.
- iii) Determine the effect of 24, 48, and 96 hours water-off events on the various stress indicator parameters (e.g., glucose, glycogen, dopamine, superoxide dismutase, total haemolymph protein) of *H. midae*.

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1.3. Hypothesis

Null hypothesis

Water-off interruptions will result in no differences in the water quality, biochemical stress indicators, growth, or survival of *H. midae*.

CHAPTER 2: MATERIALS AND METHODS

Study site

The study took place at HIK Abalone Farm (Pty) Ltd, in Hermanus (- 34.435209, 19.221237) in the Western Cape province of South Africa from September 2021 – January 2022.



Figure 2.1: Google Maps images indicating the position of HIK Abalone Farm in Hermanus South Africa (-34.435209, 19.221237).

Experimental design

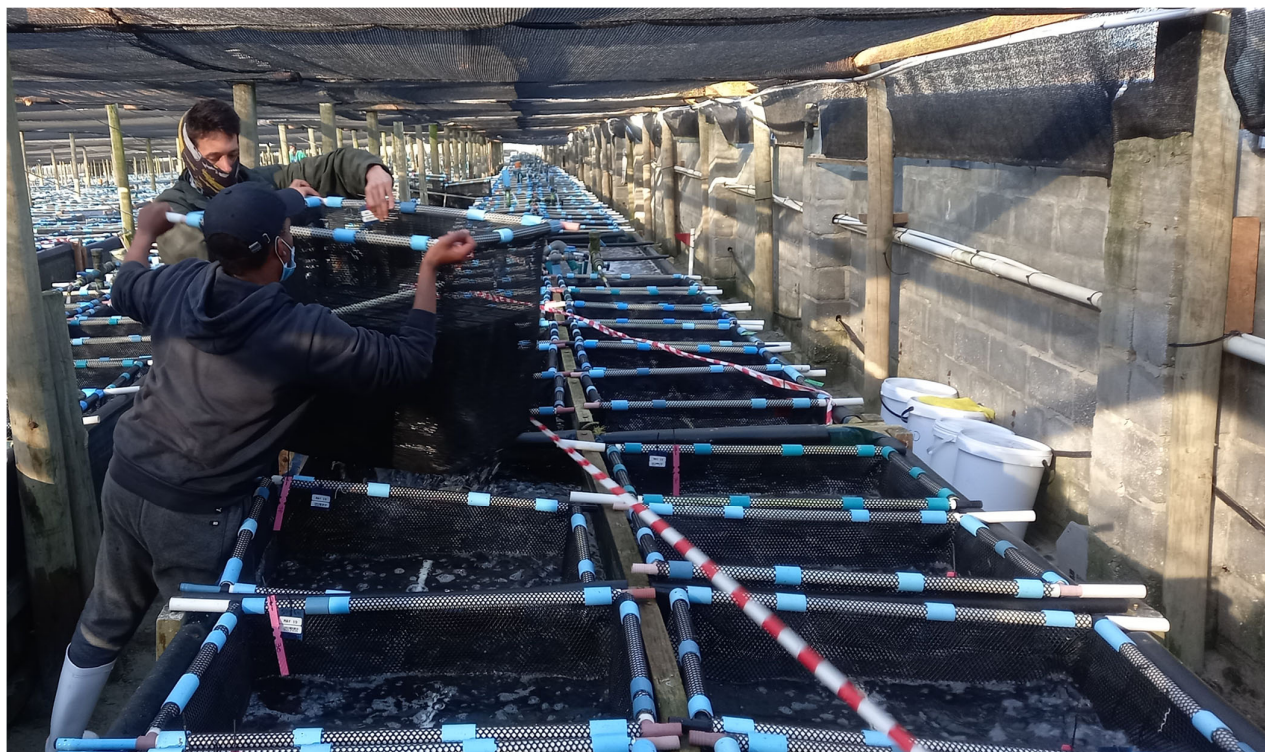


Figure 2.2: Transferring of experiment treatments tank baskets (with ± 80 abalone) to recovery tanks during 96-hour water off experiment.

Ten tanks on HIK Abalone Farm (Pty) Ltd were used for this study. Three of the tanks served as control (C) tanks, three tanks were experimental (E) treatment tanks, while four of the tanks were Initially empty and used as the recovery (R) tanks.

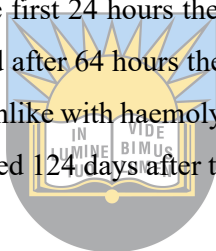
The experiment was conducted using the farm's commercial stocking densities (which were based on the size of the animals made available for this experiment). Abalone were between 45 and 55 g (approximately 29 months old) were distributed across twelve baskets in each of six tanks. Each of the baskets held a minimum of 80 abalone.

Maintaining commercial stocking densities was important for this study as the abalone conditions in which the experiment was being conducted had to be realistic to ensure the results were relevant and therefore of value to the farm.

All the animals in the control and experimental groups were weighed (g) prior to the start of the experiment using the farm's standard operation procedure. The baskets from the three treatment tanks were then divided into three groups, 24hr water off, 48hr water off, and 96hr water off group. The abalone in both the control and treatment tanks continued being fed (pelleted formulated feed (34% protein) until the start of the experiment. Thereafter, both control and treatment abalone were not fed for the duration of the water-off period.

After 24hours of acclimation the water was then switched off. In the treatment tanks water quality (temperature, pH, dissolved oxygen, and ammonia) was determined immediately prior to and after the water was switched off, Abalone were then taken from the control tanks to obtain haemolymph and muscle tissue, which served as T₀ samples. By the end of the 96-hour water off period, all baskets had been moved to the recovery tanks and a mortality count of each basket was conducted.

Within the first 24 hours of switching the water off, the water quality and abalone tissue samples were obtained at six-hourly intervals. After the first 24 hours the water quality and abalone tissue samples were obtained at eight-hour intervals, and after 64 hours the water quality and abalone tissue samples were obtained at twelve-hour intervals. Unlike with haemolymph and tissue samples, growth and long-term survival of the abalone was performed 124 days after the start of the experiment using the farm's standard operation procedure.



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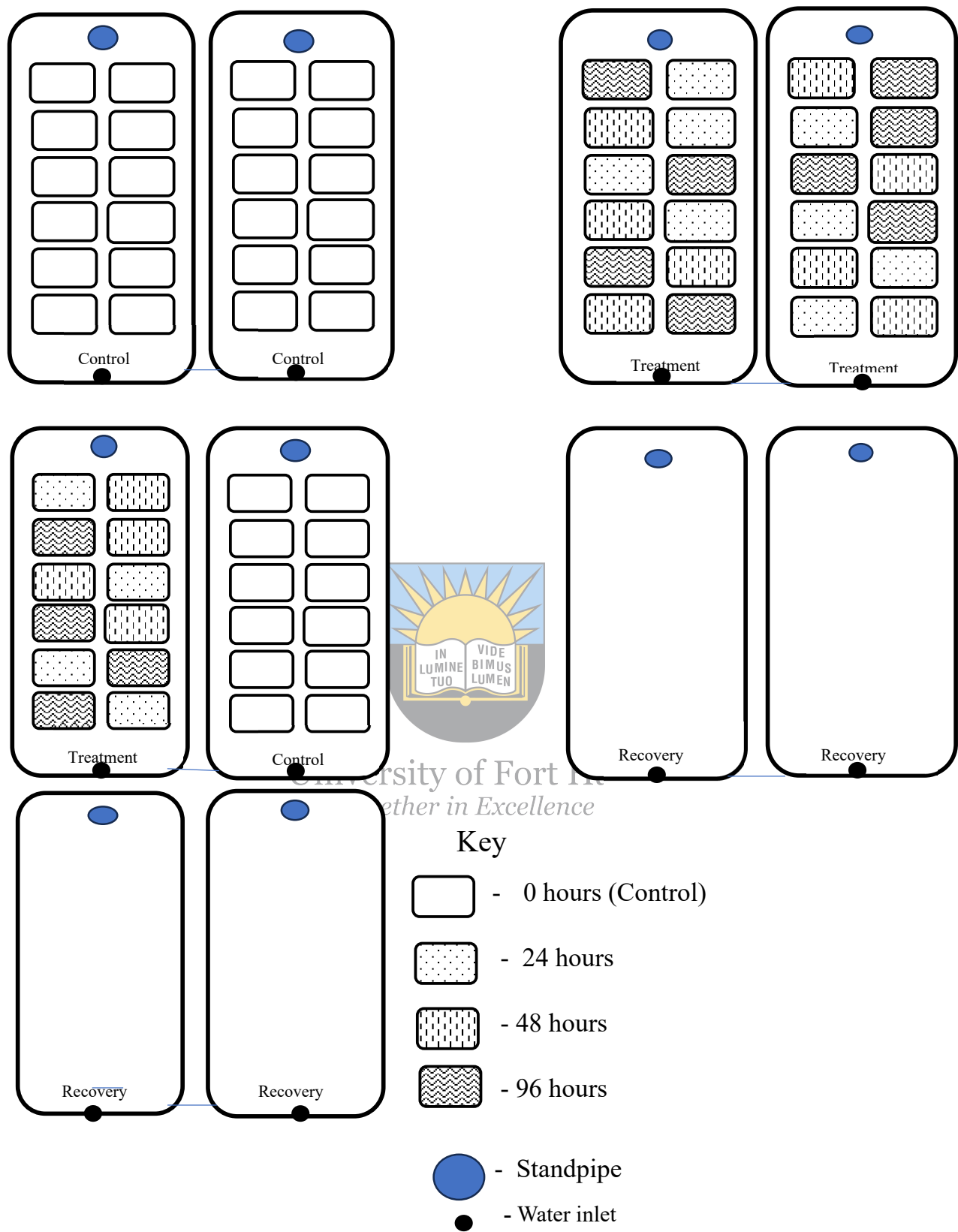


Figure 2.3: Graphical representation of basket distribution in the abalone tanks (Control, Treatment, and Recovery) at the start of the experiment.

2.1. Water Quality

Twenty-four hours after the initial weighing of the abalone, water to the treatment tanks was turned off. Water into the control and recovery tanks remained on.

Water quality parameters (Temperature, pH, Dissolved oxygen (saturation % and mg. L⁻¹), and ammonia) were measured at the outflow (standpipe) immediately after the water was switched off, and these served as T₀ samples. The water quality measurements were conducted by measuring on each of the tanks (three control tanks, three experimental tanks, and four recovery tanks) as a basis of replications. For the first 24 hours after switching the water off, water quality and abalone tissue samples were obtained at six-hour intervals. From 32 - 72h samples were taken at eight-hour intervals, and from 84 - 108h samples were taken at twelve-hour intervals.

The experiment ran for 96-hours in the Treatment tanks but continued for a further 12 hours in the Recovery tanks. An OxyGuard Handy Polaris probe was used to determine both temperature (° C) and Dissolved oxygen (Saturation% and mg. L⁻¹), while the OxyGuard Handy pH meter was used to determine the pH.

Total Ammonia Nitrogen (TAN) concentration was determined with the use of the Solorzano (1969) method with colour absorbance read by spectrophotometer. Free Ammonia Nitrogen (FAN) concentrations (µg. L⁻¹) were calculated based on Emerson *et al.* (1975) using TAN, pH and temperature. All water quality measurements were taken at the water outflow of each tank.

For TAN determination a phenol alcohol solution, and sodium nitroprusside solution (0.5%). Tri-sodium citrate solution (%), and a hypochlorite solution (%) were prepared according to Solorzano (1969). An oxidizing agent (made fresh on each sampling day) was prepared by mixing the tri-sodium citrate solution with and hypochlorite solution adjusted to the number of samples at a 4:1 ratio. To 25 mL of the water sample, 1 mL of phenol solution was added and mixed thoroughly. This was then followed by the addition of 1mL sodium nitroprusside solution to the water-phenol solution before mixing thoroughly (Solorzano, 1969).

Finally, 2.5mL of oxidizing solution was added before mixing thoroughly. The solution was left in a dark cupboard for an hour before observing absorbance at 640nm using a spectrophotometer (Secomam, Prim Light).

The TAN concentration together with the pH were incorporated in the formula (Emerson et al. 1975) to determine the NH_3 concentration:

$$1. \text{pK}_a = 0.09018 + 2729.92 / T$$

$$2. f = 1 / (10^{\text{pK}_a - \text{pH}} + 1)$$

$$3. \text{NH}_3 (\text{mg. L}^{-1}) = \text{TAN} * f$$

Where: T = temperature in Kelvin and TAN = Total ammonia nitrogen (Emerson et al. 1975).

2.2. Growth and Survival sampling

Farm standard operating procedure for abalone basket weight

Twenty-four hours before starting the experiment, Control and Treatment group abalone baskets were moved to tanks closer to the farm's splitting station. At the splitting station, empty anaesthetic tanks were filled with fresh sea water where magnesium sulphate (40.2 g. L^{-1}) was added together. The basket was then placed into the anaesthetic tank for 2 minutes before the rack in the basket was then lifted, and the abalone on the rack gently slid off into the basket.

After two minutes, the abalone were removed by hand and placed into bowls and weighed (g) in groups of 50 abalone to get an average weight of the abalone in the basket as well as the total weight of the basket. Damaged animals in the sub-sample were also counted and any shells found within the baskets were recorded as mortalities. An average abalone mass of the twelve baskets was used for statistical analysis. This process was repeated four months after the start of experiment to determine the difference in growth between the control and experimental abalone.

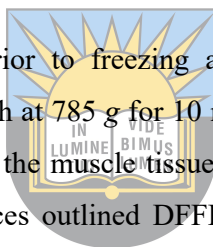
2.3. Haemolymph and Muscle Tissue

Sampling

During each sampling session, abalone were randomly selected from the control and treatment baskets. A 2 G needle and syringe were used to draw ± 2 ml of haemolymph from the foot of the abalone before the abalone were euthanized (le Bris *et al.*, 2014). Euthanasia took place by placing the abalone on its shell on ice until it no longer moved. After euthanasia, a ± 1 cm piece of tissue was removed from the posterior foot section of the abalone using scissors from a dissecting kit.

Both the 2 ml haemolymph and the muscle tissue were transferred into individual 2 ml Eppendorf tubes which were then put in a cooler box with water and ice packs to minimize enzymatic activity and degradation of the haemolymph/tissue. After all samples were collected for that time period, they were stored in a -18°C freezer before it was ready was kept in dry ice (-80°) for transportation. .

The processing of the haemolymph prior to freezing at -18°C entailed centrifuging (Hermle Labortechnik, Z 160 M), the haemolymph at 785 g for 10 minutes at 4°C (le Bris *et al.*, 2014). After drawing the haemolymph and removing the muscle tissue from the abalone, the abalone were then discarded as per current farming practices outlined DFFE Aquaculture Processing Facility permit conditions.



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After the end of the 96-hour sampling period, all the samples that were stored in the freezer were then transported in a cooler box with dry ice (Venter *et al.*, 2018) from Hermanus to Alice (Department of Biochemistry and Microbiology, University of Fort Hare) where they were then placed in a -80°C freezer until analysis (le Bris *et al.*, 2014). Samples were then thawed at room temperature before being analysed.

2.3.1. Total haemolymph protein determination

Total haemolymph protein levels of the abalone were determined using the Bradford Method for Protein quantification. The Bradford determination (Bradford, 1976) was conducted with the use of the Bradford Protein Assay kit (Product number: B6916, Merck). Bovine Serum Albumine (Product number: 10711454001, Merck) was used as a protein standard (le Bris *et al.*, 2014). Data is mean $\pm$ SD

of four replicates for each sampling period, where a total of $N = 624$ samples were obtained and the average was analysed for each sampling period.

