Acute and chronic effects of ammonia in the South African abalone, *Haliotis midae* Linnaeus (Mollusca)

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Thesis presented for the Degree of
DOCTOR OF PHILOSOPHY
In the Department of Zoology
Faculty of Science
UNIVERSITY OF CAPE TOWN

August 2006

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Frontispiece

From what little is known about the inhabitants of South Africa before the arrival of the San (Bushman) and Khoi (Hottentot) people, it appears that the marine fauna was one of their principal food sources. The finding of large mounds of shells of edible molluscs, including abalone, led to these people being called "Shellmound Man" (Stuttaford, 1997). The Khoisan style painting of a hunter-gathering fisherman on one of the experimental tanks used in the present study symbolises one of these first people to consume abalone. The markings in the middle represent the three size classes of abalone investigated in the present study and symbol of *Haliotis midae* features on the right. Insert: Abalone *Haliotis midae*.

To

my husband, Andreas

and our two sons, Hiteshin and Gajnan

For all those countless hours spent on my thesis, are the stolen hours away from my family. The result of your love, support and understanding is evident in my thesis.

To

my parents, Kaycee and Radha

For never stop believing in my dreams. Your love and encouragement has finally led to the completion of this thesis.

DECLARATION

I, Kasturi Reddy-Lopata, hereby declare that the work on which this thesis is based is my own unaided work, both in concept and execution (except where acknowledgements indicate otherwise) and that apart from the normal guidance from my supervisors, I have received no assistance (except where acknowledgements indicate otherwise). I declare that neither the whole work nor any part of this thesis has been in the past, or is being, or is to be submitted for a degree at this university or at any other university (except where acknowledgements indicate otherwise).

Parts of Chapters 2, 3 and 4 have been compiled in a shorter version in a manuscript which is in press in the Journal 'Aquaculture': Reddy-Lopata, K, Auerswald, L, Cook, P. Ammonia toxicity and its effects on the growth of the South African abalone *Haliotis midae* Linnaeus, Aquaculture (2006), doi: 10.1016/j.aquaculture.2006.06.020. The work on which this publication is based is my own unaided work, both in concept and execution, except for the normal guidance of my co-supervisor and co-author, Professor Peter Cook and statistical assistance from the co-author, Dr Lutz Auerswald.

A pilot study conducted in 1998 was one of the first studies to demonstrate the presence of heat shock proteins (HSPs) in abalone, results of which were presented in 2000 at the 4th International Abalone Symposium, Cape Town, South Africa. The results were published as an abstract in the Journal of Shellfish Research; Reddy-Lopata, K., Lopata, A.L., Cook, P.A., 2000. Toxicity and tolerance levels of ammonia in abalone (*Haliotis midae*), *J. Shellfish Res.*, 19(1), 529.

In the field of abalone and stress proteins a total of three articles has been published to my knowledge and all three since 2000 when my initial findings were presented. This is the first study to demonstrate the increase of stress proteins during ammonia stress in aquatic invertebrates and the increase of P-glycoprotein (P-gp mediated Multixenobiotic resistance) in abalone. This is also a first study to demonstrate the influence of ammonia on the growth of juvenile *H. midae* and to determine the lethal concentrations of ammonia for *H. midae*. To my knowledge there has been no record of the above mentioned studies.

I designed, assisted and co-supervised the Honours Project of Mr Louis Du Buisson entitled: Acute toxic effects of elevated ammonia concentrations on cultured abalone, *Haliotis midae*, which was part of the requirements for his Bachelor of Science (Honours) Degree, University of Cape Town, November 2005. Data from Mr Du Buisson's project have been modified and included as sub-sections in Chapter 2 (2.2.4 & 2.3.2) and Chapter 3 (3.4.4) of my thesis. These sub-sections should, therefore, be considered as a collaborative effort of Mr Du Buisson and myself.

A certain amount of repetition was unavoidable as a result of the chapters being written up as independent manuscripts to facilitate.

Chapter 6 of this thesis entitled: Stress proteins and P-glycoprotein - possible adaptations for the increased tolerance to ammonia in *Haliotis midae*, was co-supervised by my husband, Dr Andreas Lopata (Division of Immunology, University of Cape Town). This relationship was fully disclosed to the university.

I empower the University of Cape Town free licence to reproduce for the purpose of research either the whole or any part of this thesis.

I am now presenting the thesis for examination for the degree of Doctor of Philosophy, in the Department of Zoology, University of Cape Town.

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Kasturi Reddy-Lopata

August 2006

ACKNOWLEDGEMENTS

I would like to thank the following people who have contributed to the completion of this thesis:

My sincere gratitude to **Dr Coleen Moloney** for giving me the opportunity to complete this thesis after a substantial break, due to the births of my two sons. The result of her taking over supervision from Professor Peter Cook in 2005, her assistance and invaluable comments has led to the final completion of my thesis.

A special thanks to **Professor Peter Cook** for his continuous and generous support in the supervision and later co-supervision of my thesis and his belief in my abilities.

A heartfelt thanks to my husband, **Andreas Lopata**, who appeared to effortlessly cope with me as his student and his wife. I am indebted to you for your constructive criticisms on the stress protein work and for all those extra hours you spent with our two sons, allowing me sufficient time to complete my thesis.

Dr Lutz Auerswald, for his friendship, his statistical assistance and the proof reading of certain chapters.

Mr Louis Du Buisson (Honours student) for his endurance in the long hours and hard work that was demanded of the toxicity trials, the data of which are valuable in my thesis.

Ms Liesl Phigeland, **Mr Ian Davidson** and **Mr George Du Plessis**, for their technical support in the Zoology Department.

Ms Andrea Plos and **Mr Kevin Ruck** for assisting me in diving for "wild" abalone.

Ms Lerisa Govender, Ms Bartha Fenemore and **Ms Michelle van der Ventel** for their friendship and technical assistance in the protein analysis in the Division of Immunology.

Ms Lize Schoonbee (Irvin & Johnson Abalone Farms) for assisting with the farmed abalone and advice on farm related issues.

Mr Reagon Peterson, Mr Reece Marillier, Mr Dawit Yemane and **Mr David Miller** for their much needed IT support.

Ms Hayley Battle for her good secretarial skills and organising the binding of this thesis.

Ms Gillian Smith, for all her help in organizing funds, her excellent computer skills and her morning smiles to brighten my day.

I am indebted to the following institutions for their financial assistance namely, the National Research Foundation (NRF bursaries) and Eskom (funded the Marine Biology Research Institute Scholarship). I am grateful to Aquafarms (Hermanus) and Irvin & Johnson Abalone farms (Gansbaai) for donating abalone and Signet Laboratories, Inc. (Dedham, MA) for donating the C219 antibody. I am indebted to the Department of Zoology (University of Cape Town) for providing me with space, equipment and running costs for conducting my experiments. I am further indebted to the Division of Immunology (University of Cape Town) for providing me with space, equipment and running costs for the protein analysis.

Table of Contents

Abbreviations

BSA	bovine serum albumin
CE	constant environment
EC	effective concentration
EC_x	effective concentration causing x percent growth retardation
ELISA	enzyme-linked immunosorbent assay
FAN	free un-ionized ammonia
HSP	heat shock protein
ImR	immune response to *Vibrio parahaemolyticus*
LC	lethal concentration
LC_x	lethal concentration killing x percent of animals
MDR	multidrug resistance
MoAb	monoclonal antibody
MWM	molecular weight marker
MXR	multixenobiotic resistance
MXRM	multixenobiotic resistance mechanism
NH_3	free un-ionized ammonia
NH_3-N	total ammonia nitrogen ($NH_3 + NH_4^+$)
NH_4^+	ionized ammonia
NO_2-N	nitrite nitrogen
NO_3-N	nitrate nitrogen
NOEC	no observable effect concentration
OD	optical density
P-gp	P-glycoprotein
SDS	sodium dodecyl sulphate
SGR	specific growth rates
SGRL	specific growth rate for shell length
SGRSW	specific growth rate for shell width
SGRW	specific growth rate for weights
TAN	total ammonia nitrogen
TEMED	N,N,N,N' tetramethylene diamine

Abstract (for abstracting services, < 350 words)

Toxicity of ammonia, which can reach toxic levels in aquaculture systems, was investigated for *Haliotis midae*. Toxic FAN (free un-ionized ammonia) was estimated from TAN (total ammonia nitrogen) measurements. It was found that commonly used Nesslers and Palintest methods underestimated TAN. Tolerance of *H. midae* to ammonia increased with increasing size, as indicated by 36 h LC_{50} values; farmed juvenile abalone (1 - 2.5 cm shell length) had the smallest LC_{50} of 9.8 μg l^{-1} FAN, whereas LC_{50} was 12.9 μg l^{-1} FAN in wild cocktail abalone (5 - 8 cm), and 16.4 μg l^{-1} FAN in wild brood stock abalone (10 - 15 cm). *H. midae* was found to acclimatize to ammonia (LC_{50} 14.8 μg l^{-1} FAN) at sub-lethal concentrations, with this LC_{50} value for acclimatized wild cocktail-size abalone being 2.0 μg l^{-1} FAN greater than for non-acclimatized abalone. Ammonia tolerance was greater in farmed (LC_{50} 37.9 μg l^{-1} FAN) than wild (LC_{50} 12.7 μg l^{-1} FAN) *H. midae*, with a three-fold difference between the two LC_{50} values. Growth of farmed juvenile *H. midae* was inhibited during chronic exposure to sub-lethal FAN (7.4 μg l^{-1}), with mean ± s.d. specific growth rates (0.10 ± 0.03 % d^{-1}) reduced to 59 % of that in a control group (no added ammonia) (0.24 ± 0.06 % g d^{-1}). Heat shock proteins (HSPs) and/or P- glycoprotein (P-gp) could be responsible for increasing tolerance to ammonia in *H. midae*. Immunological assays showed increase of a ~257 kDa protein in the gills after ammonia exposure but not after heat shock, indicating the presence of P-gp in *H. midae*. It appeared that HSP 90, HSP 70 and HSP 60 could be implicated in both temperature and ammonia tolerance, whereas HSP 27 was solely up-regulated during heat stress. Chronic effects of ammonia on farm production were assessed using two growth models, which indicated that abalone yield could be reduced to 20% of normal yield (no ammonia stress) for sustained chronic exposure. It was proposed that HSPs and/or P-gp could be used as biomarkers indicating ammonia stress in farmed animals.

Summary

Ammonia is the major end-product of protein catabolism in abalone, and is excreted into the surrounding water. Build-up of ammonia in abalone aquaculture systems can reach high toxic levels and impact negatively on farming operations. Ammonia levels therefore have to be regulated. Accurate estimates of toxic un-ionized ammonia (FAN) from the total ammonia nitrogen (TAN) measured by photometer test kits is crucial as inaccuracies could result in major losses of abalone on the farms. The accuracy and applicability of two frequently used photometer test kits (the Nesslers method and the Palintest method) were compared. The calculated TAN concentrations were regressed against the prepared standard ammonia concentrations in seawater for both methods. The predicted slope ($\beta = 1$) if the methods were 100% accurate was compared to observed slopes using the Nesslers ($\beta = 0.8327$) and Palintest ($\beta = 0.7507$) methods. Both these test kits underestimated the TAN concentrations, but the Nesslers method was more accurate. Nitrites and nitrates are toxic to abalone at much larger concentrations than ammonia and exist at low concentrations in natural systems, therefore the sensitivity of their tests was not as important. Nitrites and nitrates were monitored using the Ferrous sulphate method and the Cadmium reduction method, respectively. The Nesslers method was used throughout this study, unless stated otherwise, and all parameters such as nitrite, nitrate, temperature, pH and salinity were monitored regularly.