For each sample, 20 μL was put in an Eppendorf tube before adding 80 μL of 0.15 N NaCl to make up a volume of 100 μL . To each tube 1 mL of Bradford reagent was added and was put on a vortex before being allowed to stand at room temperature for two minutes. From each of the samples 300 μL was taken and placed into a microplate to determine absorbance at 595 nm.

2.3.2. Deproteinization of haemolymph samples

Haemolymph samples have a high concentration of proteins (Kanost, 2009). Proteins may cause interference and inaccuracies in subsequent assays through the formation of precipitate compounds (Berth & Delanghe, 2004). After aliquots were removed for protein content determination, deproteinization was conducted on the samples whereby 200 μL of 5% trichloroacetic acid (TCA) was added to 200 μL of a sample to precipitate the haemolymph proteins. After 20 minutes at room temperature the TCA-sample was centrifuged at 13000 $\times g$ for 5 minutes before removing the clear supernatant for further analysis (Rajalingam *et al.*, 2009). The suitable concentrations of TCA were determined by comparing the glucose levels of each sample with different concentrations of TCA (5%, 1%, 0.5%, and 0%) after deproteinization.

Reference samples, with known concentrations of glucose, were also treated with varying TCA concentrations to determine interference of TCA. A control was prepared by mixing the assay reagent with TCA. Glucose levels in haemolymph samples and reference samples were calculated from a standard curve of calibration standards. TCA concentrations which have the least interference (i.e., an optical density that is similar to that of water) were used for further assays.

2.3.3. Development and optimisation of assay for measuring haemolymph glucose.

The haemolymph glucose was measured with a quantitative enzymatic GOD-POD assay kit from Sigma-Aldrich (Product code GAGO-20). Data is mean $\pm$ SD of three replicates for each sampling period, where a total of $N = 216$ samples were obtained and the average was analysed for each sampling period.

The assay test kit is designed for 20 samples when using test tubes, cuvettes, and a spectrophotometer.

However, in this experiment, the assay was modified and adapted to a 96-well microtiter plate which allow it to be highly economical and have an increased work output, and thus allow for the testing of larger sample sizes, as well as testing several stressors and their combinations. The modified assay can run more than 350 samples. The assay was adopted for the rest of the tests. Upon deproteinization of the haemolymph, 100 μ L of each sample was put in 2 mL of deionized water. This was followed by adding 300 μ L of the assay reagent to 150 μ L of the sample solution before placing in a water bath at 37° C for 20 minutes. The reaction was stopped by adding 300 μ L of H₂SO₄ before transferring 200 μ L to a microplate and reading the absorbance against a blank at 540 nm.

2.3.4. Dopamine determination

Determination of the haemolymph dopamine in *H. midae* was done using the Elabscience DA (Dopamine) ELISA kit with the product number: E-EL – 0046 (Biocom Africa) making use of the manufacturer's instructions. The dopamine assay kit makes use of a 96-well microtiter plate with which the optical density was determined, and as with the glucose kit the dopamine kit can measure numerous samples and several stressors. Data for dopamine is mean $\pm$ SD of two replicates for each sampling period, where a total of N = 384 samples were obtained and the average was analysed for each sampling period.

After preparing the reagents the wells for diluted standard blank and sample were determined. To each of the predetermined wells, 50 μ L of the standard, blank, and samples were added. This was followed by the addition of the 50 μ L Biotinylated Detection Ab working solution to each well. The microplate was then covered with the provided sealer and incubated at 37 °C for 45 minutes. After decanting the solution from each well, 350 μ L of the wash buffer was added to each well, soaking the well for 1 minute before aspirating and pat drying against absorbent paper (this step was repeated 3 times).

The HRP Conjugate working solution (100 μ L) was then added to each well and the plate was covered with a new sealer before incubating at 37 °C for 30 minutes. The solution from each well was decanted and the wells were washed 5 times before 90 μ L of the Substrate Reagent was added to each well and thereafter covering the plate with a new sealer before incubating it for 15 minutes at 37 °C. After

incubating for 15 minutes, the microplate reader was preheated, and 50 μL of the Stop Solution was added to each well. The optical density value of each well was determined at 450 nm.

2.3.5. SOD determination

The haemolymph Superoxide dismutase activity was determined using the SOD assay kit (Cayman Chemical Company, USA), making use of the manufacturer's instructions. The SOD assay kit makes use of a 96-well microtiter plate where the optical density was determined, and like the glucose kit it could measure numerous samples and several stressors.

Data is mean $\pm$ SD for each sampling period, where a total of $N = 288$ samples were obtained and the average was analysed for each sampling period. After preparing the working solutions, 20 μL of the sample solution was added to each sample and blank 2 well. While 20 μL of ultrapure H_2O got added to each blank 1 and blank 3 well. WST working (200 μL) solution was added to each well before mixing and adding 20 μL dilution buffer to each blank 2 and blank 3 well. This was followed by the addition of 20 μL enzyme working solution to each sample and blank 1 before mixing and incubating at 37 $^\circ\text{C}$ for 20 minutes. The microplate was then placed into a microplate reader to read the absorbance at 450 nm.

$$\text{SOD activity (inhibition rate \%)} = \frac{(\text{ABlank 1} - \text{ABlank 3}) - (\text{ASample} - \text{ABlank 2})}{(\text{ABlank 1} - \text{ABlank 3})} \times 100\%$$

2.3.6. Development and optimisation of an assay for muscle glycogen

The glycogen concentration of abalone was determined by first making use of the acid digestion of tissue glycogen, and subsequently measuring the glucose in solution (digestate) with the use of the modified GOD-POD assay.

To determine the effectiveness of the digestion procedure, varying concentrations (25 – 500 $\mu\text{g. mL}^{-1}$) of the pure glycogen standard (Product code GAGO-20, Sigma Aldrich) were prepared in 2M HCl. The glycogen standard was then digested in duplicates in 10 mL HCl and placed in tightly closed McCartney bottle.

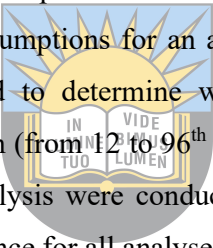
Data is mean $\pm$ SD of three replicates for each sampling period, where a total of $N = 216$ samples were obtained and the average was analysed for each sampling period. Tissue samples were boiled for

90 minutes with vigorous shaking at 30-minute intervals. About 100 mg of weighed tissue was digested in 10 mL HCl to make a determination of appropriate dilution factors for the GOD-POD assay. After boiling for 90 minutes, the samples were then cooled and neutralized by adding an equal volume of 2M NaOH solution.

The neutralized solution was centrifuged at 13000 x g for 5 minutes and the supernatant was prepared into dilutions (1:50) in deionized water. The glycogen concentrations of the diluted samples were then determined using the GOD-POD assay as outlined above in the haemolymph glucose assay (section 2.4.3).

2.4. Statistical analysis

Data from this study was analysed using Shapiro Wilk's test of normality followed by nonparametric tests due to the data not meeting the assumptions for an analysis of variance ANOVA. A Post hoc (Tukey's HSD) analysis was conducted to determine where statistically significant differences occurred on the total haemolymph protein (from 12 to 96th hour time point) with significance for all analyses set at $p < 0.05$. All statistical analysis were conducted using IBM's Statistical Program for Social Sciences (SPSS) v26 and significance for all analyses was set at $p < 0.05$.



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CHAPTER 3: RESULTS

3.1. Water Quality

3.1.1. Temperature

Temperatures differed between the control (C), treatment (T) and recovery (R) tanks throughout the experiment (Kruskal Wallis: $p = 0.04$). At 6, 18, 24, 40, 48, 64 and 96 h, the temperature in the treatment tanks was significantly lower than in the control tanks (Mann Whitney U test: $p = 0.018$), while at 32 h and 56 h (midday sampling period) the temperature in the recovery tanks was higher than in the treatment tanks ($p = 0.02$).

Figure 3 illustrates temperature as observed in the control tanks to have changed over the 96 hours (ANOVA: $p = 0.006$, $F = 20.11$, $df = 7$). Throughout the 96-h period, changes in the temperature of the treatment tanks occurred with significant differences occurring at 48 and 64 h when compared to the start (T_0) of the experiment ($p = 0.05$) when the water was turned off (Related-Samples Friedman's Two-Way Analysis of Variance: $p = 0.006$, $F = 20.11$, $df = 7$). Over the 96-h temperature in the tanks was significantly lower after 84 h compared to the temperature at the start of observation time (T_0) ($p = 0.038$).

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Water temperature rose during daytime and dropped at nighttime throughout the 96-h period for all the tanks, with the temperatures of the Control tanks following similar trends as those of the Surface-seawater temperature while the temperature of the treatment tanks followed trends similar to those of Air temperature (Figure 3.1).

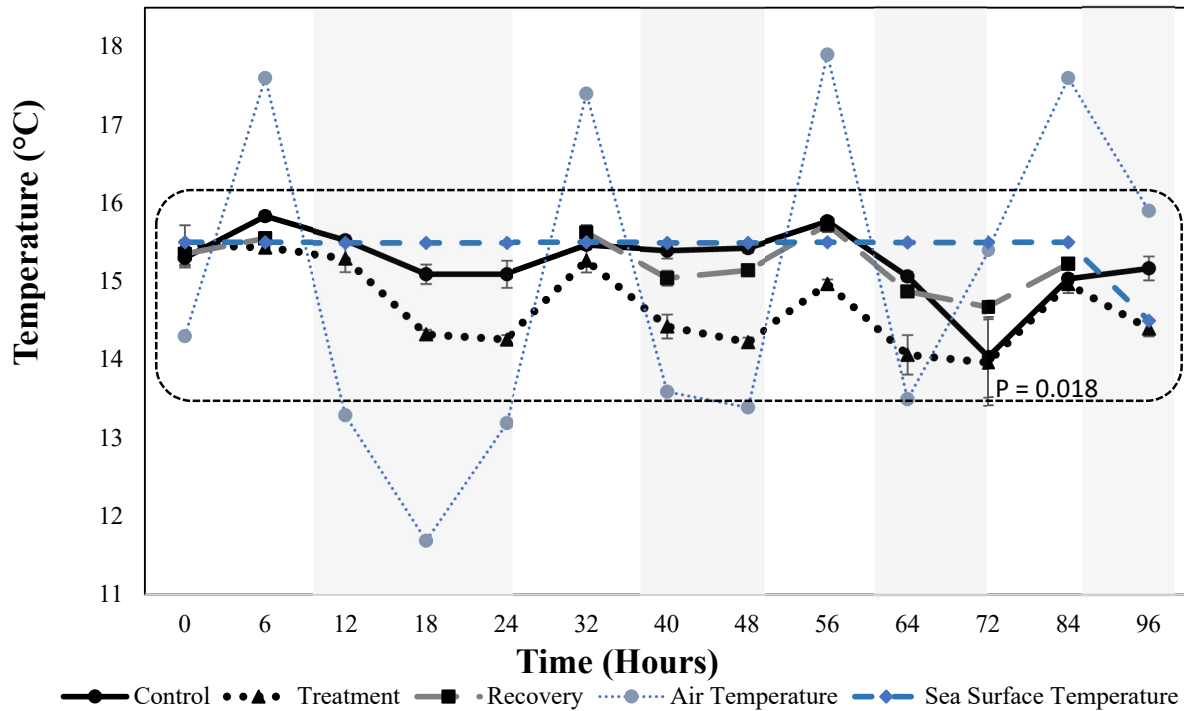


Figure 3.1: Temperature of the Air, Surface-seawater, and abalone tanks Control (C), Treatment (T), Recovery (R) over a 96-hour water-off event. Data are mean $\pm$ SD of 3 replicates. The white columns represent daytime and grey columns represent nighttime.

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3.1.2. Dissolved Oxygen (% Saturation)

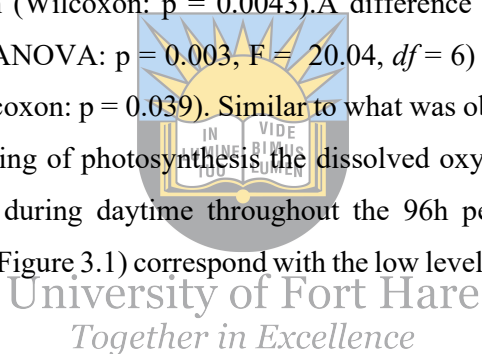
Percentage saturation of dissolved oxygen (Figure 3.2.a) changed in the control tanks (Friedman's test: $p = 0.004$, $F = 20.78$, $df = 7$) over the 96 hours with a higher %DO observed at 96 h compared to 48 h (Wilcoxon test: $p = 0.0013$) (Figure 3.2.a). The dissolved oxygen measured in control, treatment and recovery tanks varied through 56 h (Kruskal Wallis: $p = 0.046$) but was the same in all tanks at 64, 84, and 96 h.

At 32, 40, 48, and 56 h the dissolved oxygen in the Recovery tanks was significantly higher than in the treatment tanks ($p = 0.04$) (Figure 3.2.a). The DO measured in the control, treatment and recovery tanks varied throughout the 96 h (Kruskal Wallis: $p = 0.05$) but was the same in all tanks at 64 and 84h. The recovery tanks had higher %DO levels at 6, 32, 40, 48 and 56 h compared to the treatment tanks (Mann Whitney U: $p = 0.04$). The DO in the treatment tanks did not stay the same (ANOVA: $p = 0.005$, $F = 20.16$, $df = 7$), it was observed that there was an increase in %DO between 56 and 96 h

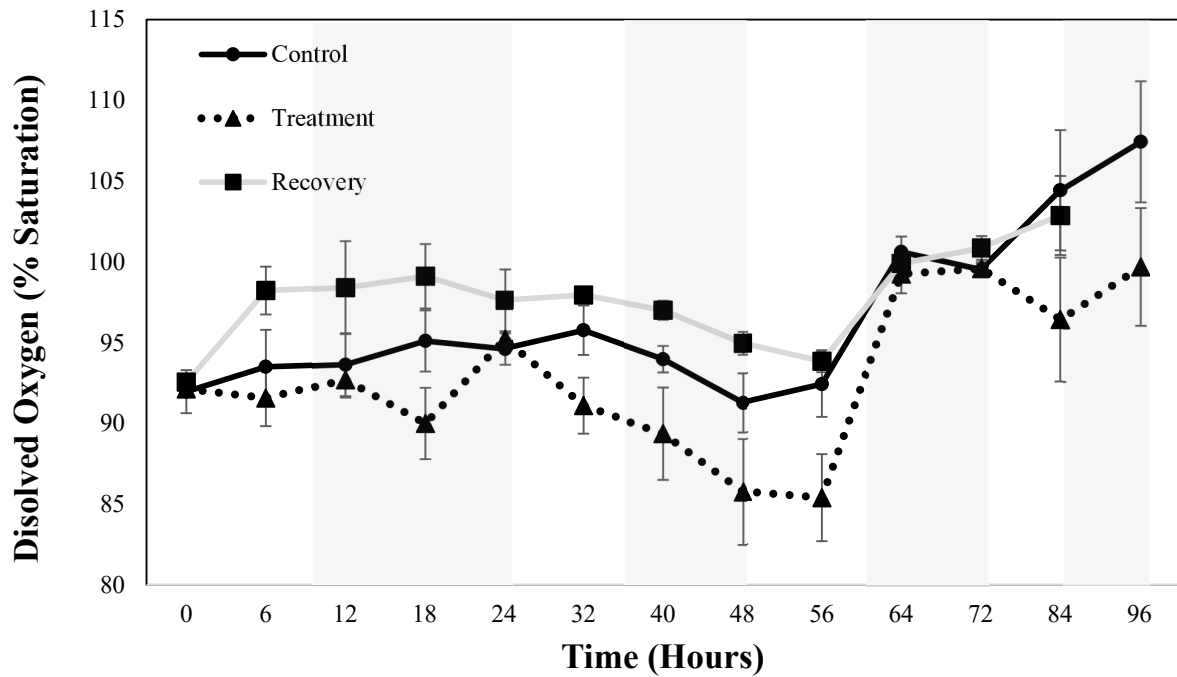
(Wilcoxon: $p = 0.04$). The DO in the recovery tanks varied over 84h (ANOVA: $p = 0.001$, $F = 23.11$, $df = 6$) as %DO at 84h was significantly higher than that observed at 48h (Wilcoxon test: $p = 0.02$). The dissolved oxygen saturation levels were higher at nighttime than during daytime throughout the 96h period. With DO at 56h being the lowest point which corresponds to the high temperature observed at 56h for the treatment tanks (Figure 3.1).

3.1.3. Dissolved Oxygen Concentration (mg. L^{-1})

The Dissolved Oxygen concentration followed a similar trend to percentage saturation of dissolved oxygen (Figure 3.2.b). control tanks DO concentration varied over the 96-h period (Friedman's test: $p = 0.006$, $F = 19.94$, $df = 7$) and were significantly different at 48 and 96 hours (Wilcoxon: $p = 0.05$) (Figure 3.2.b). There was also a difference in the measured DO concentration for treatment tanks over 96 h (ANOVA: $p = 0.006$, $F = 20$, $df = 7$) with a higher concentration of DO observed at 64 h compared to that measured 48 and 56 h (Wilcoxon: $p = 0.0043$). A difference in DO concentration was also observed for recovery tanks (ANOVA: $p = 0.003$, $F = 20.04$, $df = 6$) with high DO observed at 84h compared to 48 and 56 h (Wilcoxon: $p = 0.039$). Similar to what was observed in the %DO, due to the increased respiration and ceasing of photosynthesis the dissolved oxygen concentration in the tanks was lower at nighttime than during daytime throughout the 96h period (Datta, 2012). The high temperatures observed at 56h (Figure 3.1) correspond with the low levels of dissolved oxygen observed at 56h (Figure 3.2.b).



a.



b.

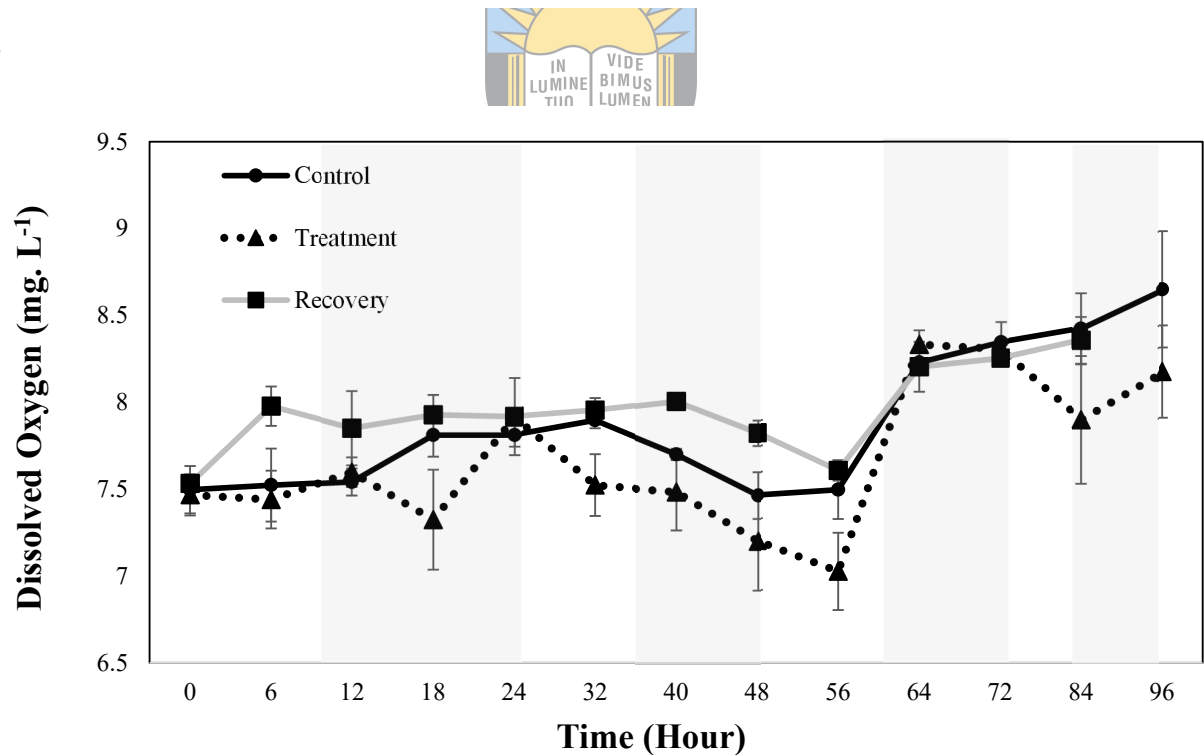


Figure 3.2: Dissolved oxygen (a. % saturation and b. concentration) of abalone Control, Treatment, and Recovery tanks over a 96-hour water-off event. Data is depicted as the Mean $\pm$ SD of three replicates. The white columns represent daytime and grey columns represent nighttime.

3.1.4. pH

pH was lower in the treatment tanks than in the control and recovery tanks. Over the 96-hour period, the pH for treatment tanks was lowest at 84 h (7.34 ± 0.05) and highest at 0 h (7.89 ± 0.05). The lower pH values in the tanks were probably due to the accumulation of carbon dioxide (CO_2) in the tanks, which then reacts with water (H_2O) to form carbonic acid (H_2CO_3^+) thereby decreasing pH levels in the water (Wedemeyer, 2001; Datta, 2012). For the control and recovery tanks the highest pH values were at 56 h (7.89 ± 0.02) and 56 (7.91 ± 0.02), while the lowest pH values were at 84 h (7.73 ± 0.01) and 64 h (7.77 ± 0.05) respectively (Figure 3.3).

In the control tanks pH varied over the 96h period (Friedmann's test: $p = 0.026$, $F=15.95$, $df=7$), at 56h was higher compared to 84 h (Wilcoxon test: $p = 0.043$). There was a change in pH measure for treatment tanks over 96 h (ANOVA: $p = 0.024$).

There was a change in pH measure in recovery tanks over 84 h (ANOVA: $p = 0.003$, $F = 19.73$, $df = 6$) as pH at 64 h was significantly lower compared to that measured at 6 h (Wilcoxon test: $p = 0.007$). The pH measure was only the same at 32 and 56 h for all tanks (Kruskal Wallis test: $p = 0.05$) but varies at other observation times (Kruskal Wallis $p = 0.038$). pH in recovery tanks was greater than in treatment tanks (Mann Whitney U test: $p = 0.032$) at 6, 40, 48, 64 and 48 h. At 96 h control tanks had higher pH when compared to treatment tanks (Mann Whitney U test: $p = 0.05$).

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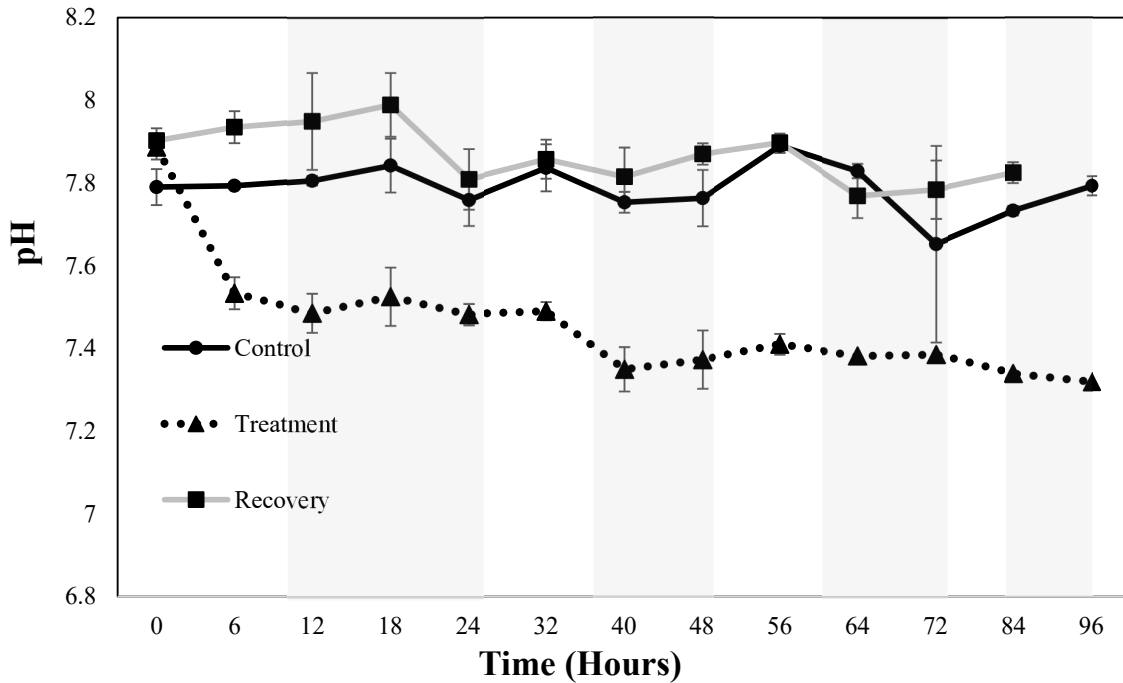


Figure 3.3: pH of abalone Control, Treatment, and Recovery tanks over a 96-hour water-off event. The pH of the of the recovery tank was only recorded up to 84 hours. Data is depicted as the Mean $\pm$ SD of three replicates. The white columns represent daytime and grey columns represent nighttime.

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3.1.5. Total Ammonia Nitrogen (TAN)

Total ammonia nitrogen levels were higher across the 96-hour period in the treatment tanks than in the control and recovery tanks (ANOVA: $p = 0.05$, $F = 3.86$, $df = 1$) (Figure 3.4). TAN levels in the treatment tanks reached their highest point at 56h (4.92 mg. L^{-1}), thereafter decreasing, and there were changes in the control and recovery tanks TAN throughout the 96-hour period. Data in control, treatment and recovery tanks in not normally distributed. Over the 96-hour period the data in the control tanks varied (ANOVA: $p = 0.003$, $F = 26.38$, $df = 10$). At 96 h Total Ammonia Nitrogen (TAN) in the control tanks was lower compared to that at 12 h ($p = 0.016$), while it was higher when compared to 6h ($p = 0.019$).

Data in the treatment tanks varied significantly over time (ANOVA: $p = 0.001$, $F = 31.10$, $df = 11$). Total ammonia nitrogen concentrations for the recovery tanks were significantly higher at 72h than a 48h ($p = 0.007$) and 108h ($p = 0.025$). The TAN values between tanks were the same at 6 and 32h (Kruskal Wallis: $p = 0.055$) but were different at other times (12h ($p = 0.050$), 18h ($p = 0.050$), 24h ($p = 0.050$), 30h ($p = 0.050$), 36h ($p = 0.050$), 42h ($p = 0.050$), 48h ($p = 0.050$), 54h ($p = 0.050$), 60h ($p = 0.050$), 66h ($p = 0.050$), 72h ($p = 0.050$), 78h ($p = 0.050$), 84h ($p = 0.050$), 90h ($p = 0.050$), 96h ($p = 0.050$)).

= 0.050), 40h ($p = 0.018$), 48h ($p = 0.043$), 56h ($p = 0.026$), 64h ($p = 0.043$), 72h ($p = 0.034$), 84h ($p = 0.050$), 96h ($p = 0.050$)). The TAN values in the treatment tanks were significantly higher than those in the control tanks at 12, 18, 24, 40, 48, 56, and 96 h ($p = 0.050$) and the TAN values at the treatment tanks were higher than those in the recovery tanks at 32 ($p = 0.055$), 64 ($p = 0.043$), and 72 h ($p = 0.034$).

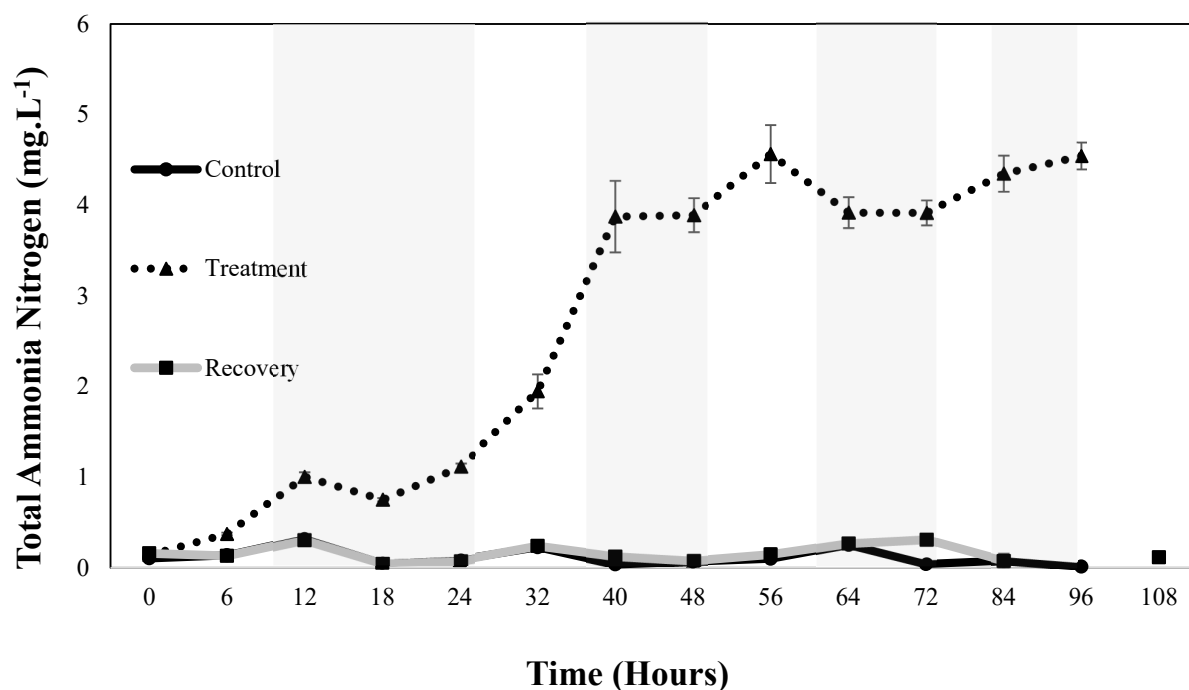


Figure 3.4: Ammonia (TAN) of the abalone tanks (Control (C), Treatment (T), Recovery (R)) over a 96-hour ff event. Data is depicted as the Mean $\pm$ SD of three replicates. The white columns represent daytime and grey columns represent nighttime.

3.1.6. Free Ammonia Nitrogen (FAN)

Free Ammonia Nitrogen was higher in the Treatment tanks than in the control and recovery tanks (ANOVA: $p = 0.05$, $F = 3.86$, $df = 1$). In the treatment tanks FAN concentration reached its maximum at 56h ($35.86 \mu\text{g. L}^{-1}$). In both the control and recovery tanks, FAN had a slight increase from 6 to 32 h, then decreased from 32 h to 40 h being slightly higher in the recovery tanks before the increase again until the 64th hour. There was no change in in FAN over 96h on the control tanks (Figure 3.5).