Toxicity levels of ammonia for South African abalone, *Haliotis midae*, have not been documented and such values are not fully presented for other abalone species. A series of experiments is described in which toxicity of ammonia to *H. midae* was investigated. Based on findings for other species, it was hypothesised (Hypothesis 1) that the tolerance of *H. midae* to ammonia should increase with increasing size, with acclimation at sub-lethal concentrations (Hypothesis 2), and in farmed as opposed to wild animals (Hypothesis 3).

Acute toxicity of ammonia to *H. midae* was investigated for three size classes relevant to mariculture operations. Tolerance to ammonia (at pH 7.8 and T = 15 °C) was found to increase with body size as indicated by 36 h LC_{50} values; farmed juvenile abalone (1 - 2.5 cm shell length) had the smallest LC_{50} of 9.8 $\mu g\ l^{-1}$ FAN, whereas LC_{50} was 12.9 $\mu g\ l^{-1}$ FAN in wild cocktail abalone (5 - 8 cm shell length), and 16.4 $\mu g\ l^{-1}$ FAN in wild brood stock abalone (10 - 15 cm shell length). When wild cocktail-size abalone were allowed to acclimatize to

sub-lethal ammonia concentrations for 48 h, their ammonia tolerance increased compared with non-acclimatized abalone of the same size: LC_{50} was 2.0 μg l^{-1} FAN greater at 14.8 μg l^{-1} FAN. Farmed cocktail-size abalone demonstrated a three-fold larger 48h-LC_{50} value (of 37.9 μg l^{-1} FAN) than wild cocktail-size abalone (12.7 μg l^{-1} FAN). Abalone are susceptible to elevated ammonia concentrations, but appear to have some ability to acclimatize.

Ammonia has been shown to retard growth in a number of abalone species. The long term impacts of sub-lethal ammonia concentrations were investigated for growth of farmed juveniles (1 – 2.5 cm shell length). It was hypothesised (Hypothesis 4) that elevated but sublethal ammonia concentrations would have a chronic effect on *H. midae* by retarding growth. Growth was inhibited during chronic exposure to sub-lethal FAN (7.4 μg l^{-1}) with mean ± s.d. specific growth rates (0.10 ± 0.03 % d^{-1}) was reduced (ANOVA: $F = 3.102$, $df = 114$, $p < 0.05$) to 59 % of that in a control group (no added ammonia) (0.24 ± 0.06 % g d^{-1}).

Two possible indicators of stress have been identified in aquatic organisms: heat shock proteins (HSPs; stress proteins) and the P- glycoprotein (P-gp) mediating multixenobiotic resistance (MXR). HSPs are known to confer tolerance by folding and refolding of partially denatured proteins while P-gp reduces toxins in the system by actively pumping them out of the cell. Neither of these responses has yet been demonstrated in ammonia-stressed abalone.

It was hypothesised (Hypothesis 5) that HSPs and/or P-gp mediated MXR are responsible for the observed increased tolerance to ammonia in *H. midae*. Four HSPs (-90, -70, -60 and -27) and the P-gp were investigated in the foot and gills of *H. midae* using immunological assays (using stress protein-specific antibodies). Induction of HSPs and P-gp by heat shock, a well known stressor of HSPs but not P-gp, was also investigated. The amount of protein present was quantified using densitometric analysis. It was demonstrated for *H. midae* that heat shock had the greatest impact in the foot (increase in HSP 90 and -70, $P < 0.05$) within the first hour, with no significant changes in the gills. In contrast, ammonia exposure had the greatest impact on the gills after 24 hours (increase in HSP 70 and -60, $P < 0.05$ and $P < 0.001$ respectively) and after 36 hours ($P < 0.01$ and $P < 0.001$ respectively). There were no significant differences in HSP 70 and -60 levels in the foot during ammonia exposure, but there appeared to be an observed increase during longer exposure for HSP 70 (after 24 and 36 hours) and HSP 60 (36 hours). HSP 90 was not present in the foot and gills of *H. midae* under

normal (control) conditions and was only present in the foot after heat shock and in the gills after ammonia shock. HSP 27 was not present in the foot and gills under normal conditions as well as during ammonia exposure but was present in the foot and gills after 24 hour heat exposure.

The presence and increase of a ~257 kDa protein (cross reacting with the C219 antibody) in the gills after ammonia exposure but not after heat shock, indicates that P-gp might be present and play a role in ammonia tolerance in *H. midae*. It appears that HSPs 90, -70 and -60 could be implicated in temperature as well as ammonia tolerance, whereas HSP -27 solely increases during heat stress and P-gp during ammonia exposure.

The findings of the present study were synthesised to demonstrate their applicability to the management of ammonia stress in farmed *H. midae*. Firstly, the potential consequences of chronic effects of ammonia on farm production were assessed using two growth models. Model 1 was applied to *H. midae* from 0.550 years to ~0.772 years (~1.89 cm; 0.730 grams), and used specific growth rates (for weights, SGRW) from the present study to calculate changes in mass over time. A purely empirical curve (model 2) which calculated growth as a function of mass, was fitted to a combination model 1 and a field-based growth model, and was applied to abalone from age 0.772 years onwards. The mass of abalone at age 4.5 years (when farmed *H. midae* are typically harvested) was calculated for different periods of exposure to sub-lethal FAN concentrations. Mass decreased with increasing periods of stress, with yield potentially reduced to 20.5% of the normal yield (no ammonia stress) for sustained chronic exposure. Monitoring of water quality can contribute to preventing ammonia stress, but these indicators of animal stress are indirect. Direct monitoring of the animals can be done using biomarkers. Both HSPs and P-gp could be used as biomarkers of ammonia stress and poor health in *H. midae*, although further research and improved (cheaper) technology would be needed to make this feasible.

Chapter 1

General Introduction –
Ammonia toxicity and abalone aquaculture

General Introduction

The increase in demand for seafood and declining global fish stocks has resulted in a dramatic increase in the farming of aquatic species (aquaculture). Aquaculture is a thriving industry but Africa's contribution in comparison to the rest of the world is considered to be fairly insignificant; in 2000 Africa produced only 0.4% (117 000 tons) of the total world aquaculture production (Stanford, 2004). Mariculture (the farming of marine species) in South Africa is more important than freshwater aquaculture because of the high-value niche-market of marine species. More than 50 % of the shellfish consumed by humans consists of scallops, mussels and abalone (Qian et al., 2001) and therefore farming these species worldwide is important. Abalone, a flat footed mollusc belonging to the family Haliotidae and the genus *Haliotis* (meaning 'sea ear') (Fallu, 1991), is one of the most valuable seafood species in the world. Its demand, especially in Asia, exceeds world supply (~22 000 tons/year) (Gordon & Cook, 2004; Stanford, 2004). This has ensured high prices but also a decline in natural stocks of abalone because of over-fishing and poaching, combined with disease and habitat loss (Gordon & Cook, 2004). Abalone farming was pioneered by Japanese farmers after World War II, as a result of problems associated with the wild fishery (Fallu, 1991).

In South Africa, the decrease in wild abalone fisheries has occurred at the same time as the increase in abalone aquaculture (Britz, 1996; Troell et al., 2006). Commercial abalone fisheries in South Africa have existed since 1949, but abalone cultivation dates from 1981, with the successful spawning of captured specimens to produce spat and juveniles (Sales & Britz, 2001). The establishment of commercial abalone farming occurred ten years later, largely as a result of research and development by the University of Cape Town, the Council for Scientific and Industrial Research, Rhodes University and certain fishing companies (Sales & Britz, 2000). It took another twelve years to build an industry, which currently comprises 13 abalone farms (estimated investment of US$12 million) along the South African coast (Sales & Britz, 2001). In 2004, the production of farmed abalone in South Africa (~800-900 tons) exceeded the wild harvest (~300 ton) (Stanford, 2004) and fetched moderate to high prices (US $30 - 36/kg) (Gordon & Cook, 2004; Stanford, 2004). Despite the animal's slow growth and resulting long culture periods, abalone aquaculture has become a viable supplier of animals worldwide.

2 - CHAPTER 1: GENERAL INTRODUCTION

Between 75 and 100 species of abalone have been identified worldwide and ~20 species are classified as commercially important (Jarayabhand & Paphavasit, 1996).

There are six *Haliotis* species indigenous to southern African waters (see Figure 1.1 for their natural distribution along the South African coast) but only *Haliotis midae* is of commercial importance (Cook, 1998). *H. midae*, also known locally as perlemoen (from the Dutch *Paarlemoer*, meaning mother-of-pearl) (Steinberg, 2005), reaches a maximum size of 20 cm shell length at over 30 years in the wild, with variation in growth rates at different areas along the coastline (Sales & Britz, 2001; Tarr, 1995). It has been found, using genetic markers (Evans et al., 2004), that the populations of *H. midae* on either side of Cape Agulhas represent two independent reproductive stocks, and this could partly explain some of the variations in growth. This herbivorous, nocturnal mollusc grazes naturally predominantly on kelp, *Ecklonia maxima* and *Laminaria pallida*, which are abundant on the west and south-west coasts (Barkai & Griffiths, 1987; Tarr, 2000). Their diet may also include a mixture of other algae such as *Plocamium*, *Ulva*, *Aeodes* and *Porphyra* depending on their distribution and abundance in the surrounding habitat (Barkai & Griffiths, 1987, 1988; Sales & Britz 2001). The smaller size classes of *H. midae* predominantly consume *Ulva*, possibly because both *Ulva* and small abalone occur in shallow water (Sales & Britz, 2001). On abalone farms, *H. midae* are reared on a mixture of diets which include *Ecklonia maxima*, other seaweeds (*Gracillaria* and *Ulva*) and Abfeed (a commercial abalone feed) (L. Schoonbee, pers. comm. 2005). Artificial feeds that contain sufficient protein are used to maximise growth (Britz et al., 1997).

Britz et al. (1997) found that the temperature range of 12 – 20 °C is optimal for farmed *H. midae* according to its growth rates, feed consumption, mortality, protein energy ratios and feed conversion ratios. This suggested temperature range corresponds well with the mean monthly minimum (12 – 13 °C) and the maximum (21 °C) sea temperatures that occur in the natural range of *H. midae* (Britz et al., 1997; Sales & Britz, 2001). This wide variation in sea temperature experienced by *H. midae* is a result of its distribution (Figure 1.1), which includes the cold waters of the Benguela upwelling system on the west coast and the warmer waters influenced by the southward flowing Agulhas Current on the South African south and east coasts (Evans et al.,

2004; Sales & Britz, 2001). The preferred temperature range of juvenile *H. midae* is 24.1 – 24.5 °C with a critical thermal maximum at 27.9 °C (Hecht, 1994).

Figure 1.1. Distribution of the six species of *Haliotis* along the coast of South Africa (http://web.uct.ac.za/depts/zoology/abnet/safrica.html).

Establishment of abalone farms is capital-intensive, although new technology has led to a reduction of costs in recent years. It takes about two-three years to establish a hatchery (L. Schoonbee, pers. comm.). Usually more than three years are required to grow an animal to the marketable cocktail size of 5-8 cm shell length (Jarayabhand & Paphavasit, 1996) and some farms aim for a larger size of 8-10 cm shell length (Macey & Coyne, 2005). Improving the running costs of aquaculture farms (specifically abalone farms) can be achieved by optimising the water quality in these farms. Water quality is the single most important factor affecting an abalone (Fallu, 1991). Water is not only a source of essential substances (such as dissolved oxygen and nutrients) but also acts as a waste disposal system. Hence, poor water quality resulting from toxic substances, or insufficient quantities of essential substances, may retard growth and cause death in abalone. Therefore, it is relevant to consider the minimum water

quality levels that an aquatic farm has to maintain. The tolerance limits of water quality depend very much on the species cultivated (Pillay, 1992).

This present study focuses on the requirements of water quality for farming the South African abalone, *H. midae*. Factors affecting water quality include dissolved oxygen, pH, temperature and concentrations of nitrites, nitrates and ammonia. Ammonia can enter natural water systems from several sources, including industrial wastes, sewage effluents, alternative fuel conversion processes, and agricultural discharges (Rand & Petrocelli, 1985). The main source of ammonia in the water of aquafarms, however, is the animal itself. Ammonia is the major end-product of protein catabolism in most aquatic animals and is excreted into the surrounding water (Wright, 1995). Build-up of ammonia resulting from the bacterial decomposition of organic matter (faeces, unconsumed food, dead animals) (Cheng et al., 2004) can result in high toxic levels and impact negatively on farm operations. This problem is exacerbated when the farm's water circulation has to be closed, for example due to harmful algal blooms in the sea (Botes et al., 2003), environmental toxins (oil spills), and high prevalence of harmful ectoparasites (Simon et al., 2004). Ammonia levels have been shown to increase with increasing stocking densities/biomass (Huchette et al., 2003; Samsukal, 2004), increase in pH and temperature (discussed later), and change from natural to artificial feed (Bredberg, 2003).

All 13 abalone farms in South Africa operate pump-ashore, land-based flow-through systems (Troell et al., 2006). In the last few years, some farms began recirculation of water to improve abalone growth by increasing the temperature of the cold water derived from the west coast of South Africa (Troell et al., 2006). There is a risk of a build-up of ammonia in these recirculation systems but the use of seaweeds to absorb ammonia has been shown to be effective in these systems (Robertson-Andersson, 2003). Of the 13 farms, seven have completely flow through systems, three farms operate on a commercial recirculation system (two farms use 100% recirculation and one farm use 25% recirculation), two farms have the possibility of 100% recirculation and use seaweeds) and one farm has the possibility of 50% recirculation and uses seaweed (D. Robertson-Andersson, pers. comm.). There are no specific guidelines for nutrient effluents from South African abalone farms but the Department of Water Affairs and Forestry has provided guidelines for the maximum TAN (total ammonia nitrogen) concentrations (1.59 mg l^{-1})

for coastal marine waters which are used by farms (Troell et al., 2006). The TAN values obtained from seven abalone farms in South Africa ranged from 0.015-0.675 mg l^{-1} (Samsukal, 2004) while the TAN value of unpolluted seawater rarely exceeds 0.18 mg l^{-1} (Samsukal, 2004). Higher values of TAN on farms have been observed (D. Robertson-Andersson, pers. comm.) but the values have not been recorded or the farmers are not willing to disclose this information.

Key questions addressed in the present study attempt to clarify the influence of ammonia on abalone: 1) Does acute toxicity of ammonia differ for different size abalone? 2) Does acute toxicity of ammonia differ for acclimatized and non-acclimatized abalone of the same size? (i.e. do abalone respond differently to sudden as opposed to steady increases of ammonia levels)? 3) Does acute toxicity of ammonia differ for wild and farmed abalone of the same size? 4) Do high concentrations of ammonia retard the growth of abalone? (i.e. what is the chronic effect of ammonia on abalone?). A fifth key question examined the cellular processes that determine the responses of animals to stressors on abalone farms: 5) Are heat shock proteins (HSPs) and/or the P-glycoprotein (P-gp) indicators of thermal and chemical stress in the South African abalone, *H. midae*?

The above five key questions have been incorporated into the overall objectives, hypotheses and design of this project, which has an overall goal of providing scientific understanding of the responses of abalone to environmental conditions that can be experienced on aquafarms. The remainder of this thesis is divided into five chapters, which are briefly described here and in more detail below. Chapter 2 is a methodology chapter, Chapter 3 addresses key questions 1), 2) and 3), Chapter 4) addresses key question 4), and Chapter 5 addresses key question 5). Finally, Chapter 6 synthesises the results and presents implications for farm management.

In Chapter 2, the results of a short methodology study are presented. Farms routinely use wet chemical tests to measure the sum of un-ionized and ionized ammonia or total ammonia nitrogen (TAN). The toxicity of ammonia, however, is determined by the proportion of free un-ionized ammonia (FAN) of the TAN. The concentration of FAN is strongly dependent on TAN, pH, temperature and salinity (Bower & Bidwell, 1978). It is important to calculate FAN when investigating ammonia toxicity as TAN can remain constant throughout the observation period

(i.e. during an experiment or during routine measurements on farms), but with change in pH and temperature, the FAN levels change. In general, at low pH and temperature, the levels of FAN are reduced (EIFAC, 1973). Two wet chemical tests are currently used commonly on South African abalone farms to measure TAN. Chapter 2 aimed to evaluate these two tests and the corresponding calculations of FAN, leading to selection of the most appropriate measure.

In Chapter 3, a series of experiments is described in which toxicity of ammonia to *H. midae* was investigated. A large body of information is available on the toxicity of ammonia to fish (Jeney et al., 1992; Knepp & Arkin, 1973; Leung et al., 1999; Randall & Tsui, 2002; Ruyet et al., 1995; Thurston et al., 1981), crustaceans (Koo et al., 2005; Young-Lai et al., 1991; Zhao et al., 1997), echinoids (Siikavuopio et al., 2004) and molluscs (Epifanio & Srna, 1975). Knowledge on the toxicity of ammonia to abalone is restricted to a few growth studies on juvenile Australian species, *Haliotis laevigata* and *H. rubra* (Harris et al., 1998a,b; Hindrum et al., 2001; Huchette et al., 2003), and standard toxicity tests (determinations of lethal concentrations of ammonia in abalone) are often not fully presented. Information from other species can be misleading because species behave differently in habitat selection and their ability to survive stressful conditions (Fallu, 1991). There is no information available for *H. midae* on ammonia toxicity (i.e. lethal- and sub-lethal concentrations, increased survival by adaptation to sub-lethal levels etc.) and the influence of ammonia on growth. In toxicity tests, LC_{50} values (mean lethal concentration killing 50 % of test animals) are used as standards for comparability (Greenberg et al., 1992), although it is also important to know at what concentration animals start to die. For such information, other values from the same experiments, such as LC_5 (5 % of animals die) to LC_{100} (all animals die), need to be recorded.

A number of hypotheses were tested that relate to the effects of ammonia on *H. midae*. Hypothesis 1 states that tolerance to ammonia in *H. midae* should increase with increasing body size. The relative surface area of an abalone decreases with increasing body size, and therefore the relatively larger surface area of small abalone make them more susceptible to external environmental stress (Fallu, 1991). Assuming the same toxicity levels of ammonia for abalone of different sizes can, therefore, be misleading. For farm management, it is necessary to establish exact LC values for a range of size classes in order to minimize stress and to ensure optimal

water quality at each life stage. Three size classes were, therefore, selected for these experiments: juveniles (1 - 2.5 cm shell length), cocktail size (5 - 8 cm shell length) and brood stock (10 - 15 cm shell length). Hypothesis 2 states that acclimatization of *H. midae* to sublethal levels of ammonia increases their subsequent resistance to lethal ammonia concentrations. Acclimatization is a process whereby an organism adjusts to various imposed conditions and it is present in many animals, including abalone. It is well established that fish respond to exposure to sub-lethal levels of ammonia by increasing their subsequent resistance to lethal concentrations (EIFAC, 1973). To date, such an increased tolerance to ammonia has not been documented in abalone. The experiments in this chapter used cocktail size abalone (5 - 8 cm shell length) to test Hypothesis 2. Hypothesis 3 states that farmed (raised in artificial conditions) *H. midae* have a higher tolerance to ammonia than wild *H. midae* of the same size. Farmed abalone are exposed to much higher levels of ammonia compared to wild abalone because of higher densities, enclosure and ammonia build-up in tanks. Experiments using cocktail size abalone were conducted to investigate differences in ammonia tolerance between wild and farmed abalone.

The effects of ammonia on abalone growth are investigated in Chapter 4. Hypothesis 4 states that high sublethal concentrations of ammonia should retard growth in *H. midae*. High ammonia concentrations are known to impact negatively on various physiological functions in a number of animals, including abalone, impairing their growth (Harris et al., 1998a,b; Hindrum et al., 2001; Huchette et al., 2003). Juvenile abalone (1 - 2.5 cm shell length) were selected for these growth experiments because they exhibit fast growth (Shepherd et al., 1995), are the least costly size class and require less logistical effort (i.e. water volume) because of their small size.

In Chapter 5, a start is made at identifying the cellular constituents that play a role in the responses of abalone to ammonia stress. It is hypothesized (Hypothesis 5) that HSPs and/or P-gp are two possible responses of ammonia stress in *H. midae*. Most organisms respond to elevated temperatures by synthesising a group of highly conserved proteins called heat-shock proteins or HSPs (Lindquist, 1981; Sanders, 1988). Such HSPs are found in organisms as diverse as bacteria, molluscs and humans (Burdon, 1982; Sanders, 1993). HSPs act as molecular chaperones, promoting the initial folding of other protein at the ribosome and the refolding of unfolded proteins when they are partially denatured (Nover & Scharf, 1997). This response is involved in

protecting organisms from damage caused by a wide variety of stressors (Sanders, 1993). HSPs are activated not only by heat but by other physiological stresses: ammonium chloride is an example of an agent that activates HSP genes by inhibiting proteolysis (Ananthan et al., 1986). In Chapters 3 and 4, ammonium chloride was the toxicant used to obtain the appropriate ammonia levels in the experiments. Compounds (such as ammonia) that can damage cells in organisms are referred to as xenobiotics. A particular response to xenobiotic exposure is to reduce the toxin in the system by the P-glycoprotein (P-gp) mediated multidrug resistance (MDR) or multixenobiotic resistance mechanism (MXRM) (Eufemia & Epel, 2000). This protein and members of its family (ABC-proteins) have been found in almost all organisms investigated (Higgins,1992), including in a number of freshwater and marine species such as fish (Doi et al., 2001, Luckenbach et al, 2003), mussels (Eufemia & Epel, 2000; Hamer et al., 2004; Luedeking & Koehler, 2004; Minier et al., 2000; Smital et al., 2004), limpets (Smital et al, 2000) and clams (Archard et al., 2004; Kurelec et al., 1996; Legeay et al, 2005). There are no records, however, of P-gp induction in abalone, and there are no studies on aquatic invertebrates demonstrating ammonia as an inducer of P-gp. This chapter investigates whether HSPs and P-gp are produced in response to both ammonia and heat stress.

Chapters 3 and 4 describe the responses of abalone to ammonia stress, whereas Chapter 5 describes the responses at the cellular level. In Chapter 6, the various results at the organismal level are synthesized and expressed in terms that can be used for farm management. The potential losses in productivity that can be caused by different ammonia environments are calculated, and recommendations are made for water quality monitoring and management. The possible use of HSPs and P-gp as biomarkers of stress in abalone aquaculture is explored, with recommendations for future study.

Abstract

Ammonia is toxic to abalone and therefore the amount of ammonia in seawater has to be regulated by monitoring water quality parameters such as salinity, temperature and pH, as well as by controlling other factors such as the amount of feed, stocking densities and water flow rates. Accurate measurements of toxic un-ionized ammonia (FAN) from the total ammonia nitrogen (TAN) measured by photometer test kits is crucial as inaccuracies could result in major losses of abalone on the farms. Different methods of calculating ammonia in seawater using appropriate tables are investigated. The accuracy and applicability of two frequently used photometer test kits (the Nesslers method and the Palintest method) were compared. The calculated TAN concentrations were regressed against the prepared standard ammonia concentrations in seawater for both methods. The predicted slope ($\beta = 1$) if the methods were 100% accurate was compared to observed slopes using the Nesslers ($\beta = 0.8327$) and Palintest ($\beta = 0.7507$) methods. Both these test kits underestimated the TAN concentrations, but the Nesslers method was more accurate. Nitrites and nitrates are toxic to abalone at much larger concentrations than ammonia and therefore the sensitivity of their tests was not as important. Nitrites and nitrates were monitored using the Ferrous sulphate method and the Cadmium reduction method, respectively. It was concluded that the Nesslers method should be used throughout the present study and all parameters such as nitrite, nitrate, temperature, pH and salinity were monitored regularly.