Free Ammonia Nitrogen in treatment tanks varied significantly over time (ANOVA: $p = 0.002$, $F = 22.83$, $df = 7$). At 72h FAN was significantly higher than at 48h ($p = 0.015$). The FAN concentration between tanks were significantly different throughout the experiment Kruskal Wallis: 6h ($p = 0.095$), 12h ($p = 0.050$), 18h ($p = 0.050$), 24h ($p = 0.050$), 32h ($p = 0.057$), 40h ($p = 0.18$), 48h ($p = 0.043$), 56h ($p = 0.018$), 64 ($p = 0.043$), 72h ($p = 0.034$), 84h ($p = 0.050$), 96h ($p = 0.050$).

In treatment tanks, FAN concentration was significantly higher than in Control tanks at 12, 18, 24, 40, 48, 56 and 96 h ($p = 0.05$) while the treatment values were higher than those in recovery tanks at 32, 64 and 72 h ($p = 0.05$) (Figure 3.5). Values in treatment tanks were significantly higher than in control tanks at 12, 18, 24, 40, 48, 56 and 96 h ($p = 0.05$) and treatment values were higher than those in recovery tanks at 32, 64 and 72 h ($p = 0.034$).

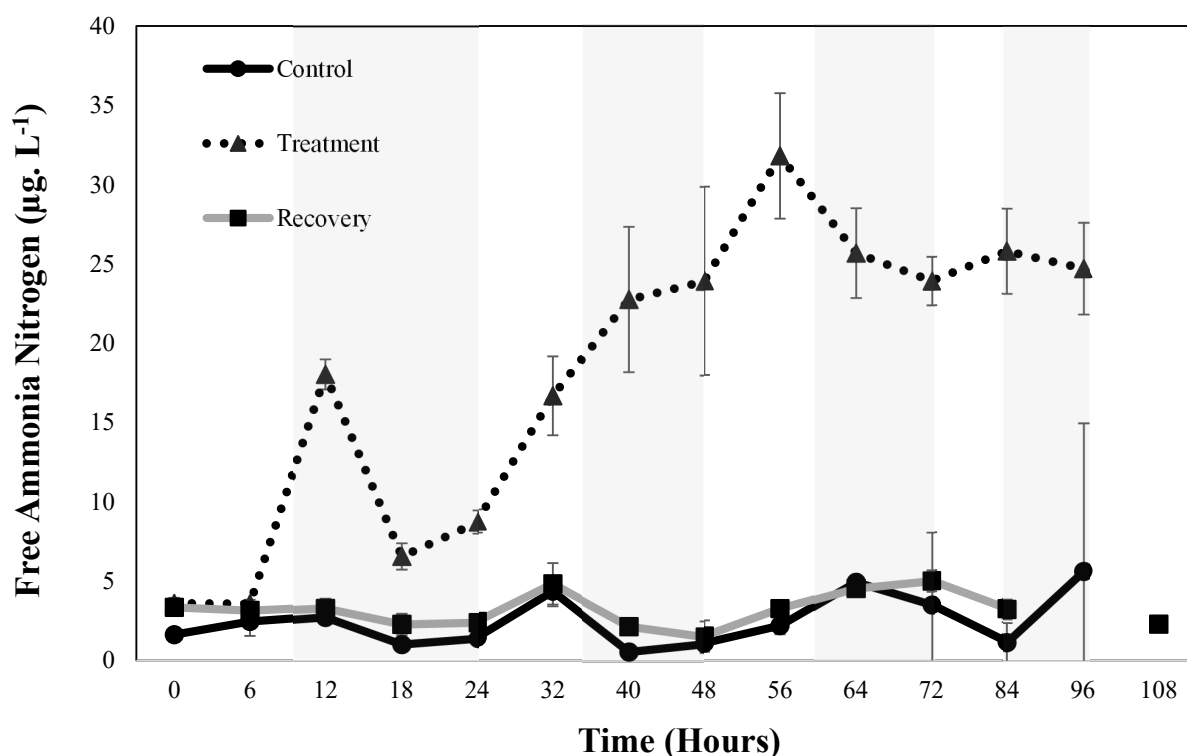


Figure 3.5: Ammonia (FAN) of the abalone Control, Treatment, Recovery tanks over a 96-hour off event. Data is depicted as the Mean $\pm$ SD of three replicates. The white columns represent daytime and grey columns represent nighttime.

3.2. Abalone Survival and Growth

Four months after the start of the experiment there was a 100% survival for the abalone in the control and treatment tanks. The statistical tests showed no significant differences, thus suggesting that all treatments can be considered as similar. The growth data was not normally distributed (Kolmogorov-Smirnov test: $p = 0.2$) and therefore non-parametric tests were used. The distribution of average weight gain (Kruskal-Wallis: $p = 0.39$, $F = 3.00$, $df = 3$) and survival ($p = 1$, $F = 0$, $df = 3$) were the same across all treatments.

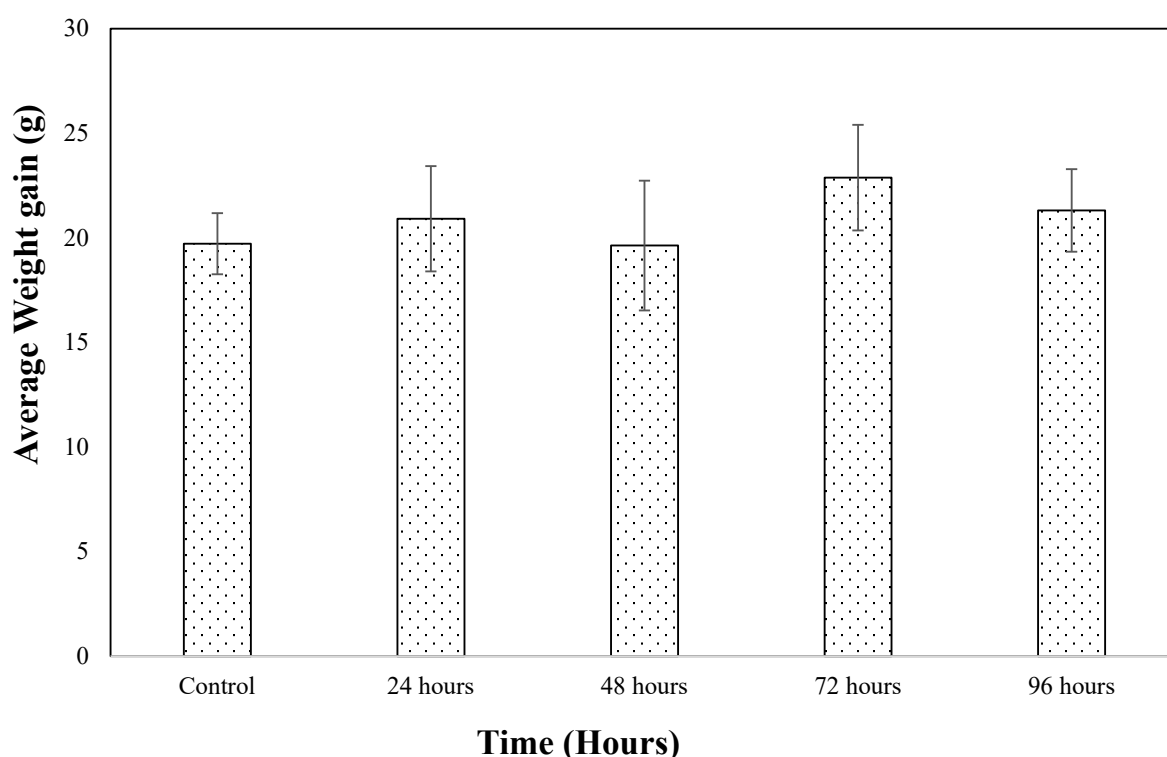


Figure 3.6: Average weight gain (g) of abalone measured four months after exposure to three (24, 48, 72, and 96hr) water off periods and the Control (0h). Data is mean $\pm$ SD. Where an average weight of 36 baskets of abalone was used for the control and an average weight of 12 baskets of abalone was used per treatment for the 24, 48, 72, and 96 hours water-off treatments ($N = 84$).

3.3. Abalone biochemical stress indicators

3.3.1. Glucose

Haemolymph glucose concentration did not change over the 96-hour for the control (Friedman's test: $p = 0.231$, $F = 5.60$, $df = 4$), treatment (Friedman's test: $p = 0.334$, $F = 3.40$ $df = 3$), and recovery tanks (Friedman's test: $p = 0.472$, $F = 1.50$ $df = 2$). There was also no difference in the glucose concentration between the three treatment tanks (control, treatment, and recovery) for each of the 24-hour sampling periods.

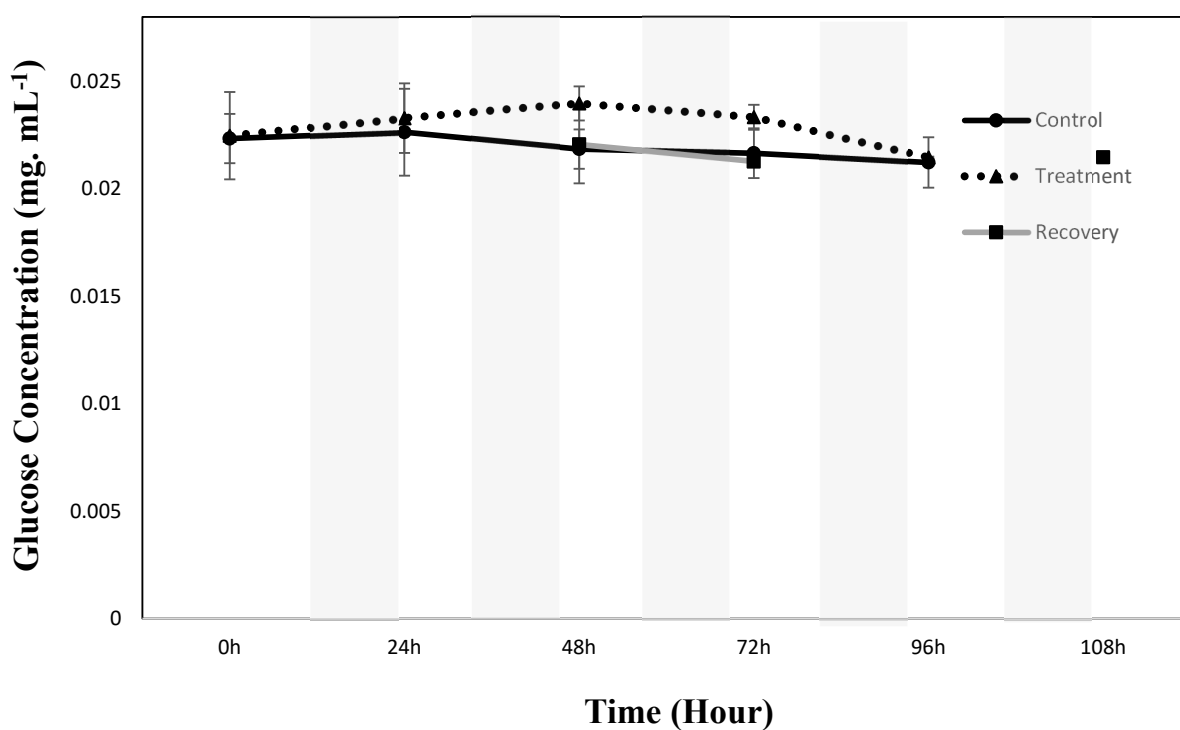


Figure 3.7: Haemolymph glucose concentration (mg. mL⁻¹) of Abalone in control, treatment and recovery tanks exposed to water off treatment for 96 hours. Data is mean $\pm$ SD of three replicates for each sampling period, where a total of $N = 216$ samples were obtained and the average was analysed for each sampling period. The white columns represent daytime and grey columns represent nighttime.

3.3.2. Glycogen

For tissue glycogen concentration, there was no difference between the control (Friedman's test: $p = 0.483$, $df = 4$, $F = 3.467$), treatment (Friedman's test: $p = 0.241$, $df = 3$, $F = 4.200$), and recovery tanks (Friedman's test: $p = 1$, $df = 2$, $F = 0$) over the 96-hour period. There was also no difference in between tanks, for glycogen 24h (Independent-Samples Kruskal Wallis test: $p = 0.827$), 48h ($p = 0.184$), 72h ($p = 0.978$), 96h ($p = 0.827$) (Figure 3.8), with a glucose concentration range being $0.41 - 0.76 \text{ mg. g}^{-1}$.

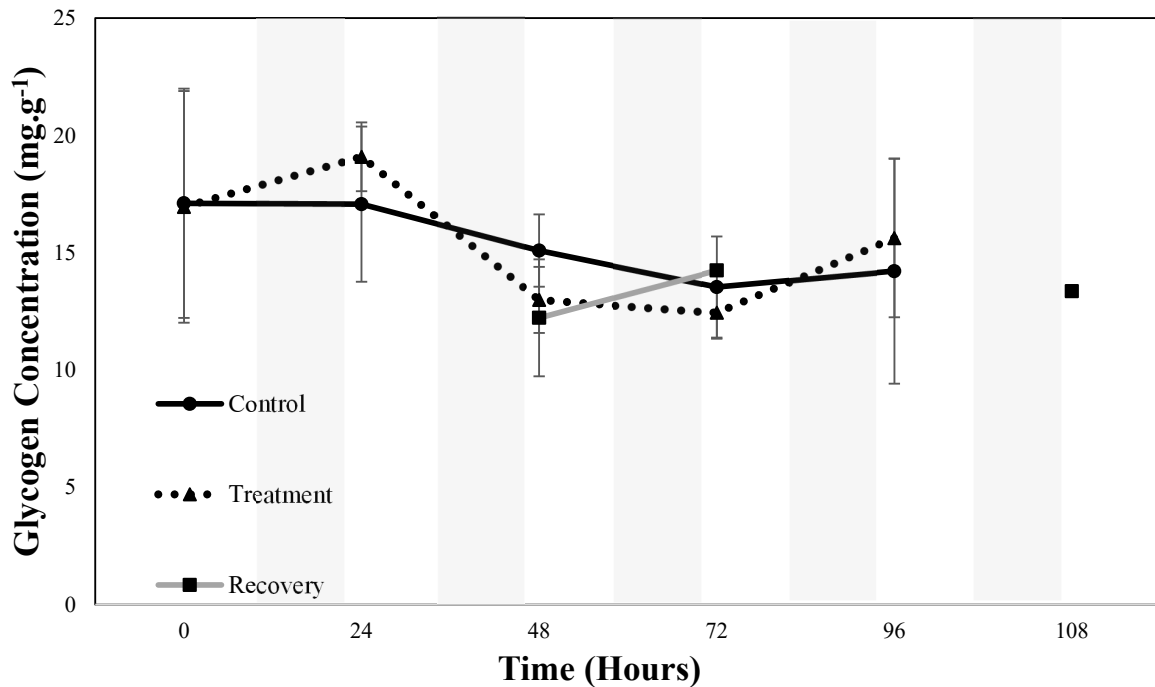


Figure 3.8: Muscle tissue glucose concentration (mg. g^{-1}) of Abalone in Control, Treatment, and Recovery tanks exposed to water off treatment for 96 hours. Data is mean $\pm$ SD of three replicates for each sampling period, where a total of $N = 216$ samples were obtained and the average was analysed for each sampling period. The white columns represent daytime and grey columns represent nighttime.