2.1. Introduction

The establishment of aquaculture farms is capital-intensive. Availability of new technology has, however, led to a reduction in costs in recent years. A good management programme for water quality can further improve the running costs of aquafarms as well as reduce the number of deaths of the species cultivated. Requirements for water quality include certain levels of dissolved oxygen, pH, temperature, nitrites, nitrates and ammonia. The tolerance limits of these water quality requirements are specific and differ strongly among species (Pillay, 1992). Ammonia is known to be toxic to fish, crustaceans and molluscs, including abalone (Colt & Armstrong, 1981; Harris et al., 1998a, b; Jeney, et al., 1992; Leung et al., 1999; Meade & Watts, 1995). This project was designed to investigate the impacts of elevated concentrations of (especially) ammonia in the South African abalone, *H. midae*.

Ammonia would usually be removed from the water by bacteria (Pathway 1, Figure 2.1) or taken up directly by plants (Pathway 2, Figure 2.1). In pathway 1 (Figure 2.1) ammonia excreted by abalone or released by their feed is converted to nitrite by *Nitrosomonas* bacteria, which is in turn converted to nitrate by *Nitrobacter*. Plants utilize the nitrates as nutrients (Francis-Floyd & Watson, 1990; Hargreaves, 1998). Pathway 1 utilizes oxygen and if there are insufficient levels of oxygen, this pathway can break down (Francis-Floyd & Watson, 1990). Nitrites are considered more toxic than nitrates in abalone (Basuyaux & Mathieu, 1999; Harris, et al., 1997), clams and oysters (Epifanio & Srna, 1975), fish (Hamlin, 2006) and crayfish (Meade & Watts, 1995).

Alternatively, in pathway 2 (Figure 2.1) algae such as kelp convert ammonia-nitrogen into plant protein-nitrogen, in the presence of light and dissolved inorganic carbon (either as dissolved CO_2 or HCO_3). Kelp can be utilized as a food source for abalone (Demetropoulos & Langdon, 2004b; Langdon et al., 2004), and this has led to the establishment of co-culture systems of abalone with seaweed in most parts of the world (Demetropoulos & Langdon, 2004 a, b, c; Langdon et al., 2004; Neori et al., 2004). Its potential in South Africa is currently being investigated (Troell et al., 2006).

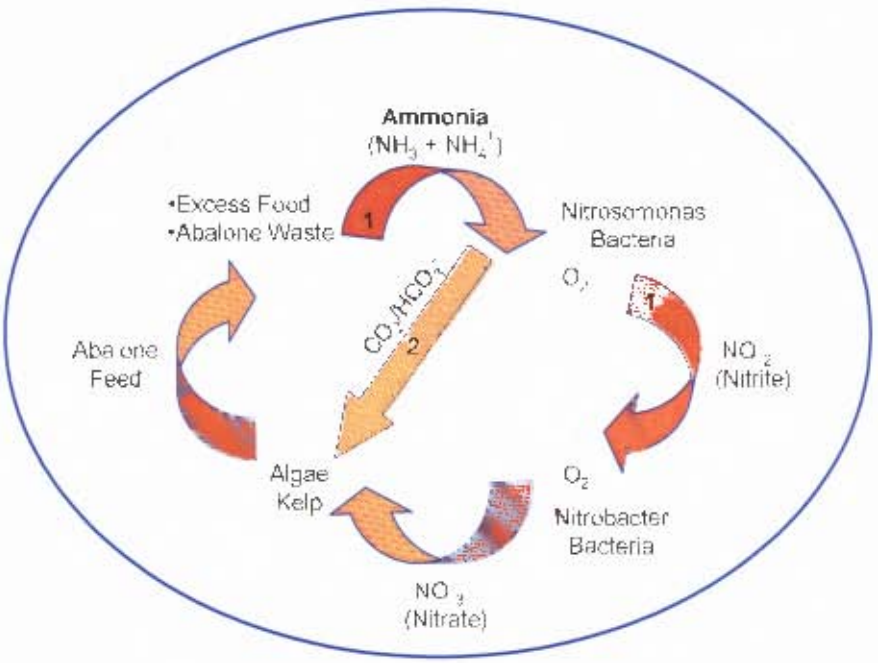

Figure 2.1. The ammonia/nitrogen cycle in abalone farms, illustrating the removal of ammonia from seawater by organisms. Adapted and modified from Francis-Floyd & Watson, 1990).

Toxicity of ammonia is caused by the un-ionized proportion (FAN) of total ammonia nitrogen (TAN) (see Figure 2.2), so that TAN measurements need to be converted to FAN to be useful in a monitoring situation in an aquaculture facility. Farms routinely use wet chemical tests such as the traditional Nesslers method (Palintest Information sheet, 1999) or the more recent Palintest (J. Venter, pers. comm.) to measure TAN which is the sum of FAN and ionized ammonium. Both these photometer test kits are designed to provide quick, accurate and high precision results (Anon, Palintest Instruction Manual). When analyzing seawater samples containing small amounts of ammonia nitrogen (0.33-2.92 mg l^{-1} in the present study), the method chosen should be accurate and reproducible, especially when small changes in the ammonia concentration can have detrimental or lethal effects on the animals. Photometer test kits are also available to measure a wide range of other chemicals (including nitrites and nitrates) in various media (freshwater, seawater and wastewater) (Anon, 1988).

In aqueous ammonia solutions, un-ionized ammonia (NH_3) exists in equilibrium with the ammonium ion (NH_4^+) and the hydroxide ion (OH^-).

This equilibrium can be written as:

$NH_{3(g)} + nH_2O_{(l)} \leftrightarrow NH_3.nH_2O_{(aq)} \leftrightarrow NH_4^+ + OH^- + (n\text{-}1)H_2O_{(l)}$ (Emerson et al., 1975)

Wet chemical tests measure the following:

Total ammonia nitrogen = Un-ionized ammonia + Ionized ammonium

TAN	=	NH_3	+	NH_4^+	(Huguenin & Colt, 1989)
		Toxic		Less toxic	

Figure 2.2. Chemical equilibrium of ammonia in water.

This chapter aims to determine the applicability of tests used in nitrogen (ammonia, nitrate and nitrite) analysis, to compare the accuracy and precision of two different methods (Nesslers and Palintest) routinely used on farms for ammonia analysis, and to select the most accurate method to calculate FAN in seawater.

2.2. Materials and methods

A range of nitrite concentrations (0-3 $mg.L^{-1}$ NO_2-N) and nitrate concentrations (0-5 $mg.l^{-1}$ NO_3-N) were prepared to test the accuracy and precision of the methods. The samples were prepared in de-ionized water (according to manufacturer's specifications) using analytical grade $NaNO_2$ (Basuyaux & Mathieu, 1999; Harris et al., 1997) and analytical grade $NaNO_3$ (Basuyaux & Mathieu, 1999), respectively. Measurements of the samples were performed in triplicates. Three sample concentrations were used for nitrites and four sample concentrations were used for nitrates.

2.2.1. Nitrite analysis

Nitrite concentrations were determined colourmetrically using the Ferrous sulphate method, which detects nitrites at a high range of 0-150 $mg.l^{-1}$ NO_2-N. Nitrite in the sample is reduced to nitrous oxide by ferrous sulphate in an acidic medium. The nitrous oxide complexes with the ferrous ions to form a greenish brown colour and its intensity is in direct proportion to the nitrite present (Anon, 1988; http://www.hach.com):

$$2FE^{2+} + 4H^{+} + 2NO_2^{-} \rightarrow 2FE^{3+} + 2NO + 2H_2O$$

$$NO + FeSO_4 \rightarrow FeSO_4 \bullet NO$$

The reaction time, once the reagents are added, is ten minutes. The nitrite concentrations in the samples were quantified by measuring absorbance at 585 nm with the Hach DR/2000 spectrophotometer. The same spectrophotometer was used throughout all experiments.

2.2.2. Nitrate analysis

Nitrate concentrations were determined colourmetrically using the Cadmium reduction method, which detects nitrates at a high to medium range of 0-30.0 mg l^{-1} NO_3-N. Nitrate in the sample is reduced to nitrite by cadmium metal (reaction 1, Figure 2.3). The nitrite in turn reacts with sulfanilic acid forming an intermediate diazonium salt (reaction 2, Figure 2.3) which in turn couples with gentisic acid (reaction 3, Figure 2.3) to form an amber colour. Its intensity is in direct proportion to the nitrate present (Anon, 1988; http://www.hach.com). The reaction time, once the reagents are added, is five minutes. The nitrate concentrations in the sample were quantified by measuring absorbance at 500 nm with the Hach DR/2000 spectrophotometer.

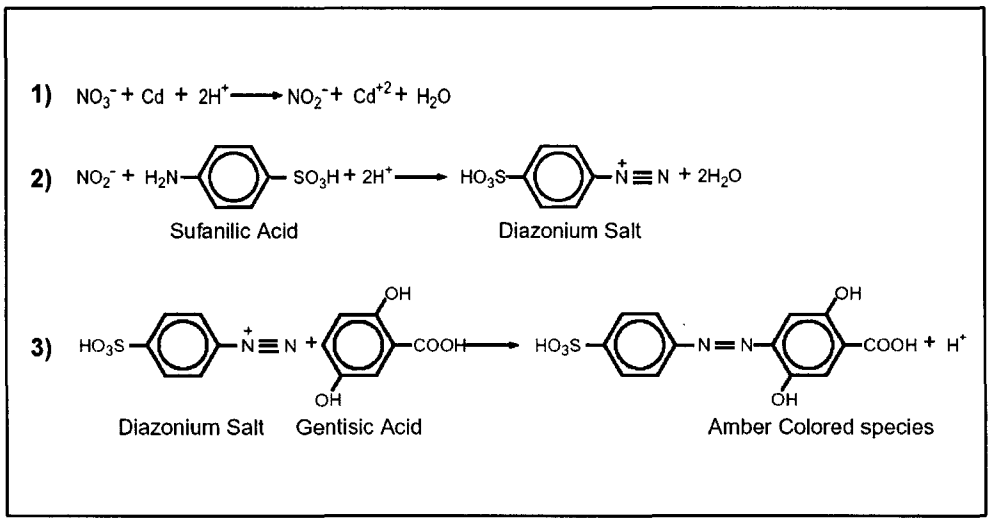

Figure 2.3. Three step reaction of the Cadmium reduction method used to determine the Amount of nitrates in seawater (adapted from http://www.hach.com.).

2.2.3. Ammonia analysis

Dissolved ammonia concentrations were determined using the Nesslers and/or Palintest colourmetric methods. Seawater samples were analyzed immediately because storage of seawater can cause variability in the amount of ammonia present (Degobbis, 1973).

2.2.3.1. Nesslers method

The Nesslers method detects total ammonia at a range of 0.02-2.50 mg l^{-1} NH_3-N. The method involves adding Rochelle salt (polyvinyl alcohol, potassium sodium tartrate, propionic acid, and demineralised water) to the sample to complex hardness and act as a dispersant. Nesslers reagent (sodium hydroxide, sodium iodide, demineralised water) is then added, and it reacts with the ammonium ion to produce a yellow colour in proportion to the total ammonia concentration (Anon, 1988). The reaction time, once the reagents are added, is one minute. If necessary, a mineral stabiliser (potassium sodium tartrate, sodium citrate, demineralised water) is added initially to the sample to complex the high magnesium and calcium concentrations found in seawater, which can interfere with the Nesslers reagent causing turbidity (Anon, 1988). The total ammonia concentration (TAN) in the sample is quantified by measuring absorbance at 425 nm with the Hach DR/2000 spectrophotometer.

2.2.3.2. Palintest method

The Palintest method detects ammonia at a range of 0 - 1mg l^{-1} NH_3-N. The test is based on the Indophenol blue method (Anon Palintest Instruction manual) and involves the addition of two reagents (tablet form): potassium nitroprusside/salicyclic acid and lithium hydroxide. A conditioning reagent powder (lithium hydroxide) is added initially to the sample to complex the high magnesium and calcium concentrations found in seawater, which can cause turbidity. Ammonia reacts with alkaline salicylate in the presence of chlorine and forms a green-blue Indophenol complex. The reaction time, once the reagents are added, is ten minutes. The percentage transmittance was measured colourmetrically with a Milton Roy spectrophotometer (Spectronic 20D$^+$) at a wavelength of 640nm. Total ammonia nitrogen (TAN) in the sample was determined from a calibration chart Table 2.1.

Table 2.1. Calibration chart for the Palintest method used to determine total ammonia nitrogen (TAN) from transmission (% T) reported by the spectrophotometer. (Adapted and modified from Palintest instruction manual, Anon.). Unit values for % T are given in the columns.

	Total ammonia nitrogen (mg l^{-1} TAN)									
%T	9	8	7	6	5	4	3	2	1	0
80	-	-	-	-	-	0.00	0.00	0.01	0.01	0.02
70	0.02	0.03	0.03	0.04	0.04	0.05	0.05	0.06	0.06	0.07
60	0.07	0.08	0.09	0.09	0.10	0.11	0.11	0.12	0.13	0.13
50	0.14	0.15	0.16	0.16	0.17	0.18	0.19	0.20	0.20	0.21
40	0.22	0.23	0.24	0.25	0.26	0.27	0.28	0.29	0.30	0.31
30	0.32	0.33	0.34	0.36	0.37	0.38	0.39	0.41	0.42	0.44
20	0.45	0.47	0.48	0.50	0.51	0.53	0.55	0.57	0.59	0.61
10	0.63	0.66	0.68	0.71	0.74	0.77	0.80	0.83	0.87	0.91
0	0.96	1.00	-	-	-	-	-	-	-	-

2.2.3.3. Calculations of toxic un-ionized ammonia

Experimental concentrations of free un-ionized ammonia (FAN) in all experiments were calculated using the total ammonia nitrogen (TAN) concentration, pH, temperature and salinity values (Huguenin & Colt, 1989):

$$\text{Un-ionized ammonia } (\mu g/l \text{ as } NH_3\text{-}N) = 1000(a)\,(\text{TAN})$$

where (a) is the mole fraction of ammonia and is equal to the proportion of un-ionized ammonia in a sample. This proportion is extracted from a pH-, temperature- and salinity-specific table (Table 2.2) adapted from Bower & Bidwell (1978). The salinity of the seawater during all experiments was 35 ‰.

Table 2.2. Percent un-ionized ammonia in seawater (Salinity = 32-40 ppt) at different temperatures and pHs, used to calculate free ammonia nitrogen concentrations. (Adapted and modified from Bower & Bidwell, 1978)

	pH										
Temp (°C)	7.5	7.6	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4	8.5
0	0.218	0.275	0.346	0.435	0.547	0.687	0.863	0.863	1.36	1.71	2.14
1	0.235	0.296	0.372	0.468	0.589	0.740	0.930	0.930	1.47	1.84	2.30
2	0.253	0.319	0.401	0.504	0.634	0.797	1.00	1.00	1.58	1.98	2.48
3	0.273	0.343	0.432	0.543	0.683	0.858	1.08	1.08	1.70	2.13	2.66
4	0.294	0.370	0.465	0.585	0.735	0.924	1.16	1.16	1.83	2.29	2.86
5	0.317	0.398	0.501	0.630	0.792	0.995	1.25	1.25	1.97	2.46	3.08
6	0.341	0.429	0.540	0.678	0.852	1.07	1.34	1.34	2.11	2.65	3.31
7	0.367	0.462	0.581	0.730	0.918	1.15	1.45	1.45	2.27	2.85	3.56
8	0.396	0.498	0.626	0.787	0.988	1.24	1.56	1.56	2.45	3.06	3.82
9	0.426	0.536	0.674	0.847	1.06	1.34	1.68	1.68	2.63	3.29	4.11
10	0.459	0.577	0.726	0.912	1.15	1.44	1.80	1.80	2.83	3.54	4.41
11	0.495	0.622	0.782	0.982	1.23	1.55	1.94	1.94	3.04	3.80	4.74
12	0.533	0.670	0.842	1.06	1.33	1.67	2.09	2.09	3.27	4.08	5.08
13	0.574	0.721	0.906	1.14	1.43	1.79	2.25	2.25	3.51	4.38	5.46
14	0.618	0.777	0.976	1.23	1.54	1.93	2.42	2.42	3.78	4.71	5.85
15	0.665	0.836	1.05	1.32	1.66	2.07	2.60	2.60	4.06	5.05	6.28
16	0.717	0.900	1.13	1.42	1.78	2.23	2.79	2.79	4.36	5.42	6.73
17	0.772	0.970	1.22	1.53	1.92	2.40	3.00	3.00	4.68	5.82	7.22
18	0.831	1.04	1.31	1.64	2.06	2.58	3.23	3.23	5.02	6.24	7.73
19	0.895	1.12	1.41	1.77	2.22	2.78	3.47	3.47	5.39	6.69	8.28
20	0.963	1.21	1.52	1.90	2.39	2.98	3.73	3.73	5.78	7.17	8.87
21	1.04	1.30	1.63	2.05	2.57	3.21	4.01	4.01	6.20	7.69	9.49
22	1.12	1.40	1.76	2.20	2.76	3.45	4.30	4.30	6.65	8.23	10.1
23	1.20	1.51	1.89	2.37	2.97	3.71	4.62	4.62	7.13	8.81	10.8
24	1.29	1.62	2.04	2.55	3.19	3.98	4.96	4.96	7.64	9.43	11.6
25	1.39	1.75	2.19	2.74	3.43	4.28	5.32	8.32	8.18	10.1	12.4

2.2.4. Comparing the Nesslers and Palintest methods

The experiments described here were carried out by a BSc Honours student (du Buisson, 2005) under my supervision.

A number (11) of standard ammonia concentrations (ranging from 0-2.45 mg.l^{-1} TAN) were prepared in seawater, using reagent grade ammonium chloride as toxicant (Basuyaux & Mathieu,

1999; Thurston et al., 1981). These standard solutions were used for comparing the Nesslers and Palintest methods. The two methods were tested within their respective detection ranges. The total ammonia concentrations were calculated using the absorbance and percentage transmittance readings for the Nesslers and Palintest methods, respectively.

2.2.4.1. Statistical analysis

The calculated TAN concentrations were regressed against the prepared standard ammonia concentrations in seawater. The observed regression slopes were compared with the expected slope ($\beta = 1$) using students t distribution (STATISTICA v7, StatSoft, Inc., 2004).

2.3. Results

2.3.1. Calibrations of nitrite and nitrate analyses methods

The measured nitrites and nitrates gave identical readings to the prepared samples (Table 2.3). There was no variability among the triplicates (Table 2.3) probably because the precision of the measurements of the Hach spectrophotometer is to the nearest 0.04 mg l^{-1}.

Table 2.3. Measurements of nitrites and nitrates in the prepared samples.

Nitrites (NO_2-N mg l^{-1})		*Nitrates (NO_3-N mg l^{-1})*	
sample	**Mean ± s.d.**	**Sample**	**Mean ± s.d.**
0.8	0.8 ± 0.0	0.6	0.6 ± 0.0
1.5	1.5 ± 0.0	1.3	1.3 + 0.0
3.0	3.0 ± 0.0	2.5	2.5 ± 0.0
		5.0	5.0 ± 0.0

2.3.2. Comparing the Nesslers and Palintest methods

The regression lines obtained for the Nesslers and Palintest methods resulted in significant, strong positive correlations (Figure 2.4) between the calculated TAN concentrations and the standard concentrations ($r = 0.998$ and 0.999, respectively). However, the slopes of both lines were less than 1 (Nesslers: $t = -3.910$, $df = 9$, $p < 0.01$, and Palintest: $t = -19.803$, $df = 4$, $p < 0.01$), indicating negative bias in the TAN measurements.

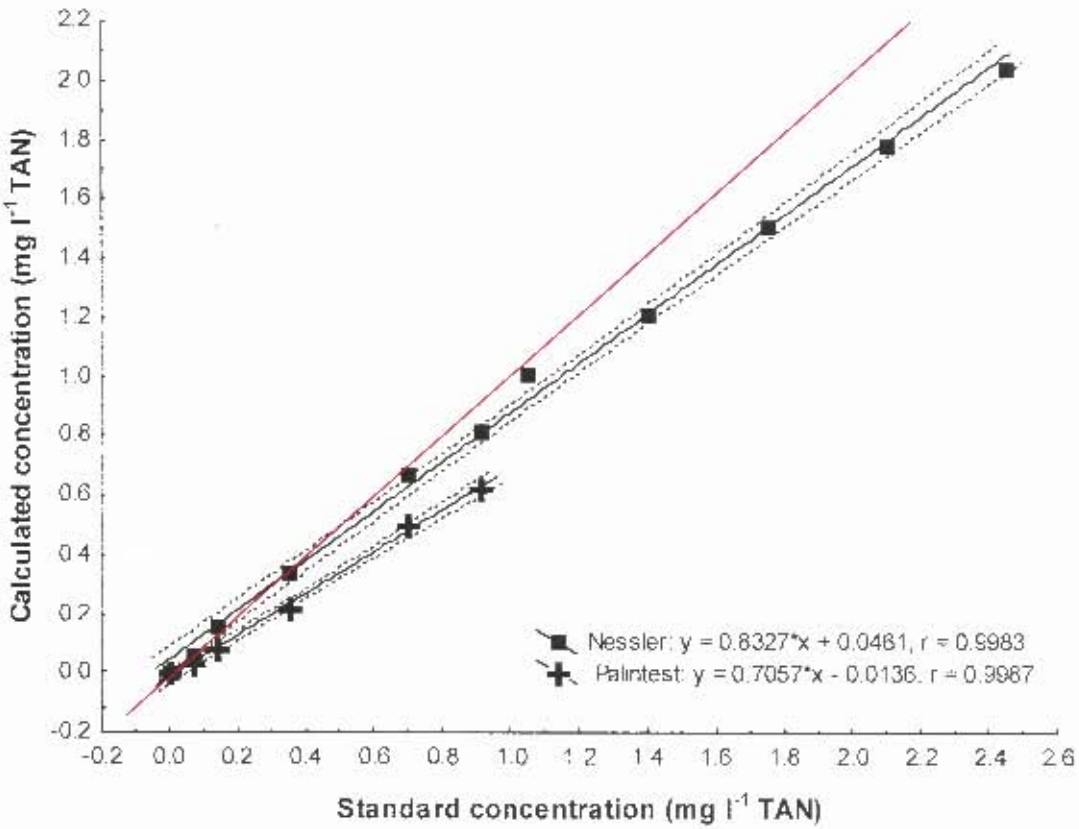

Figure 2.4. Calculated total ammonia nitrogen (TAN) concentrations plotted against standard ammonia nitrogen concentrations for Nesslers and Palintest methods in seawater. Faint dotted lines indicate 95% confidence limits. Graphs obtained using data from du Buisson, (2006). The expected slope ($\beta = 1$) is indicated by the red line.

2.4. Discussion

The Nesslers method was found to be more accurate than the Palintest method, although both underestimated the standard concentrations. Ammonia has been measured using the complete Indophenol blue method (Koroleff, 1983) in a number of growth studies on abalone (Basuyaux & Mathieu, 1999; Harris et al., 1998a,b; Huchette et al., 2003). However, when ammonia concentrations need to be monitored at regular intervals and when changes in ammonia can occur rapidly (within hours), the Nesslers method is preferred. The Nesslers method has a reaction time of one minute and a preparation time of ~10-15 minutes compared to the complete Indophenol blue method which has a reaction time of 24 hours (although this can be reduced to ~two hours; H. Waldron, pers. comm.). Additional benefits of the Nesslers method are that it is portable, it has a large ammonia detection range, and it is much simpler compared to the Indophenol blue and Palintest methods. The Nesslers method can be used by scientists (for toxicity testing and water analysis) as well as by abalone farmers, who are interested in a quick, easily accessible, fairly accurate and reproducible method for ammonia measurements in seawater.