3.3.3. Dopamine

Dopamine data was not normally distributed ($p \geq 0.05$), and therefore non-parametric tests were used. Throughout the first 48-hours, dopamine concentration for abalone in the control tanks remained the same ($p = 0.453$, $df = 7$, $F = 6.768$) and similarly in the treatment tanks with the related-samples Friedman's two-way ANOVA ($p = 0.619$, $df = 7$, $F = 5.333$).

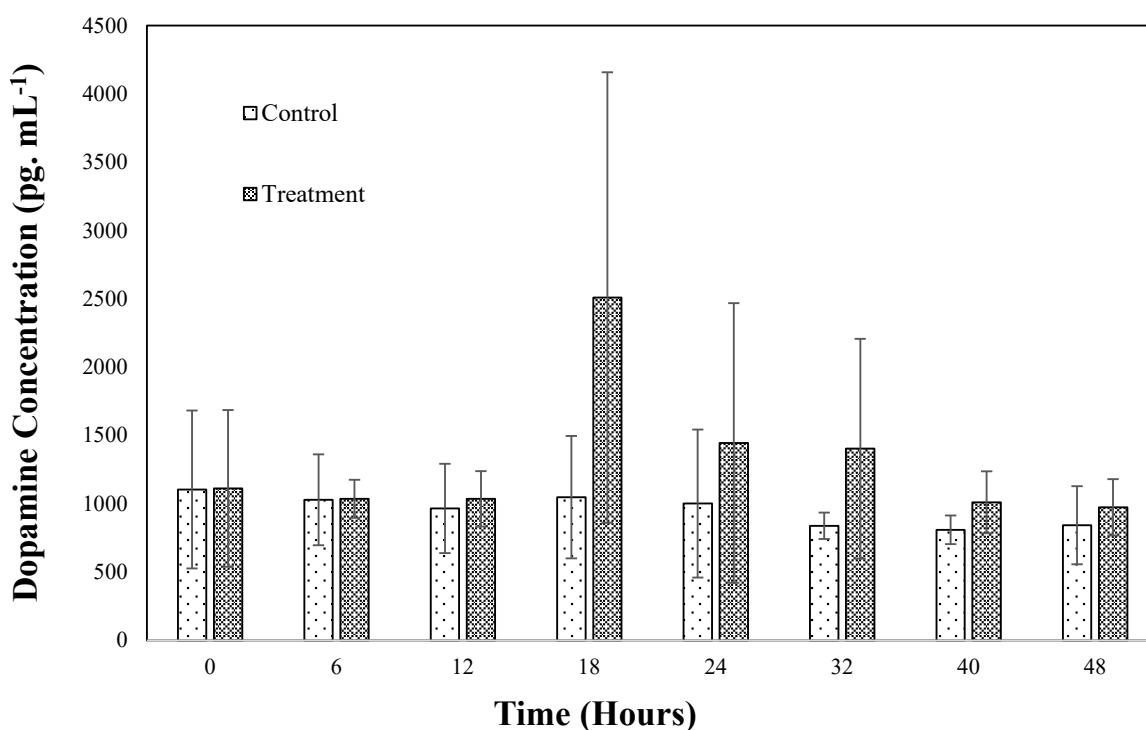


Figure 3.9: Dopamine concentration (pg. mL⁻¹) of abalone in Control and Treatment tanks exposed to a water off period 48 hours. Data is mean $\pm$ SD of two replicates for each sampling period, where a total of $N = 384$ samples were obtained and the average was analysed for each sampling period.

3.3.4. Superoxide Dismutase

The superoxide dismutase inhibition for control and treatment tanks was not normally distributed. For superoxide dismutase levels measured at different times the related-samples Friedman's two-way ANOVA showed no differences in the control ($p = 0.269$, $F = 8.78$ $df = 7$) and treatment ($p = 0.125$, $F = 10.00$, and $df = 6$) tanks over the 96-hour time period (Figure 3.10).

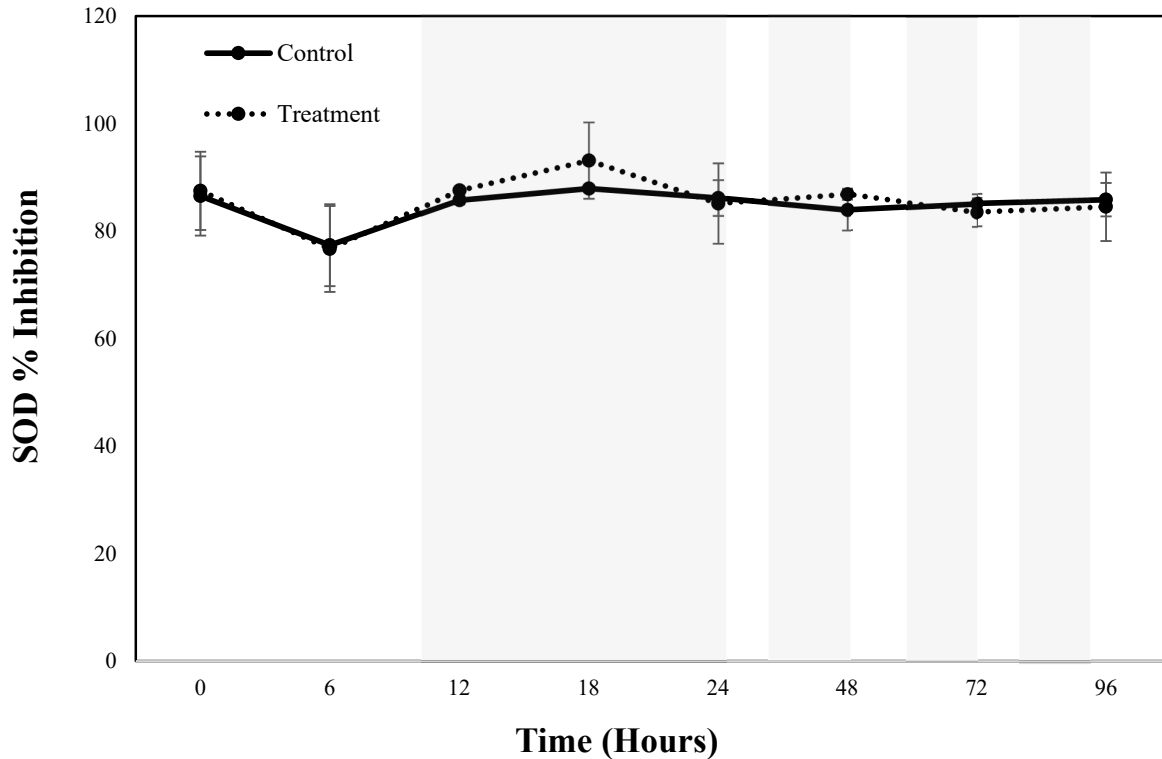


Figure 3.9: Superoxide dismutase % inhibition of Abalone in Control and Treatment tanks exposed to water off treatment for 96 hours. Data is mean $\pm$ SD for each sampling period, where a total of N = 288 samples were obtained and the average was analysed for each sampling period. The white columns represent daytime and grey columns represent nighttime.

3.3.5. Total Protein

Protein values declined over time in the control tanks (ANOVA: $p = 0.018$, $F = 22.91$, $df=11$) and was different between 6h and 72 hours. Similarly, in the treatment tanks protein levels decreased over time (ANOVA: $p = 0.005$, $F = 25.39$, $df = 10$) with differences occurring at 6h and 84h ($p = 0.049$). In the recovery tanks there was a decrease in total haemolymph protein (Friedman's test: $p = 0.004$, $F = 18.96$, $df = 6$), and there was significant difference between values at 32h and 72h ($p = 0.022$).

The total protein concentration for control, treatment and recovery tanks was lowest at 72h, suggesting that the water off events may have not been the only determining factor for the amount of total protein in the experiment.

Between treatments, the total protein was the same except for at 12h ($p = 0.05$), 40h ($p = 0.024$) and 56h ($p = 0.038$). At 12h, protein values in the control tanks were significantly higher than in treatment tanks ($p = 0.05$, $F = 3.86$, $df = 1$), at 40h ($p = 0.024$, $F = 7.44$, $df = 2$), while at 56h the concentration of total protein was higher in the recovery tank than those in control and treatment tanks ($p = 0.038$, $F = 6.56$, $df = 2$) (Figure 3.11). A t-test was also conducted to assess whether there were any differences in the total protein between day and night time for the treatment and no differences were detected with $p = 0.0632$.

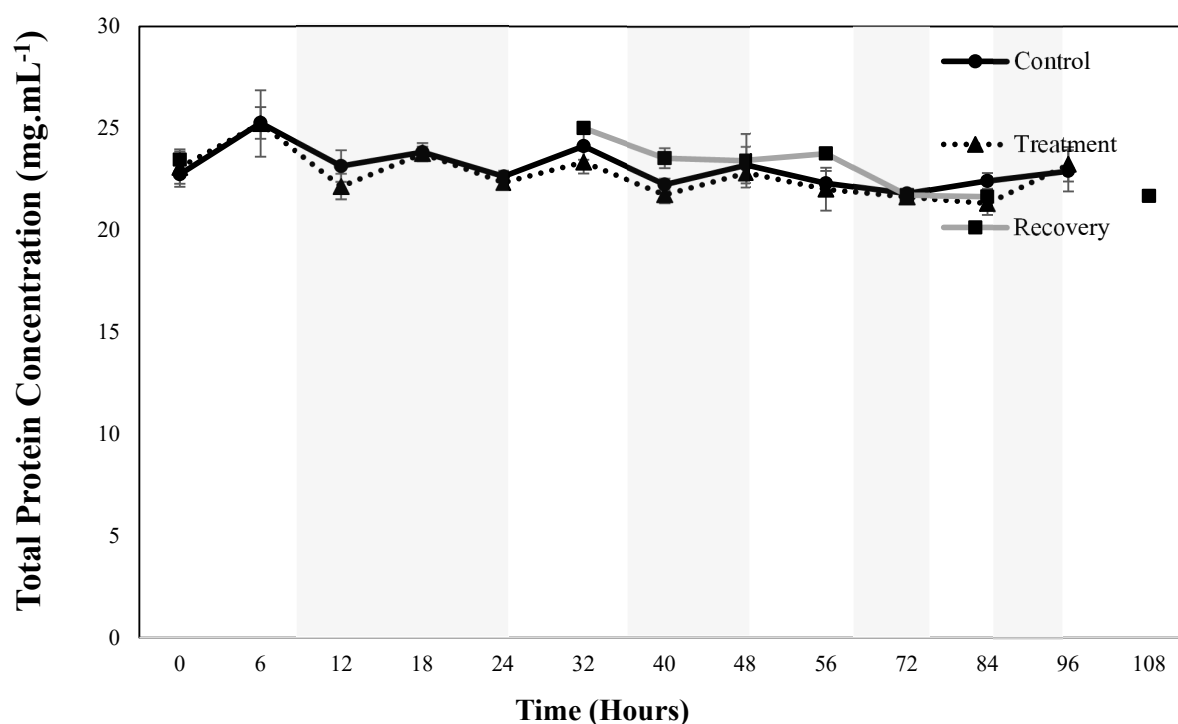


Figure 3.11: Protein concentration (mg. mL⁻¹) of Abalone in Control, Treatments, and Recovery tanks exposed to water off treatment for 96 hours. Data is mean $\pm$ SD of four replicates for each sampling period, where a total of $N = 624$ samples were obtained and the average was analysed for each sampling period. The white columns represent daytime and grey columns represent nighttime.

CHAPTER 4: DISCUSSION

4.1. Water quality

Understanding the effects of water quality parameters on abalone growth and biochemical indices during water-off events plays a critical role in helping managers make decisions during these periods (Yearsley, 2008). Water temperature is an environmental determining factor for the metabolism of poikilotherms Fry (1971), and has a positive correlation with the growth of abalone (*H. midae*) in the 12 - 20°C range where their metabolic rate increases with an increase in temperature (Britz *et al.*, 1997).

Due to the flow through system design of most abalone aquaculture facilities, farmed abalone are exposed to environmental water temperatures (Kang *et al.*, 2019). For the 96-hour duration of the study, temperatures varied diurnally across all tanks as expected. When comparing the temperatures in the tanks against ambient and sea surface temperature (Figure 3.1), it can be observed that the temperature of water in the treatment tanks followed trends similar to those of ambient temperatures. Whilst temperature of water in the control and recovery tanks followed trends similar to those illustrated by sea surface temperatures.

Due to the cooler ambient temperatures, the water in the control and recovery tanks were warmer during the night than that in the treatment tanks which dropped to $14.06 \pm 0.25^{\circ}\text{C}$ at 64h (2 a.m.) after the water was turned off.

During the 96-hour water-off period, low water temperatures in all tanks were experienced during the night. Whereas the highest water temperatures recorded in the treatment tanks were $15.43 \pm 0.05^{\circ}\text{C}$, $15.26 \pm 0.15^{\circ}\text{C}$, and $14.96 \pm 0.05^{\circ}\text{C}$ and were experienced at 6 (14:00), 32 (18:00), and 56 (16:00) hours respectively after the water had been turned off. These higher water temperatures occurred during the day. The highest water temperatures for the control and recovery tanks were $15.83 \pm 0.05^{\circ}\text{C}$ and $15.80 \pm 0.09^{\circ}\text{C}$ at 6 and 56 hours respectively.

Yearsley (2008) determined the annual mean of influent water temperature at 09:00 and 16:00 for HIK Abalone Farm as $14.91 \pm 0.25^{\circ}\text{C}$ and $15.61 \pm 0.26^{\circ}\text{C}$ respectively. Lee *et al.* (2015) reported that the optimum water temperature for Pacific abalone is 18 °C – 22 °C. Green *et al.* (2011) reported that *H.*

midae growth is dependent on water temperature, and that the growth declined when exposed to temperatures (24 °C) higher than optimal temperatures (12 – 20 °C) (Britz *et al.*, 1997). Gilroy & Edwards (1998) stated that the optimum temperatures for *H. rubra* and *H. laevigata* growth are 17.0 °C and 18.3 °C respectively. Kim *et al.* (2023) shows that high temperatures (25 °C) specifically result in apoptosis by causing an increase of in the apoptosis-related gene caspase-3 expression. The current study showed that even though water was turned off for 96 hours, the water temperatures in the raceway tanks still remained within an optimal temperature range for farmed *H. midae*. Water temperatures have an effect on several physiological processes in aquaculture systems such as feed consumption, ammonia excretion and oxygen consumption (Uki & Kikuchi, 1975; Barkai & Griffiths, 1987; Lyon, 1995).

A low dissolved oxygen concentration in the culture water is a primary limiting factor in flow through systems (Colt & Orwicz, 1991). Understanding the properties of dissolved oxygen in raceway tanks of abalone farms is important as the consumption of oxygen is a major constraint to the carrying capacity of these intensive farms (Lyon, 1995). The stress caused by the lack of oxygen affects growth more than the stress caused by increased ammonia (Haskell, 1955; Speece, 1973).