The concentration of toxic unionized ammonia (FAN) is dependent on and can be calculated from measurements of TAN, pH, temperature and salinity. FAN increases with increasing pH and temperature and decreases with increasing salinity (Bower & Bidwell, 1978). As a consequence, TAN can remain constant throughout an observation period (i.e. during an experiment or during routine measurements on farms), but with changes in pH and temperature, the FAN concentrations will change (Table 2.4).

Table 2.4. Demonstration of the sensitivity of FAN to temperature, pH and TAN using a single measured value from one of the later experiments (see Chapter 3, Table 3.2 for the sublethal value observed for juvenile abalone). The hypothetical change of factors is shown in grey shading, giving rise to the resulting FAN concentration. FAN concentrations are calculated according to Bower and Bidwell (1978).

Factors influencing FAN			**Resulting FAN** ($\mu g\ l^{-1}$)
TAN ($mg\ l^{-1}$)	pH	temperature	
Sub-lethal level*			
0.56	7.8	15 °C	7.4
Increase of TAN by 0.1 $mg\ l^{-1}$			
0.66	7.8	15. °C	8.7
Increase of pH by 0.1			
0.56	7.9	15 °C	9.3
Increase of T by 1 °C			
0.56	7.8	16 °C	8.0
Increase of TAN (0.1 $mg\ l^{-1}$), pH (0.1) and T (1 °C)			
0.66	7.9	16 °C	11.2

Concentrations of FAN in the present study were calculated following Bower & Bidwell (1978) (Table 2.2). Other methods are less reliable. For example, the mole fractions of un-ionized ammonia in seawater provided by Huguenin & Colt (1989) are based on freshwater constants obtained from Emerson et al. (1975) and cover a wide salinity range (5 - 40‰). The tables provided by Trussell (1972) and Emerson et al. (1975) are not reliable for estimating FAN in seawater because the percent un-ionized ammonia at different temperatures and pH values are calculated for freshwater, omitting the effect of salinity on the ammonia-water equilibrium

(Bower & Bidwell, 1978). The main advantage of the Bower & Bidwell (1978) method is that they used a number of different salinity ranges (18 - 22‰, 23 - 27‰, 28 - 31‰ and 32 - 40‰) in constructing their tables.

Nitrites and nitrates are toxic to abalone in relatively large amounts, compared to the toxicity of un-ionized ammonia. Nitrite is toxic in concentrations > 5 $mg.l^{-1}$ (Basuyaux & Mathieu, 1999; Harris et al., 1997) and nitrate is toxic in concentrations of 100 - 250 mg l^{-1} (Basuyaux & Mathieu, 1999; Harris et al., 1997). The tests used for nitrite and nitrate analyses do not need to be very sensitive, and the Ferrous sulphate and Cadmium reduction methods, which gave precise results to 0.1 mg l^{-1} are adequate for the present study. Both these methods are quick (total preparation time = ~10 - 15minutes), use the Hach spectrophotometer (which is portable) and are reproducible, making them useful for both scientist and farmer.

The Nesslers method is appropriate for the toxicity and growth tests used in the present study, because of its wide detection range and its ease of use. Although it showed some bias underestimating TAN compared with the standards (Figure 2.4), it was more accurate than the Palintest. It was therefore the method of choice throughout the study, except in certain experiments with the farmed "cocktail" size abalone (as discussed in Chapter 4). All calculations of FAN throughout the study were based on measured TAN, pH, temperature and salinity, according to Bower & Bidwell (1978).

Chapter 3

Acute effects of ammonia in three size classes of abalone, *Haliotis midae*.

Abstract

Ammonia has been shown to be toxic to a number of aquatic invertebrates, including abalone. Toxicity levels of ammonia for South African abalone, *Haliotis midae*, have not been documented and such values for other abalone species typically do not record a range of LC values. A series of experiments is described in which toxicity of ammonia to *H. midae* was investigated. It was hypothesised (Hypothesis 1) that the tolerance of *H. midae* to ammonia should increase with increasing size, with acclimation at sub-lethal concentrations (Hypothesis 2), and in farmed as opposed to wild animals (Hypothesis 3).

Acute toxicity of ammonia to *H. midae* was investigated for three size classes relevant to mariculture operations. Tolerance to ammonia (at pH 7.8 and T = 15 °C) was found to increase with body size as indicated by 36 h LC_{50} values; farmed juvenile abalone (1 - 2.5 cm shell length) had the smallest LC_{50} of 9.8 μg l^{-1} FAN, whereas LC_{50} was 12.9 μg l^{-1} FAN in wild cocktail abalone (5 - 8 cm shell length), and 16.4 μg l^{-1} FAN in wild brood stock abalone (10 - 15 cm). When wild cocktail-size abalone were allowed to acclimatize to sub-lethal ammonia concentrations for 48 h, their ammonia tolerance increased compared with non-acclimatized abalone of the same size: LC_{50} was 2.0 μg l^{-1} FAN greater at 14.8 μg l^{-1} FAN. Farmed cocktail-size abalone demonstrated a three-fold larger 48h-LC_{50} value (of 37.9 μg l^{-1} FAN) than wild cocktail-size abalone (12.7 μg l^{-1} FAN). Abalone are susceptible to elevated ammonia concentrations, but appear to have some ability to acclimatize.

3.1. Introduction

Commercial mariculture of the South African abalone, *H. midae*, employs high density rearing in onshore systems (Sales and Britz, 2001). These systems usually operate as an open flow-through system to ensure sufficient supply of oxygen and removal of excretory products. Excretory products, however, can reach high toxic levels and can impact negatively on farm operations. This problem is exacerbated when the circulation has to be closed, for example during periods when harmful algal blooms occur in the sea (Botes et al., 2003) or when there is an increase in pH or temperature, resulting in a build-up of toxicants. One major toxicant is the nitrogenous waste, ammonia, which can result from bacterial decomposition of organic matter (faeces, unconsumed food, dead animals) (Cheng et al., 2004) and is also produced through the metabolism of the animal itself.

Ammonia is the end-product of protein catabolism in most aquatic invertebrates, such as abalone. Abalone excretes ammonia via the right kidney (Andrews, 1985) into the mantle cavity (which houses the gills) and is passed out in the exhalent currents through the holes in the shell (Fallu, 1991). Due to its small molecular size and high solubility, ammonia can diffuse rapidly through any surface in contact with water (such as the gills) and need not be excreted by the kidney (Schmidt-Nielsen, 1983). Ammonia is one of the most toxic waste products (Fallu, 1991; Schmidt-Nielsen, 1983). The other nitrogenous wastes that may accumulate, resulting in poor water quality, are nitrites and nitrates. Nitrite is an intermediate in the conversion of ammonia to nitrate (see Chapter 2, Figure 2.1) and appears to be more toxic than nitrate in *H. tuberculata* (Basuyaux & Mathieu, 1999) and the greenlip abalone, *H. laevigata* (Harris et al., 1997).

In aqueous solutions, ammonia exists in a pH-, temperature- and salinity-mediated equilibrium between the unionized (NH_3) and ionized (NH_4^+) forms, of which the unionized form is the more toxic (Russo & Thurston, 1991) (see Chapter 2, Figure 2.2). Toxicity levels of ammonia have been established for aquatic animals such as fish, crustaceans and molluscs (Colt & Armstrong, 1981; Jeney et al., 1992; Leung et al., 1999; Meade & Watts, 1995).

Ammonia has been shown to affect the immune response of Taiwan abalone, *H. diversicolor supertexta* (Cheng et al., 2004) and kidney structure in greenlip abalone, *H. laevigata* (Harris et al., 1998a), ultimately influencing the health and growth of the animals. A number of workers have investigated the influence of ammonia on the survival and growth of Australian abalone (Harris et al., 1998b; Basuyaux and Matthieu, 1999; Hindrum et al., 2001; Huchette et al., 2003), but they have tended to concentrate mostly on the growth of juveniles. Standard toxicity tests (determinations of lethal concentrations of ammonia in abalone) are often not fully presented. Such toxicity tests are well documented for fish at a wide range of temperatures and pHs (Thurston et al., 1981; Knepp & Arkin, 1973). For abalone, however, such data are scarce. In toxicity tests, LC_{50} values (mean lethal concentration killing 50 % of test animals) are used as standards for comparability (Greenberg et al., 1992). However, for the abalone farmer, it is important to know at what concentration the animals start to die. For such information, other values from the same experiments, such as LC_5 (5 % of animals die) to LC_{100} (all animals die) should be calculated. The determination of LC_5 is also important in estimating the possible safe concentrations of ammonia for abalone.

There is little information available to South African abalone farmers on ammonia toxicity; lethal- and sub-lethal concentrations, and increased survival by adaptation to sub-lethal concentrations are not known for the locally farmed species *H. midae*, but are assumed from data for foreign species. It is important that tests on ammonia toxicity in *H. midae* are conducted in order to evaluate the risks associated with ammonia and the impact ammonia has on water quality. However, there are nuances in the potential response of *H. midae* to acute ammonia toxicity. According to Fallu (1991), the ability of abalone to withstand stress increases with body size. It is hypothesised that this is true also for *H. midae*, so that assuming the same toxicity levels for abalone of different size classes can be misleading and result in losses of animals. In the present study a range of sizes has been used in experiments: farmed juveniles (1 - 2.5 cm shell length), farmed and wild cocktail size (5 - 8 cm) and wild brood stock (10 - 15 cm) (Figure 3.1). LC values of ammonia in farmed juvenile abalone were calculated to allow farmers to improve water quality of their environment, hence, increasing their survival rate. Spat and larval stages of *H. midae* were not part of the study. *H. midae* can reach a maximum size of about 20 cm shell length at an age of 30 years in the wild (Sales & Britz, 2001), but farms concentrate on the

marketable cocktail size of 5-8 cm shell length (just over three years), although recently they have aimed for a larger size of ~10 cm shell length (Macey & Coyne, 2005). To maintain a healthy brood stock (taken from the wild), the present study calculated LC values of ammonia of wild brood stock abalone ranging from 10-15 cm shell length.

It has been shown that exposure of freshwater and seawater fish to sub-lethal concentrations of ammonia increases their subsequent resistance to lethal concentrations (EIFAC, 1973). It is hypothesized that similar acclimatization processes occur in *H. midae*, so that animals exposed to sub-lethal but elevated concentrations should increase their tolerance, implying also that farmed animals should have greater tolerance than wild animals. This chapter investigated the acute effects of ammonia on *H. midae*. It aimed to test three hypotheses. The first was that LC values of ammonia should increase with size; this was tested using three size classes of *H. midae*: juveniles, cocktail size and brood stock. The second hypothesis was that ammonia tolerance should increase after acclimatization to sublethal concentrations of ammonia; this was tested using cocktail size *H. midae*. The third hypothesis was that ammonia tolerance should be greater in farmed than in wild abalone; this was tested by comparing LC values of ammonia for wild and farmed cocktail size *H. midae*.

3.3. Materials and Methods

The first phase of the present study was initiated in 1998 and used farmed juvenile, wild cocktail size and wild brood stock size abalone for toxicity tests. At that time, farmed abalone of the two latter size classes were not available because of high costs and the large numbers required for the experiments, and because the farms were newly established (less than five years old) with limited stocks. Acclimatization experiments were conducted using wild cocktail size. *H. midae*. The second phase of the present study was carried out in 2005 when a commercial farm donated farmed cocktail size *H. midae*. Toxicity tests were carried out on these abalone, allowing for a comparative study on the effect of ammonia on wild versus farmed cocktail size abalone.