In this study, dissolved oxygen concentration and saturation dropped to their lowest (7.02 ± 0.2 mg. L⁻¹) and (85.4 %) respectively in treatment tanks at 56h. This comparatively low dissolved oxygen corresponded with the high temperature observed at the same time. Harris *et al.* (1999) reported that decreasing dissolved oxygen in juvenile *H. laevigata* lead to growth reduction by 5% and 50% at 7.36 and 5.91 mg. L⁻¹ of O₂ respectively. The optimum dissolved oxygen level for *H. midae* is not known (Vosloo *et al.*, 2009). However, Yearsley (2008) reported a 20 – 30 mg growth after 104 days at median concentrations (7.0 – 7.2 mg. L⁻¹ O₂) of dissolved oxygen for *H. midae*. Hutchinson & Vandeppeer (2006) reported to have observed abalone farms with a minimum DO of 4 mg. L⁻¹ (56 % saturation), however the amount of time the farms had this low DO concentration is not known. Lee *et al.* (2015) reported that the optimum dissolved oxygen for the Pacific abalone is 6 – 8 mg. L⁻¹. The absence in growth differences in the current study is contradictory to what was reported in Harris *et al.* (1999) and Yearsley (2008). The reason for this contradiction could possibly be that abalone used for the current study were approximately 29 months and had more tolerance to environmental stress than the juvenile abalone in Harris *et al.* (1999), and that the abalone in Yearsley (2008) were being fed throughout the experimental period unlike those in the current study. It can thus be said that because the lowest concentration of dissolved oxygen observed in this study is still within a range that showed good abalone growth in Yearsley (2008). Turning off water for 96-hours will not have significant

negative effects on the dissolved oxygen concentration and saturation of raceway tanks. The dissolved oxygen concentration in this experiment did not drop below $7.02 \pm 0.2 \text{ mg. L}^{-1}$ during the water-off event. A drastic drop in of dissolved oxygen during a water-off event is unlikely due to the air circulation not being turned off during these events, and the dissolved oxygen can therefore be of limited consideration when turning water off in farms.

While dissolved oxygen levels may remain elevated during the water-off event, the carbon dioxide levels may change as a result of respiration which could impact on the water pH (Crim *et al.*, 2011). An increase in carbon dioxide in water may result in the formation of carbonic acid and thus a decrease in pH (Wurts, 2003; Datta, 2012). Shell forming organisms such as abalone have been described to fare poorly when exposed to lower water pH (Kroecker *et al.*, 2010, 2013). In this study, it can be seen that the water pH in the recovery and control tanks was higher than that of the water in the treatment tanks. While pH for both the control and recovery tanks were within the 7.7 – 7.9 range, the pH in the treatment tanks decreased and reached a pH of 7.3 . Dixon *et al.* (1991) described *H. midae* water pH as optimal at 8.1. While Khuzwayo (2014) reported that the water pH range for the acclimatization of East and West coast *H. midae* was 8.1 – 8.3 and 7.7 – 8.3 respectively for their study. Wessel *et al.* (2018) stated that for pH levels lower than 7.7 and 7.6 the shell of *H. tuberculata* had a reduced mineralization. A decrease in water pH below the optimum range might have a negative effect on shell formation, and this might lead to energy not being used for the growth of the abalone (Kim *et al.*, 2013; Cunningham *et al.*, 2015). Avignon *et al.* (2020) also reported that maintaining abalone metabolism at low pH conditions occurs at the cost of growth and shell integrity for adult abalone (*H. tuberculata*). However, this might not have been detected during the four-month growth trial of the current study as it is more likely to impact on smaller abalone. The control and recovery tank pH are with a similar range to those of tropical abalone which showed that the optimal pH range for those species (*Haliotis asinina*, *Haliotis ovina*, *Haliotis varia*) is 7.9 – 8.1 (Jaraybhand & Paphavasit, 1996) .

Yearsley (2008) found that the daily pH of incoming tank water ranged from pH 8.06 to 8.13 while that of the outflowing water was 7.90 to 7.95. Decrease in water pH can occur due to an increased presence of carbon dioxide (CO₂) which forms carbonic acid, the resulting carbonic acid dissociates and releases bicarbonate as well as hydrogen ions (H⁺) thus lowering the pH (Sanni & Forsberg, 1996). The lower pH at the outflow for (Yearsley, 2008) study was similar to that measured in the treatment tanks in the current study, and this is due to carbon dioxide accumulation as a result of the animals respiring and the water in the tanks not flowing out quick enough.

Maintaining a lower pH value in water is important as it helps reduce the proportion of NH_3 or Free ammonia nitrogen (FAN) (Aguado *et al.*, 2022) which can be harmful to abalone in the water (Bower & Bidwell, 1978; Thurston *et al.*, 1981), however very low pH values can distort growth in younger abalone (Kim *et al.* 2013, Cunningham *et al.* 2015). For example, Auzoux-Bordenave *et al.* (2020) showed how low pH (7.6) negatively impacts on abalone shell after a 3 months exposure to reduced pH levels.

Increased ammonia levels can occur due to several factors which include overfeeding of the animals and excess feed decay which ends up liberating toxic ammonia gas (Datta, 2012). At 84 h in the current study, Total Ammonia Nitrogen (TAN) and FAN levels of the control tanks were higher than those of the recovery tanks. The FAN levels in the treatment tanks of the current study did reach $35.9 \mu\text{g. L}^{-1}$ but this was for less than 16 hours, after which the FAN levels had dropped to $25.7 \mu\text{g. L}^{-1}$. Lyon (1995) reported that acute exposure (12 h) to FAN concentrations below 0.88mg. L^{-1} does not have long term effect on the growth of *H. midae* L. De Prisco (2020) reported that after acute exposure (=36 h) of *H. midae* to FAN concentrations of $23.8 \mu\text{g. L}^{-1}$ there were no differences in growth and survival, while acute exposure (=36 h) of *H. midae* (5.0 – 8.0 cm) to $> 12.9 \mu\text{g. L}^{-1}$ concentration of FAN affected the growth and mortality (Reddy-Lopata *et al.*, 2006a).

The increase of FAN in the Treatment tanks to $35.9 \mu\text{g. L}^{-1}$ may have been due to the accumulation of sludge from abalone faeces throughout the water-off period as a result of the lack of water flow as well as the tanks not being cleaned. Yearsley *et al.* (2009) reported that FAN increases when there is no sludge removal from a raceway tank with abalone. The drop in of both TAN and FAN after reaching peak concentrations may have been due to the presence of other potential sinks for total ammonia such as the loss from water to air, being taken up by algae, and conversion to nitrite by nitrifying bacteria (Yearsley *et al.*, 2009).

Similar to De Prisco (2020) and Lyon (1995) growth and survival of abalone in this study was not affected by the high FAN concentration. The accumulation of FAN in the Treatment tanks suggests that the effects of the water-off on the ammonia levels appear to be negligible in raceway tanks of an abalone farm. This may be due to acclimatization to high levels of ammonia (Reddy-Lopata, 2006)Click or tap here to enter text..

4.2. Survival and Growth

Survival was 100% across all the treatments. This suggests that even though there may have been acute changes in the water quality of the tanks due to the different exposure periods to the water-off events, these changes did not have a chronic effect on the abalone survival and did not result in any mortalities for the farmed abalone. All water quality parameters remained within optimal range for *H. midae*, thus there was 100% survival across all the treatments (Dixon *et al.*, 1991; Lyon, 1995; Britz *et al.*, 1997; Lee *et al.*, 2015). To ensure that commercial production is efficient, successful, and viable, it is important that management prioritizes and maximizes the growth of the abalone (Keesing & Wells, 1989; Capinpin (Jr) *et al.*, 1999; Lee, 2004; Setyono, 2005; Naidoo *et al.*, 2006).

Due to the slow growth rate of abalone growth was only measured after four months since the start of this study. Growth can be described as the outcome of food that is being ingested later being converted to body tissue (Barnabé, 1994).

Over the 124-day (4 months) growth trial there was no difference in growth between the control and treatment tanks. While an acute water-off event (such as that in this study) would appear to not have an effect on abalone growth, the long-term effect of reduced water flow does. For example (Naylor *et al.*, 2011) suggested that $7.2 - 9.0 \text{ L} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ of abalone's the lowest flow rate required to maintain acceptable growth in *H. midae* (50–60 g). Similarly, a study conducted by Yearsley (2008) where water quality, abalone growth and the potential for integrated mariculture on a *H. midae* farm was investigated and suggested a flow of $9.7 - 14.5 \text{ L} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ be maintained to ensure growth is not impacted on 20–30 g *H. midae*. The reason this study unlike the Yearsley (2008) and Naylor *et al.* (2011) didn't show any reduced growth could be that the trials for both the latter studies took place over an extended period and the abalone were thus exposed to chronic stress which has been shown to impact on growth (Lyon, 1995). Lyon (1995) reported that even though it does not take place as often as acute exposure, chronic exposure of *H. midae* to high levels of ammonia could have negative effects on the growth.

Reddy-Lopata *et al.* (2006) investigated the acute and chronic toxicity of sub-lethal levels of ammonia on the growth rate of juvenile *H. midae* which is directly proportional to body size. Juvenile (1–2 cm) *H. midae* had a 36 h LC_{50} of $9.8 \mu\text{g} \cdot \text{L}^{-1}$ Free Ammonia Nitrogen (FAN) which increased to $12.9 \mu\text{g} \cdot \text{L}^{-1}$ FAN for “cocktail” size (5–8 cm shell length) abalone. (Reddy-Lopata *et al.*, 2006) stated that when juvenile (1–2.5 cm shell length) and “cocktail” size (5–8 cm shell length) abalone experience chronic

exposure to sub-lethal FAN levels their growth is inhibited (Reddy-Lopata *et al.*, 2006). De Prisco (2020) argued that after the acute exposure (36 h) of *H. midae* to FAN concentrations of 23.8 $\mu\text{g. L}^{-1}$ there were no differences in growth and survival.

Water quality analysis during this study (Figure 3.5), illustrates that despite an acute increase in FAN during the experiment growth and survival were not negatively affected. This is likely because exposure to increased FAN during the study was only for 16 hours and therefore did not have any significant effect on the growth of the abalone (Lyon, 1995; De Prisco, 2020).

4.3. Biochemical indicators of abalone health

Various biochemical stress indices (haemolymph glucose, muscle glycogen, dopamine, superoxide dismutase, and total haemolymph protein) of the abalone were measured before and during the 96-hour water-off period. These stress indices were measured during the water-off period due to indices such as dopamine and glucose going through a rapid change when the abalone are exposed to a stressor (Ekanayake *et al.*, 2006; Lacoste *et al.*, 2001; Lee *et al.*, 2023; Malham *et al.*, 2003).

In abalone, haemolymph glucose is derived mainly from ingested feeds which when exceeding the energy requirements of the animal are stored as glycogen in the animal's tissues (Koyama *et al.*, 2020). When animals are fasting, glucose levels are maintained by breaking down glycogen (glycogenolysis) or protein reserves (proteolysis) (Liu *et al.*, 2024). The conversion of glucogenic amino acid to glucose is another significant source of haemolymph glucose (Venter, L *et al.*, 2018). Haemolymph glucose levels can spike during stressful conditions (Baek *et al.*, 2014), and for this reason it was chosen as a stress indicator for the current study.

The haemolymph glucose concentration of the abalone in the treatment, control, and recovery tanks did not show any significant differences from each other throughout the 96-hour water-off period. The glucose concentration of the abalone in the treatment tanks did however increase from 24h to 48h until it reached its highest point ($3.02 \pm 1.75 \text{ mg. mL}^{-1}$) at 48h. Similarly, the abalone in the recovery tanks also had their highest glucose concentration ($3.04 \pm 1.59 \text{ mg. mL}^{-1}$) at 48h.

The results of the current study are different from those of a study where the analysis of the physiological responses of the Pacific abalone was conducted after the abalone had been exposed to

various stressors (low temperatures, high temperatures, low salinity, and air exposure) (Lee *et al.*, 2023). The result on the study by Lee *et al.* (2023) showed an immediate increase in haemolymph glucose concentrations to $2.26 \pm 0.45 \text{ mg. mL}^{-1}$, $2.53 \pm 0.06 \text{ mg. mL}^{-1}$, $2.35 \pm 0.20 \text{ mg. mL}^{-1}$, and $2.79 \pm 0.03 \text{ mg. mL}^{-1}$ when exposed to low water temperatures, high water temperatures, low salinity, and air exposure respectively. The difference in glucose concentration between the two studies might be due to sampling of the different treatments (in the current study) only taking place before and 6 hours after the initial exposure to the stressor which then suggests that the spike in the glucose concentration might have been missed. When the abalone (*H. discus hannai*) are exposed to different stressors, the haemolymph glucose concentration rises rapidly and typically peaks immediately (within an hour) after the exposure to a stressor (Lee *et al.*, 2023).

Cheng *et al.* (2004) reported that after being exposed to 2.11 and 3.08 mg. L⁻¹ of dissolved oxygen for 12 hours, abalone (*H. diversicolor*) showed an increase in haemolymph glucose levels from 0.018 to 0.035 mg. mL⁻¹. The results observed in Cheng *et al.* (2004) contradict the previous claim stating that a spike in glucose concentrations may only be detected immediately after the exposure to stress. Which the leads to another possible reason for the glucose concentration not showing differences in the current study being that even though there might have been changes in the water quality parameters, these changes were not enough to sufficiently stress the abalone and therefore cause changes in the glucose concentration. Vosloo *et al.* (2013) reported that after being exposed to low ($6.74 \pm 0.002 \text{ mg. L}^{-1}$) and high ($8.96 \pm 0.011 \text{ mg. L}^{-1}$) concentrations of dissolved oxygen for a month there were no changes in the haemolymph glucose concentration ($0.0418 \pm 0.0036 \text{ mg. mL}^{-1}$) of *H. midae*. The glucose results observed in Vosloo *et al.* (2013) confirm those of the current study and suggests that the water quality parameters during the 96-hour water-off period were within a safe range for *H. midae*.

Similar to the haemolymph glucose, average muscle glycogen concentration ($59.75 \pm 12.81 \text{ mg. g}^{-1}$) for the abalone in the current study did not show any differences between the abalone in the control, treatment, and recovery tanks throughout the 96-hour water-off period. Vosloo & Laas (2010) reported that the average concentration of muscle glycogen in young (38 months) *H. midae* is $81 \pm 2.37 \text{ mg. g}^{-1}$, which is within the same range to what was observed in the current study. The findings of the current study however differ from those of Omolo *et al.* (2003) where the concentration of muscle glycogen decreases from 39.96 to 19.98 mg. g⁻¹ when exposed to anoxia at 7°C (Omolo *et al.*, 2003). Li *et al.* (2018) reported that after exposure to chronic acidification (pH = 7.7), the muscle glycogen of *H. discus hannai* was 29 % higher in the control treatment than in those with the pH = 7.7. Chiou *et al.* (2002) reported that the concentration of muscle glycogen of *H. diversicolor* decreases from $18.26 \pm$

1.47 mg.g⁻¹ to 12 mg.g⁻¹ after exposure to air at 5°C for 3.5 days. Baldwin *et al.* (1992) reported that after exerting exercise stress on *H. iris* the foot muscle glycogen decreases from 49.55 ± 9.59 mg. g⁻¹ wet weight in the control to 17.72 ± 6.26 mg. g⁻¹ wet weight. Baldwin *et al.* (1992), Chiou *et al.* (2002), Omolo *et al.* (2003) and Li *et al.* (2018) all show a decrease in abalone glycogen concentrations under different acute and chronic stressful conditions, which suggests that the stress applied by the water-off event during this study was not enough to cause changes in the glycogen concentrations of *H. midae* and an increase on the duration may result in a potential starvation response where there'd be a lower glycogen concentration which would suggest a decreased anaerobic energy production (Sheedy *et al.*, 2015)

Glucose and glycogen take on different roles within the animal's body (Vosloo & Laas, 2010). Glucose provides energy for the metabolic processes of cells and normal functioning of muscle tissue, whereas glycogen serves as a major energy storage form in abalone tissue due to their metabolism being carbohydrate based (Baldwin *et al.*, 1992; Braid *et al.*, 2005; Gómez-Montes *et al.*, 2003; Picos-García *et al.*, 2000; Vosloo & Laas, 2010). The results for muscle glycogen confirm what was observed with the haemolymph glucose in that the muscle tissue glycogen did not get broken down causing a spike in the haemolymph glucose as it would when abalone are stressed (Ambade *et al.*, 1998; Baeck *et al.*, 2014).

This is an indication that should there be a shortage of energy for cellular metabolism there would be a breakdown or decrease of glycogen and an increase in tissue glucose to meet the metabolic needs of the animals. This suggests that the abalone in this study did not have an increased demand of glucose for metabolic needs and therefore no muscle glycogen breakdown took place to increasing haemolymph glucose during their most stressful period to cause low levels of muscle glycogen during that period. These results may have been due to water quality parameters such temperature, pH, and dissolved oxygen remaining within a safe range for *H. midae*, as well as FAN only reaching high concentrations (35.9 µg. L⁻¹) only for a short period of time (16 hours), which may not have had impact on the muscle glycogen of the abalone during the experimental period (Reddy-Lopata *et al.*, 2006).

Dopamine has a function in motor control sensory gating and as a neurotransmitter (Montague *et al.*, 1996; Sawin *et al.*, 2000; Schultz, 2010; Strausfeld & Hirth, 2013). There were no differences between treatments or over time in the dopamine concentrations over the 96-hours of water-off event, with the maximum level being reached in the treatment tanks was 2509.74 pg. mL⁻¹ at 18h. However, this concentration wasn't significantly different from those observed in the Control tank.

These results are different to those of a study on *H. tuberculata* (Malham *et al.*, 2003) . The study examined the possibility of stress altering immune functions, the first line of defence against disease causing microbes, in a mollusc, *H. tuberculata*, which examined the effect of stress on altering immune functions, the first line of defence against disease causing microbes (Malham *et al.*, 2003). During the application of the stressor, immune parameters such as dopamine increased significantly ($> 2\text{ng. mL}^{-1}$) but decreased to the initial values ($0.2 - 0.5 \text{ ng. mL}^{-1}$) within 15–30 min after the end of the disturbance (Malham *et al.*, 2003). Zhou *et al.* (2011) reported an increase in *Chlamys farreri* (Scallops) dopamine concentration to $480.4 \text{ pg. mL}^{-1}$ after being exposed to bacteria *Vibrio anguillarum*. Pan *et al.* (2014) and Zhao *et al.* (2016) both reported that the haemolymph dopamine of the arthropod *Litopenaeus vannamei* (Whiteleg shrimp) peaked 24 and 12 hours respectively after being exposed to changes in salinity. Chen *et al.* (2008) stated that the dopamine levels of *C. farreri* decreased upon a 72-hour exposure to low salinity and high temperature (28°C), which led to a controversial conclusion stating that dopamine variations are sensitive to the environmental stressor.

This study suggests that even though there may have been changes in the water quality during the water-off period, these changes did not have a significant effect on the dopamine concentration of the abalone. This means that the water-off event did not cause stress that would result in a catecholamine release which would constitute a form of neuroendocrine response to stress in the abalone, a response which is expected molluscs such as abalone and oysters (Lacoste *et al.*, 2001).

Another early indicator of stress in abalone is Superoxide dismutase, a metalloenzyme responsible for detoxifying and scavenging on free radicals that are products of endogenous and exogenous substances, to stop lipid peroxidation, and to delay senescence thus protecting the organism (Che *et al.*, 2016; Fukai & Ushio-Fukai, 2011). Superoxide dismutase is often used as a potential marker for assessing environmental stress and water pollution, due to its ability to improve phagocytosis and viral resistance in cells (Achuba, 2002; Penicaud *et al.*, 2017; Ren *et al.*, 2017).

There were no differences in superoxide dismutase % inhibition between the control and treatment tanks, over the 96-hour water-off period suggesting that the stress of the 96-hours water-off event wasn't sufficient to cause an increase in free radicals which would have led to increased production of the Superoxide dismutase metalloenzyme. The results are however contradictory to those of a study by Yang & Min (2019) which investigated at how sub-optimal or reduced temperature and salinity decrease antioxidant activity and cellularity in the haemolymph of the Pacific abalone (*H. discus hannai*) (Yang & Min, 2019).

Yang & Min (2019) showed that salinity induced stress resulted in an increase of superoxide dismutase to $5.5 - 6 \text{ U. mL}^{-1}$ in *H. discus hannai* exposed to temperature and salinity stressors for 7 days, which was double that of the control ($2 - 3 \text{ U. mL}^{-1}$) which is considered to be baseline for the species (Yang & Min, 2019). Kim *et al.* (2023) reported that there was an increase in *H. discus hannai* SOD levels exposed to low or high temperatures, as well as under lower pH levels. Zhang *et al.* (2023) reported that the haemolymph SOD activity was lower in the treatment than that of the control 12 hours *H. discus hannai* was exposed to one stressor (hypoxia or *Kerenia mikimotoi*), however, the SOD activity became higher in the treatment at all points in time when the abalone were exposed to both stressors. Yasa *et al.* (2019) reported an increase in SOD activity of *H. squamata* exposed to a temperatures of 32°C for 12 hours.

Yasa *et al.* (2019) also revealed that the SOD activity decreased after 24h of exposure to 32°C and remained stable for 96h. The stability in SOD activity in Yasa *et al.* (2019) is similar to what was observed in the current study, which could be explained by a reduction in the production of O_2^- as a result of limited availability of oxygen. The difference between the current and the cited studies may have been due to the 96-hours of water-off events not resulting in the deterioration of the water quality in such a manner that may cause stress which may alter the biochemical health indices of the abalone. Another reason for the difference in the response in the two studies may be due to the two species (*H. midae* and *H. discus hannai*) in these studies being adapted to different environment, at conditions where for example, *H. midae* prefers temperatures of $12 - 20^{\circ}\text{C}$ according to Britz *et al.* (1997) while *H. discus hannai* prefers temperatures of $18 - 22^{\circ}\text{C}$ (Lee *et al.*, 2015). It is also possible that as SOD is regarded as an early stress response, and any changes that may have taken place could have done so soon after the animals had been exposed to the stressor which were not detected in the sampling methodology.

The last biochemical health indicator that was looked at during this study was the total haemolymph protein. Proteins are the largest macromolecules found in cells and the polymers of amino acids (Cox & Nelson, 2013). There weren't any differences in the haemolymph protein between treatments for the 96h water-off period., Even though figure 3.11 appears to have diel fluctuations in the protein concentrations in all the tanks for the 96h period, the statistical analysis does not support that with the control and treatment tanks following a very similar trend for the 96h period, as well as a decrease in the protein concentration for all treatments, suggesting that another factor (proteolysis and starvation) may be affecting the haemolymph protein of the abalone (Sheedy *et al.*, 2016; Venter *et al.*, 2018). However, due to the duration of the starvation period being shorter in the current study when compared

to studies such as those conducted by Sheedy *et al.*, (2016), starvation may have not caused any changes in the total haemolymph protein of the current study.

The decrease in the total haemolymph protein of the abalone over the 96-hour water-off period can be explained by the fact that the abalone were not being fed throughout this period. An example of this can be seen in a study by (Akanke *et al.*, 2010) the biochemical effects of aestivation and starvation on *Bulinus globosus* and *Bulinus rohlfsi* was being investigated. (Akanke *et al.*, 2010) reported that the total haemolymph protein of *B. globosus* and *B. rohlfsi* decreases during starvation. The total haemolymph protein concentration ranged at Control ($21.78 \pm 0.16 - 25.26 \pm 0.78$ mg. mL⁻¹), Treatment ($21.31 \pm 0.56 - 25.23 \pm 1.63$ mg. mL⁻¹), and Recovery ($21.64 \pm 0.49 - 25.01 \pm 0.74$ mg. mL⁻¹). These protein values are similar to those observed in a study on *H. iris* which measured how they are affected by a pathological shell condition (Nollens *et al.*, 2004).

Normal total haemolymph protein concentration of *H. iris* had a range of 18.6 – 21.2 g. L⁻¹. However, unlike the results of the current study which showed a decrease in the total haemolymph protein range for the abalone in the treatment tanks, the results observed in Nollens (2004) showed an initial decrease in total haemolymph protein where the un-stressed abalone had a concentration of 19.9 ± 0.7 g. L⁻¹, and the abalone that weren't severely affected abalone had a concentration of 19.8 ± 0.8 g. L⁻¹. The severely affected abalone however showed an increase in total haemolymph proteins by having a protein concentration of 21.1 ± 1.0 g. L⁻¹ (Nollens *et al.*, 2004).

Similarly, in a study by Hooper *et al.* (2014) investigating the effects of severe heat stress on the immune function, biochemistry, and histopathology on the hybrid *H. laevigata* and *H. rubra*, the total haemolymph protein concentration of control animals was higher than that of the treatment animal on the first two days of being exposed to heat stress with control animals having a protein concentration of 9.8 g. L⁻¹ while stressed animals were 9.1 g. L⁻¹ on day one of the experiment, (Hooper *et al.*, 2014). On the seventh day of exposure to stress, the protein concentration of the Control being 8.6 g. L⁻¹ while the concentration of the treatment animals was 8.8 g. L⁻¹, a difference which may be attributed to the longer (7 days) exposure to heat (stress) on Hooper (2014), while the exposure to the water-off event was 96-hours (4 days).

The fluctuation of total haemolymph concentration values may be attributed to changes in light and the secretion of digestion enzymes (proteins) when the animals anticipate being fed. Gao *et al.* (2021) investigated the relationship between light/dark cycles and digestive enzyme activity (Gao *et al.*, 2021).

Abalone increased their food intake at night (dark) compared to daytime(light), therefore higher digestive enzyme secretion at night than during the day, suggesting that abalone have an internal automatic regulatory system which forms as a result of long-term evolutionary adaptation, to provide synchrony between the periodic environmental changes and the animal's behavior and may have variation in protein secretion between day and night (Gao *et al.*, 2021). The differences in how the animals in the current study and those in Gao (2021) responded to stress could be due to the animals in the current study not being fed for the duration of this study.

4.4. Conclusion and Recommendations

The water quality parameters in the raceway tanks were affected by lack of water flow, and ambient temperatures. The temperature of the water was explained by the changes in air temperature as well as changes in the water flowing into the control tanks. Water temperature did remain within the safe range for *H. midae* throughout the 96-hour water-off event (Britz *et al.*, 1997; Yearsley, 2008). Dissolved oxygen concentration and saturation dropped to their lowest ($7.02 \pm 0.2 \text{ mg. L}^{-1}$) and (85.4 %) respectively in treatment tanks at 56h. This comparatively low dissolved oxygen corresponded with the high temperature observed at the same time. However, as with the water pH, the dissolved oxygen concentration and saturation were also within the safe range for *H. midae*. Unlike other water quality parameters measured during this study, both TAN and FAN increased and reached alarming levels, but this was for less than 16 hours. The TAN and FAN levels then dropped from $35.9 \text{ } \mu\text{g. L}^{-1}$ to $25.7 \text{ } \mu\text{g. L}^{-1}$. The increase in temperature and decrease in dissolved oxygen require further investigation in other seasons when SST may be closer to upper thermal limits for *H. midae*.

Survival, growth, and biochemical health indices (haemolymph glucose, muscle glycogen, dopamine, superoxide dismutase, and total haemolymph protein) were used as stress indicators for the current study. There weren't any differences in the different treatments for each of the abalone stress indicators throughout the study period. This suggests that it safe for farm managers to implement a 96-hour water-off events.

It can therefore be suggested that due to the current loadshedding issue faced by South African abalone farms at the moment, there is the potential that farms may have regular water-off. However due since announced power-off period (loadshedding) may result in both water-off and air-off event, which

unlike controlled water-off events may result in acute (biochemical) effects and may be sufficient to induce chronic (growth) effects.

While the current study looked at a single 96-hour water-off event and found no effect on abalone health and growth, there may be just cause to do an experiment to test the effect of repeated short-term water off events (simulating loadshedding) and whether that has any effect on health and growth, the effects of longer water-off periods on health and growth and evaluating the possible implications of not feeding the abalone and not pumping in water to the farm during the water-off period. It is also recommended that future studies should be conducted focusing on the optimum dissolved oxygen levels for *H. midae* as well as the effects of different culture-induced stressors on the biochemical stress indicators of *H. midae*.



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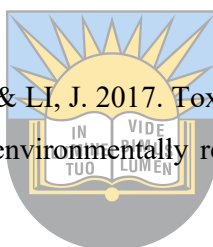
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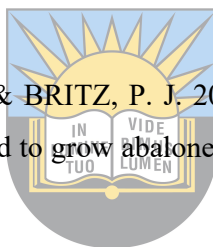
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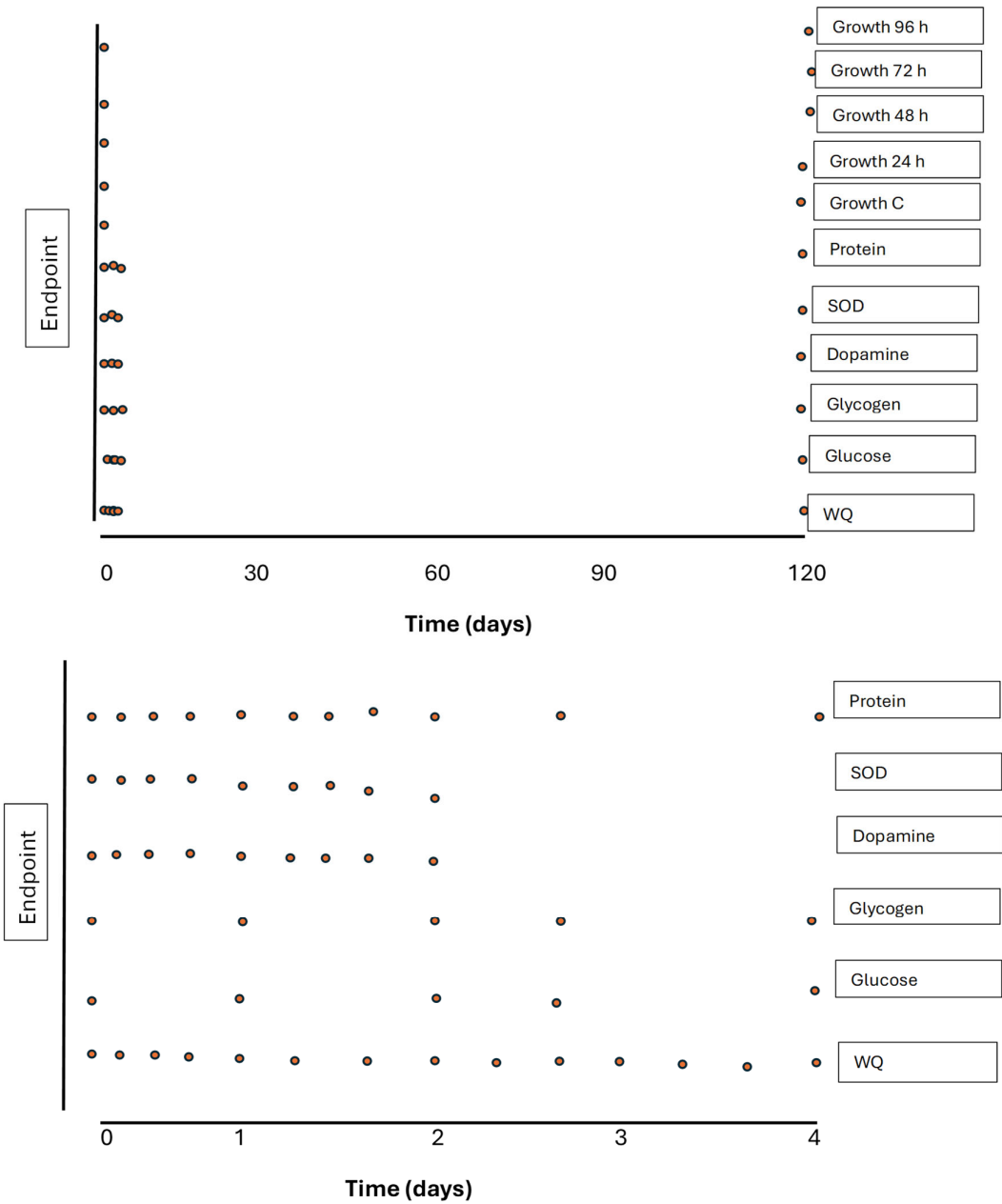
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Appendix A

Graphical representation of the sampling that took place during the experiment.