3.3.1 Experimental animals

In total, 180 juvenile abalone (1.0 - 2.5 cm shell length; ~1 year old) (Figure 3.1a) were provided by a commercial abalone farm (Aquafarms) in Hermanus on the southwest coast of South Africa. Another 180 cocktail abalone (5.0 – 8.0 cm shell length) (Figure 3.1b) were donated by another commercial abalone farm (Irvin and Johnson Ltd) at Danger Point in Gansbaai on the southwest coast of South Africa. Two other size classes of abalone (cocktail and brood stock size (10.0 – 15.0 cm shell length)) (brood stock, Figure 3.1c) were collected from the sea in Gansbaai and termed wild abalone. The farmed juveniles derived from brood stock that had been previously collected from the sea off the southeast coast of South Africa, within 50 km of the area where the two other size classes were caught (A. Hattingh, Aquafarms, Hermanus, pers. comm.).

All experiments were conducted in the Zoology Department at the University of Cape Town, South Africa. The farmed abalone had been reared on the farms with a mixture of diets which included *Ecklonia maxima*, seaweeds (*Gracillaria* and *Ulva*) and Abfeed (a commercial abalone feed) (L. Schoonbee, I & J Farms, pers. comm.). One month prior to experiments, the farmed abalone were fed exclusively on *E. maxima*, their predominant food in their natural habitat (Branch et al., 1994). The wild abalone were also fed exclusively on *E. maxima* during the one month holding period. All abalone were held in holding tanks with a closed-circulation system that used sand-filtered seawater. The abalone were kept in the holding tanks for a minimum period of three weeks and a maximum period of one month. The system contained aerated and continuously flowing natural seawater at 14 – 16 °C. This temperature range is well within the optimal range of 12 – 20 °C for this species (Britz et al., 1997; Sales & Britz, 2000). Each animal was only used once and was not included in any further experimentation.

Figure 3.1. Photos illustrating the three size classes of *H. midae* investigated in the toxicity experiments. a) juveniles, b) cocktail and c) brood stock. (Courtesy of Irvin and Johnson Abalone Farms).

3.3.2. Experimental design and protocol

The experiments were carried out in a constant environment (CE) room (humidity = 50% and ambient temperature = 15 °C). Natural daylight conditions were achieved using fluorescent lighting. An 11 hour light (L):13 hour dark (D) photoperiod was chosen instead of the 16L:8D suggested by Greenberg et al. (1992), to simulate the 11 hour photoperiod observed on the farms during the winter season (June-August), the same time of the experiments. Round pots were used as experimental tanks (Edwards, 2003) (to allow for the even distribution of oxygen) and were coated with epoxy paint (to prevent fluctuations in pH of the water). Clay pots were used to simulate the natural substratum of abalone (Figure 3.2). Abalone were easily removed from the pots, by inserting a thin plastic card underneath the foot (Harris et al., 1999) without the use of anaesthetics (Hindrum et al., 2001). Abalone were gently scrubbed with a small, soft nail brush (Lu et al., 1999) to remove any organisms and foreign particles that could cause changes in ammonia concentrations. Water levels in each pot were kept to a maximum and mesh lids (Figure

3.2) were tied down to prevent 'crawl-outs' observed in another study on *H. midae* (Britz et al., 1997). Each tank was individually aerated using air stones.

Mean (± s.d.) dissolved oxygen (7.90 ± 0.07 ppm; range: 7.68 – 8.20 ppm) was monitored using a dissolved oxygen meter (YSI DO200, ± 0.01) (Figure 3.2). Temperature (15 °C) and mean pH (7.8 ± 0.04; range 7.78 – 7.85) were monitored using a pH meter (YSI pH100, temperature ± 0.1 °C and pH ± 0.01) (Figure 3.2) and calibrated with Crison buffers. The pH was maintained using reagent grade HCL and NaOH pellets. Mean salinity (34.6 ± 0.1 ppt) was monitored with a salinity meter (YSI EC300, ± 0.1 ppt). The baseline readings of the seawater used (T = 15 °C, pH = ~7.8, salinity = ~35 ppt) are listed in Table 3.1. There were no measurable differences in the above parameters between individual tanks. There were no measurable differences in the concentrations of TAN and FAN among individual holding tanks, because of the small numbers of animals kept and the large volumes of re-circulated water, especially compared with differences observed on farms as a result of high stocking densities and commercial feed.

Table 3.1. Baseline readings of seawater used (T = 15 °C, pH = ~7.8, salinity = ~35 ppt).

	Nitrogen compounds	**Mean (±s.d) concentration (range)**
Before experiment:	TAN (mg l^{-1})	0.35 ± 0.02 (0.33 - 0.38)
	FAN (µg l^{-1})	4.65 ± 0.29 (4.36 - 5.02)
	Nitrite (mg l^{-1})	0.53 ± 0.13 (0.39 - 0.7)
	Nitrate (mg l^{-1})	1.54 ± 0.29 (1.3 - 1.9)
During experiment:	Nitrite (mg l^{-1})	0.89 ± 0.20 (0.37 - 1.02)
	Nitrate (mg l^{-1})	1.90 ± 0.40 (1.1 - 2.5)

Figure 3.2. Experimental set up of an individual tank (clay pot) in the constant environment (CE) room. The seawater in the tank is aerated and dissolved oxygen, pH and temperature are monitored with appropriate meters.

Range-finding acute toxicity tests were carried out for a wide range of ammonia concentrations, using two abalone per concentration tested (Greenberg et al., 1992). These tests were used to determine the approximate concentration range to be included in the definitive acute toxicity experiments. The definitive acute toxicity experiments were carried out using standard toxicity tests as outlined by Greenberg et al. (1992). The experiment consisted of one control pot (no toxicant added) and two pots containing the toxicant at the same concentration (Figure 3.3). Reagent grade ammonium chloride was used as the toxicant (Thurston et al., 1981, Barimo & Walsh, 2005). Ten abalone were gently scrubbed, placed into each pot and fed the day prior to the experiments to minimize handling stress at the start of the experiments. The pots containing the abalone remained in the aquarium as part of the continuous flow system and were transported to the CE (constant environment) room just before the experiments. Abalone in each pot were exposed to specific ammonia concentrations for 48 hours. The 48-hour exposure incorporated both the inactive (day) and active (night) phases of *H. midae* (Barkai & Griffiths, 1987). Inclusion of the active (night) phase, when more water is taken up via the gills as a result of the

increased demand of oxygen, could cause an increase in the absorption of the toxin (ammonia) across the gills. Different animals were used for each ammonia concentration. The treatments were applied in concentration sequence from lowest concentration to highest concentration. Mortality was recorded every two hours for the first 12 hours and then at 24, 36 and 48 hours. Animals that started gaping or showed loss of tenacity (holding grip) by gentle touching were considered dead or unlikely to survive (Drew et al., 2001). A final mortality test was performed on the abalone whereby an abalone that lost tenacity or started gaping was placed on the vertical surface of the tank and allowed 15 seconds to attach; if it failed to attach it was considered dead. Equal amounts of kelp were weighed and placed in each tank during the night as *H. midae*, like other abalone, is a nocturnal grazer (Barkai & Griffiths, 1987). This ensured that starvation did not act as an additional stressor (Greenberg et al., 1992).

Figure 3.3. Toxicity experiments consisted of one control pot (no toxicant added) and two experimental pots (toxicant added). The pots containing the abalone remained in the aquarium as part of the continuous flow system (above) and were transported to the CE room just before the experiments.

In one experiment, some animals were pre-acclimatized to sub-lethal concentrations of ammonia. The experiment consisted of one control pot (no toxicant added) and two pots containing the toxicant. Ten animals were placed in each pot and the animals were allowed to pre-acclimatize to an ammonia concentration of 0.76 mg TAN l^{-1} (10.03 µg FAN l^{-1}) for 48 hours. These pre-acclimatised animals were then tested for each ammonia concentration, and mortality was determined as described above. Experimental concentrations of FAN in all experiments were calculated using TAN, pH, temperature and salinity values following Bower & Bidwell (1978) (see Chapter 2, section 2.2.3.3) and adjusted using reagent grade ammonium chloride. Dissolved ammonia concentrations were determined by the traditional Nesslers method (see Chapter 2, section 2.2.3.1). However, the Palintest (see Chapter 2, section 2.2.3.2) was used to determine dissolved ammonia for the first two concentrations of the toxicity tests for farmed cocktail size

abalone as the mineral stabiliser required for the Nesslers method was not available at the time. The ammonia concentrations, measured every 2 hours for the first 12 hours, and thereafter every 12 hours until the end of the experiment (48 hours), did not fluctuate outside the range stated and adjustments were not necessary. LC values were recorded for a wide range of ammonia concentrations, however, only the concentrations which caused mortalities plus the highest concentration that did not cause any deaths are presented in Tables 3.2 – 3.6. Nitrite and nitrate concentrations were determined by ferrous sulphate and cadmium reduction methods respectively (both these methods are described in Chapter 2, sections 2.2.1 and 2.2.2, respectively), and measured every 12 hours.

3.3.3. Statistical analysis

Mortality rates from acute toxicity tests were analysed by probit analysis (Cramer, 2004; Greenberg et al., 1992; Wardlaw, 1995). This involves transformation of the percentage mortality values into probit values, and subsequent fitting of the (now linearised) sigmoid response curve and estimation of LC values. The LC values were calculated using Microsoft XLSTAT 7.5.3-Dose, as were 95% confidence limits. Linearized best-fit curves were constructed from data obtained from probit analysis of 36 h and 48 h, and LC_{50} values were compared using one-way ANOVA, and a post-hoc Bonferroni test (Figure 3.4) or a log rank test (Figures 3.5 and 3.6). Both these post-hoc tests were performed using GraphPad Prism (statistical analysis for laboratory and clinical research).

3.4. Results

3.4.1. LC values of ammonia for farmed juvenile abalone

A FAN concentration of 8.7 $\mu g\ l^{-1}$ was the lowest ammonia level that caused mortality and the experimental concentration below that level (7.4 $\mu g\ l^{-1}$ FAN) was regarded, therefore, as the sub-lethal level (Table 3.2). LC_{50} values range from 9.3 to 10.2 $\mu g\ l^{-1}$ FAN, depending on exposure time. The LC_5 of 8.8 $\mu g\ l^{-1}$ FAN and LC_{100} of 11.4 $\mu g\ l^{-1}$ FAN were estimated from probit analysis for the exposure time of 36 h.

Table 3.2. Toxicity of different ammonia concentrations to juvenile abalone. Lower case letters a and b indicate parallel experiments (n = 10 each) under same conditions. TAN and FAN concentrations are given as median with concentration ranges in brackets.

TAN	FAN	Number of dead abalone at:																	
mg l^{-1}	µg l^{-1}	2 h		4 h		6 h		8 h		10 h		12 h		24 h		36 h		48 h	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
0.56 (0.51-0.60)	7.4 (6.73-7.92)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.66 (0.61-0.70)	8.7 (8.05-9.24)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
0.76 (0.71-0.80)	10.0 (9.37-10.56)	0	0	1	2	1	2	3	3	4	3	5	6	5	6	6	6	6	6
0.86 (0.81-0.90)	11.4 (10.69-11.88)	3	2	5	5	6	7	9	8	10	10	10	10	10	10	10	10	10	10
0.96 (0.91-1.00)	12.7 (12.01-13.2)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
LC_{50} (FAN; µg l^{-1}):												10.2		10.2		9.8		9.3	
95% confidence limits:												9.8-10.6		9.8-10.6		9.4-10.1		8.9-9.7	

3.4.2. LC values of ammonia for wild cocktail size abalone

A FAN concentration of 11.4 µg l^{-1} was the lowest ammonia level that caused mortality and 10.0 µg l^{-1} FAN was regarded as the sub-lethal level for this size class (Table 3.3). LC_{50} values range from 12.7 to 13.9 µg l^{-1} FAN. The LC_5 is 11.2 µg l^{-1} FAN and LC_{100} level is approximately 15.8 µg l^{-1} FAN for 36 h of exposure.