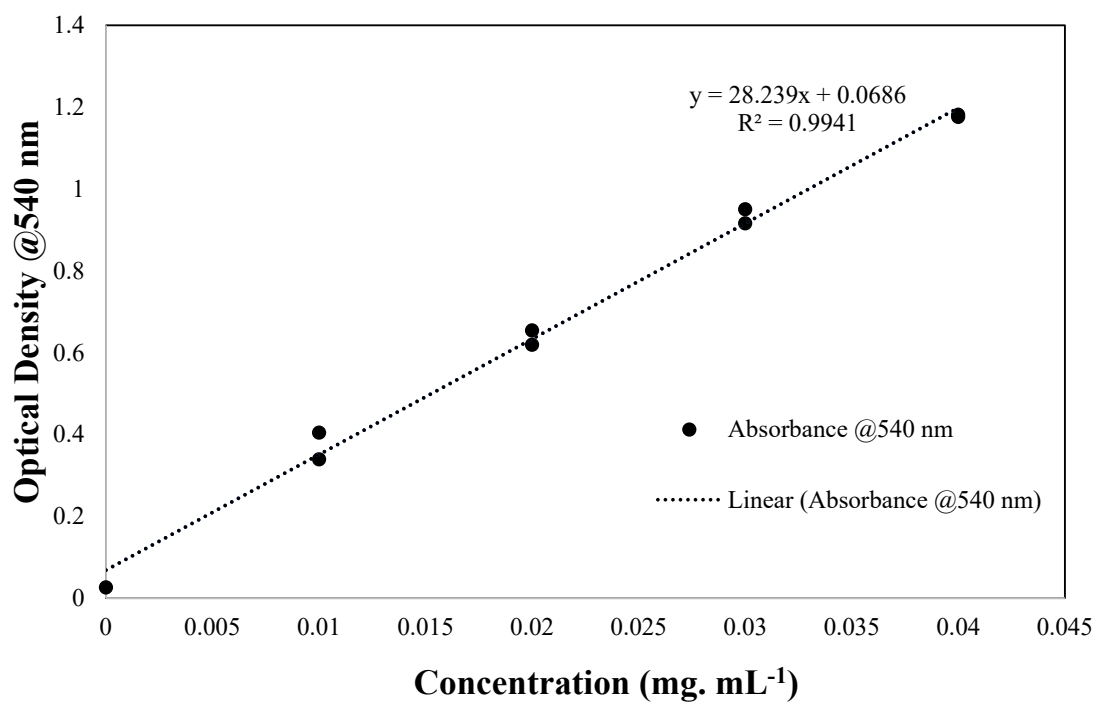
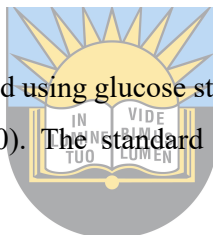


Figure A - Glucose standard curve prepared using glucose standard from the GOD-POD assay kit from Sigma-Aldrich (Product code GAGO-20). The standard curve was used to develop glucose and glycogen graphs (CHAPTER 2).



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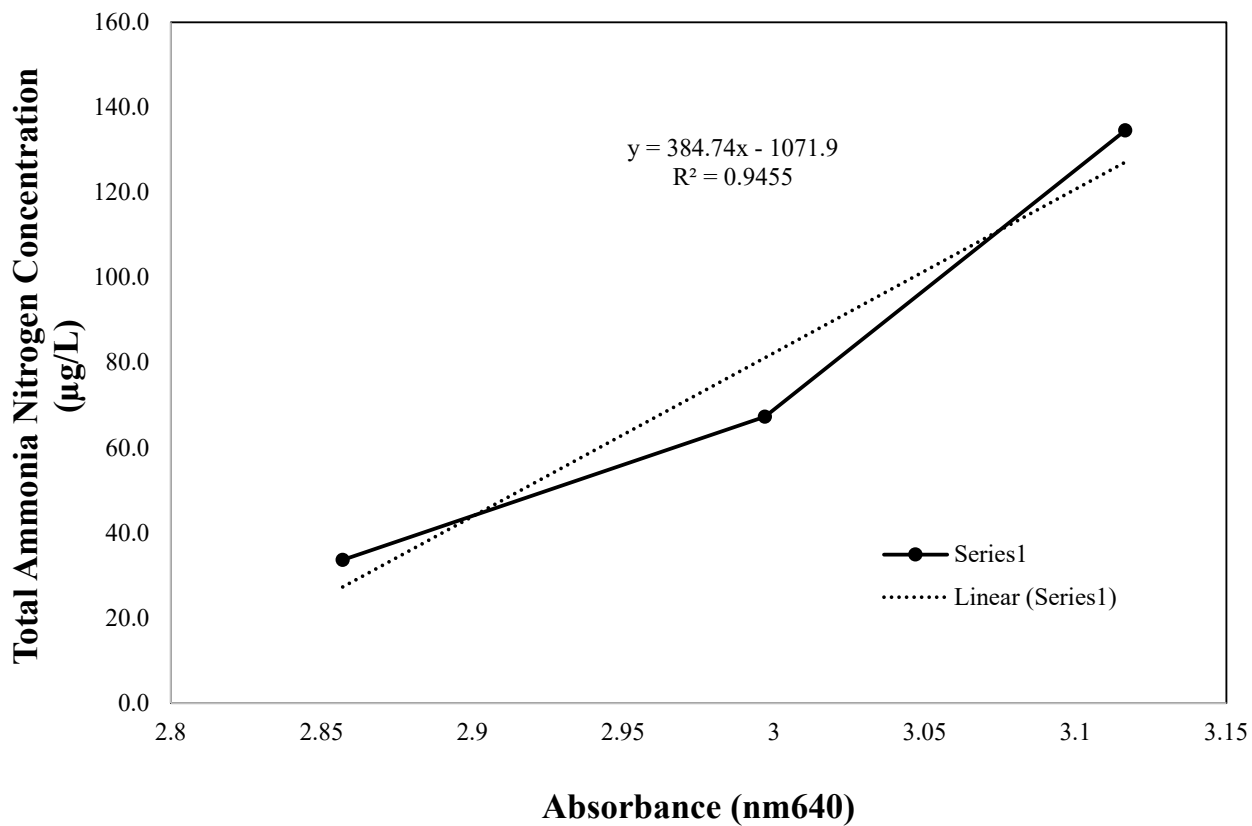
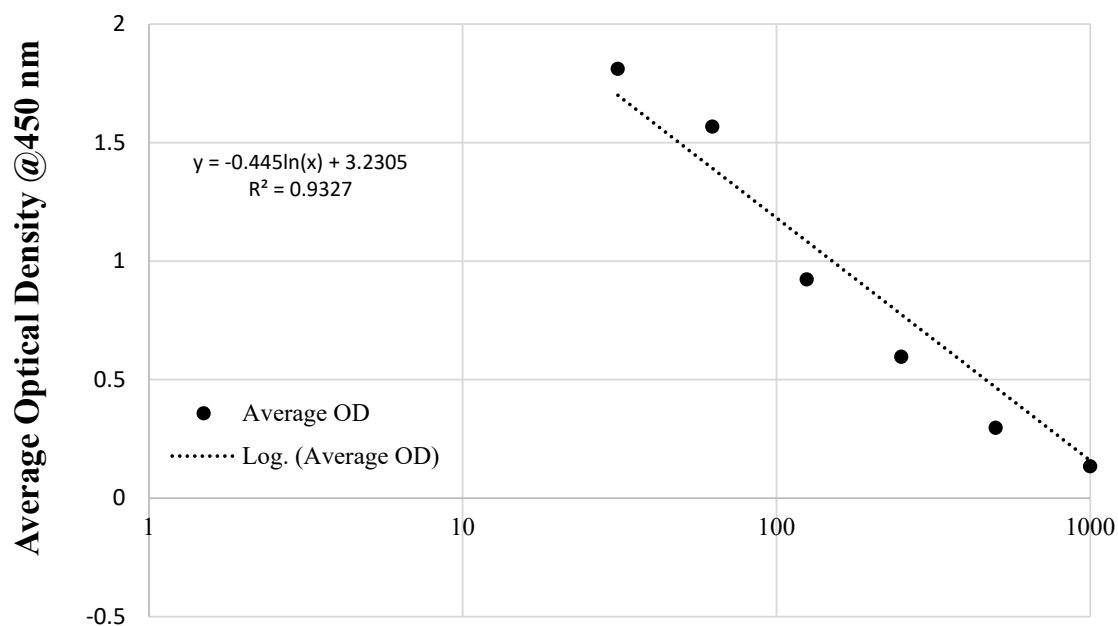


Figure B - Total ammonia nitrogen standard curve developed using the Solorzano (1969) method with colour absorbance read by spectrophotometer. The TAN standard curve was used to develop the TAN and FAN graphs (CHAPTER 2).



Dopamine Concentration (pg. mL⁻¹)

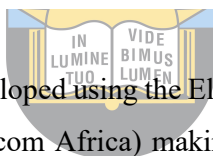


Figure C - Dopamine standard curve developed using the Elabsience DA (Dopamine) ELISA kit with the product number: E- EL – 0046 (Biocom Africa) making use of the manufacturer's instructions. The standard curve was used to develop the dopamine graph (CHAPTER 2).

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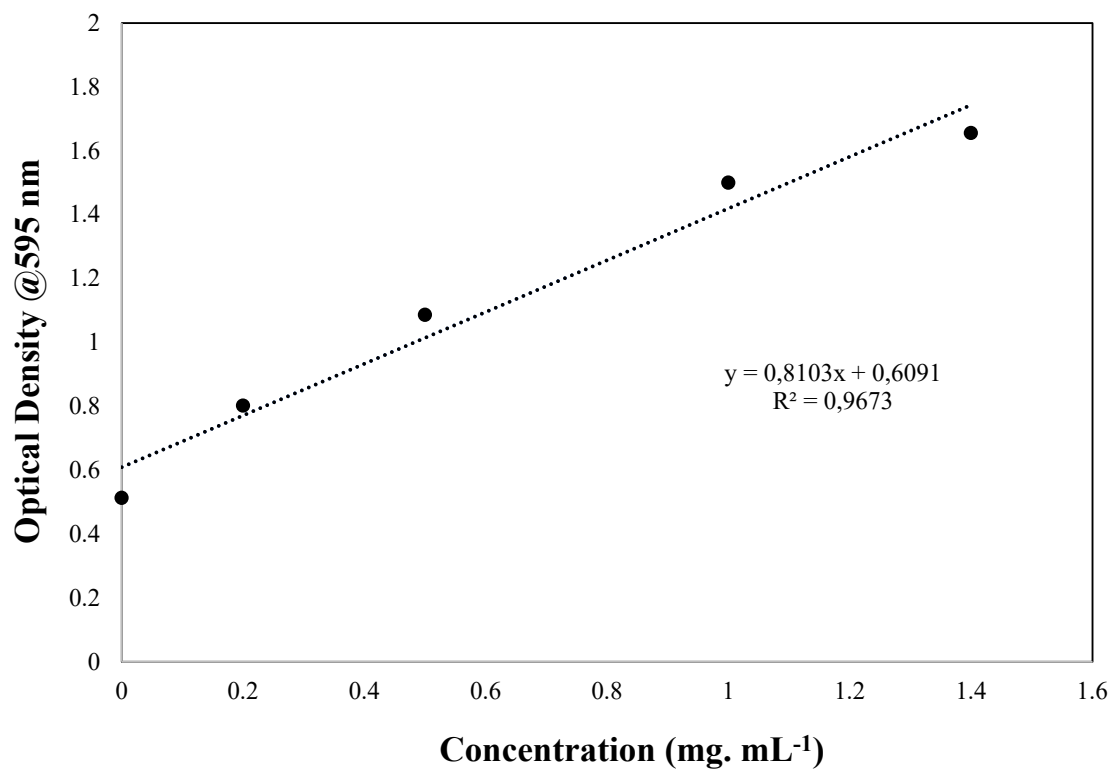


Figure D - Protein standard curve prepared with the use of Bovine Serum Albumine (le Bris et al., 2014). The standard curve was used to develop the protein graph (CHAPTER 2).

Table A - Stepwise comparisons (post-hoc tests) of haemolymph protein concentration over the 96-hour water off period.

Hours	Treatment	p-value	statistic	df
12	C-T	0.05	3.857	1
18	C-T	0.05	3.857	1
24	C-T	0.05	3.857	1
32	T- (C and/or R)	0.057	5.727	2
40	C-T	0.014		
48	C-T	0.046		
56	C-T	0.014		
64	R-T	0.039		
72	R-T	0.034		
84	C-T	0.05		
96	C-T	0.05		

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Appendix B

Ethical Clearance certificate.



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ETHICAL CLEARANCE CERTIFICATE AREC-150311-008

Certificate Reference Number: VIN041SMNG01/21/A

Project title: **The effects of water off events on the growth, survival, and biochemical stress indicators of the South African abalone *Haliotis midae***

Nature of Project: Lower invertebrates (MSc)

Principal Investigator: Prof N.G. Vine

Applicant: Mr B.L. Mngqushu

Supervisor: Prof N.G. Vine

On behalf of the University of Fort Hare's Animal Research Ethics Committee (AREC) I hereby give ethical approval in respect of the undertakings contained in the above-mentioned project and research instrument(s). Based on the information provided no additional permits or permissions besides those provided are required for this research. Should any other instruments be used these require separate authorization. The Researcher may, therefore, commence with the research as from the date of this certificate, using the reference number indicated above. This certificate will allow the project to continue for a period no longer than one year where after a progress report is required for project renewal.

Number of approved animals:

- Not applicable

Project Duration:

- 1 July 2021 – 31 December 2022 (Progress report due 30 April 2021)

Special conditions:

- None

Please note that the AREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the document;
- Any changes in personnel related to the approved project;
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AREC in the prescribed format, **no later than 10 months from the date of this certificate**, and at the end of the project, in respect of ethical compliance.

The AREC retains the right to

- Withdraw or amend this Ethical Clearance Certificate if
 - Any unethical principal or practices are revealed or suspected;
 - Relevant information has been withheld or misrepresented;
 - Regulatory changes of whatsoever nature so require;
 - The conditions contained in the Certificate have not been adhered to.
- Request access to any information or data at any time during the course or after completion of the project.
- In addition to the need to comply with the highest level of ethical conduct principle investigators must report back annually as an evaluation and monitoring mechanism on the progress being made by the research. Such a report must be sent to the Animal Research Ethics Committee so that these can be discussed and forwarded to the Dean of Research's office.

The Animal Research Ethics Committee wished you well in your research.

Yours sincerely



Dr Craig Tambling
AREC Chairperson

1 July 2021