Table 3.3. Toxicity of different ammonia concentrations to wild cocktail size abalone. Lower case letters a and b indicate parallel experiments (n = 10 each) under same conditions. TAN and FAN concentrations are given as median with concentration ranges in brackets.

TAN	FAN	Number of dead abalone at:																	
mg l^{-1}	µg l^{-1}	2 h		4 h		6 h		8 h		10 h		12 h		24 h		36 h		48 h	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
0.76 (0.71-0.80)	10.0 (9.37-10.56)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.86 (0.81-0.90)	11.4 (10.69-11.88)	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2	2	1
0.96 (0.91-1.00)	12.7 (12.01-13.2)	0	0	0	0	1	2	1	0	1	2	2	2	2	3	3	3	4	6
1.06 (1.01-1.10)	14.0 (13.32-14.52)	0	0	0	1	2	2	3	4	4	6	5	7	6	7	9	9	9	10
1.16 (1.11-1.20)	15.3 (14.65-15.84)	7	8	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
LC_{50} (FAN; µg l^{-1}):												13.9		13.7		12.9		12.7	
95% confidence limits:												13.5-14.3		13.3-14.1		12.5-13.3		12.3-13.1	

3.4.3. LC values of ammonia for wild brood stock size abalone

The sub-lethal level of FAN was 12.7 µg l^{-1}, the first mortalities occurred at a concentration of 14.0 µg l^{-1} (Table 3.4). LC_{50} values range from 16.2 to 17.5 µg l^{-1} FAN. The LC_5 is 14.2 µg l^{-1} FAN and LC_{100} level is approximately 18.9 µg l^{-1} FAN for 36 h of exposure.

Table 3.4. Toxicity of different ammonia concentrations to wild brood stock abalone. Lower case letters a and b indicate parallel experiments (n = 10 each) under same conditions. TAN and FAN concentrations are given as median with concentration ranges in brackets.

TAN	FAN	Number of dead abalone at:																	
mg l^{-1}	µg l^{-1}	2 h		4 h		6 h		8 h		10 h		12 h		24 h		36 h		48 h	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
0.96 (0.91-1.00)	12.7 (12.01-13.2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.06 (1.01-1.10)	14.0 (13.32-14.52)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
1.16 (1.11-1.20)	15.3 (14.65-15.84)	0	0	0	0	1	0	1	0	1	0	2	1	2	1	2	2	2	2
1.26 (1.21-1.30)	16.6 (15.97-17.16)	0	0	0	1	1	1	2	3	2	3	3	4	5	5	6	5	6	6
1.36 (1.31-1.40)	18.0 (17.29-18.48)	0	0	2	3	2	3	5	4	6	6	7	8	8	8	8	8	9	8
1.46 (1.41-1.50)	19.3 (18.60-19.8)	4	3	7	6	9	10	9	10	10	10	10	10	10	10	10	10	10	10
LC_{50} (FAN; µg l^{-1}):												17.5		16.6		16.4		16.2	
95% confidence limits:												17.1-17.9		16.2-17.0		16.0-16.9		15.8-16.6	

3.4.4. LC values of ammonia for farmed cocktail size abalone

Two different methods were used to measure TAN concentrations in Table 3.5 (see Section 3.3.2). The first two concentrations were measured using the Palintest and the last three concentrations were measured using Nesslers method. This is the reason why the second concentration (27.09 µg l^{-1}) measured was higher than the third concentration (24.72 µg l^{-1}). A comparative analysis of both methods (see Chapter 2, section 2.3.2) showed that the Nesslers method was more accurate than the Palintest in measuring dissolved ammonia in seawater. No mortalities were recorded for the first two concentrations (Table 3.5). A FAN concentration of 24.72 µg l^{-1} was the lowest ammonia level that caused mortality and 19.69 µg l^{-1} FAN was regarded as the sub-lethal level for this size class (Table 3.5). An LC_{50} value of 37.9 µg l^{-1} FAN for 48 h was obtained. The LC_5 is approximately 24.72 µg l^{-1} FAN and LC_{100} level is approximately 49.87 µg l^{-1} FAN for 48 h of exposure (Table 3.5). There were insufficient data points to obtain the LC_{50} for the 12, 24 and 36 h exposure.

Table 3.5. Toxicity of different ammonia concentrations to farmed cocktail size abalone (5.0 – 8.0 cm shell length). Lower case letters a and b indicate parallel experiments (n = 10 each) under same conditions. TAN and FAN concentrations are given as median with concentration ranges in brackets. (Data obtained from du Buisson, 2005).

TAN	FAN	Number of dead abalone at:																	
mg l^{-1}	µg l^{-1}	2 h		4 h		6 h		8 h		10 h		12 h		24 h		36 h		48 h	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
1.03 (0.80-1.14)	19.69(14.12-27.07)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.32 (0.87-1.60)	27.09(18.07-33.04)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.13 (1.01-1.30)	24.72(20.86-29.26)	0	0	0	0	0	0	0	0	1	0	1	0	2	0	3	0	3	0
1.81 (1.61-2.00)	38.44(27.15-50.83)	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	4	3
2.40 (2.20-2.92)	49.87(39.80-60.42)	0	0	0	0	0	0	0	0	0	0	0	0	1	0	7	7	9	10
LC_{50} (FAN; µg l^{-1}):																		37.9	
95% confidence limits:																		34.7-42.4	

3.4.5. Acclimatization of wild cocktail size

This treatment lowered the sensitivity to ammonia in the acute toxicity test when compared with animals of the same size that were not pre-acclimatized (see Table 3.3). The sub-lethal level of FAN was 12.7 µg l^{-1}, and the first mortalities occurred at a concentration of 14.0 µg l^{-1} (Table 3.6). LC_{50} values range from 14.4 to 18.2 µg l^{-1} FAN. The LC_5 is 13.5 µg l^{-1} FAN and LC_{100} level is approximately 16.8 µg l^{-1} FAN for 36 h of exposure

Table 3.6. Toxicity of different ammonia concentrations to wild cocktail size abalone previously acclimatized to sublethal concentrations of ammonia. Abalone were allowed to acclimatize in a sub-lethal ammonia concentration (TAN = 0.76 mg l^{-1}, FAN = 10.0 µg l^{-1}) for 48 h prior to experimentation. Lower case letters a and b indicate parallel experiments (n = 7-10 each) under same conditions. TAN and FAN concentrations are given as median with concentration ranges in brackets.

TAN	FAN	Number of dead abalone at:																	
mg l^{-1}	µg l^{-1}	2 h		4 h		6 h		8 h		10 h		12 h		24 h		36 h		48 h	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
0.96 (0.91-1.00)	12.7 (12.01-13.2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.06 (1.01-1.10)	14.0 (13.32-14.52)	0	0	0	0	0	0	0	0	0	0	1	1	2	1	2	2	2	3
1.16 (1.11-1.20)	15.3 (14.65-15.84)	0	0	0	1	2	1	2	2	2	2	4	4	4	5	4	6	8	7
LC_{50} (FAN; µg l^{-1}):												15.3		15.0		14.8		14.4	
95% confidence limits:												15.0-15.8		14.6-15.4		14.4-15.2		14.0-14.8	

3.4.6. Comparison of LC values of ammonia for three size classes of abalone

Best-fit curves, constructed from the 36 h probit data, for all three size classes were significantly different from each other, as were LC values (ANOVA, F = 0.497, df = 39; post-hoc Bonferroni test, $p < 0.05$, df = 128) (Figure 3.4). Farmed juveniles were the most sensitive to ammonia in the environment. Wild abalone of cocktail size were less sensitive to ammonia than juveniles. Wild abalone of brood stock size were the size group that was least sensitive to ammonia in their environment.

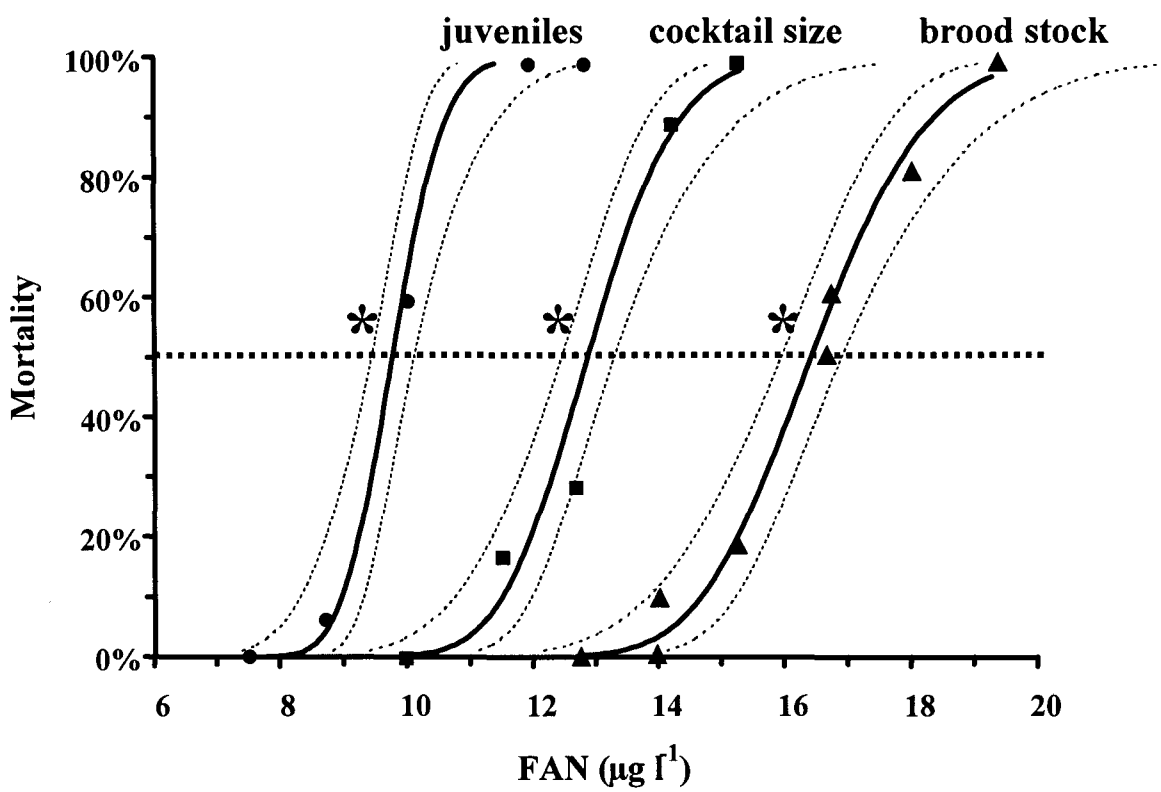

Figure 3.4. Comparison of ammonia tolerance of juvenile abalone (1.0 – 2.5 cm shell length), cocktail-size abalone (5.0 – 8.0 cm) and brood stock abalone (10.0 – 15.0 cm) in a 36 h acute toxicity test. Curves were constructed using data from Tables 3.2-3.4 and probit analyses thereof. Faint dotted lines indicate respective 95% confidence limits. Goodness of fit of curves for juvenile abalone: $X^2 = 0.368$, $R^2 = 0.999$; cocktail-size abalone $X^2 = 3.386$, $R^2 = 0.986$ and brood stock abalone $X^2 = 1.307$, $R^2 = 0.998$. *Curves are significantly different from each other (Bonferroni test, df = 39, $p < 0.05$).

3.4.7. Comparison of LC values of ammonia for acclimatized versus non-acclimatized wild cocktail size abalone

Best-fit curves for non-acclimatized and acclimatized wild cocktail size abalone, constructed from the 36 h probit data, were significantly different, as were the LC values (log rank test, $p < 0.05$, $df = 1$) (Figure 3.5).

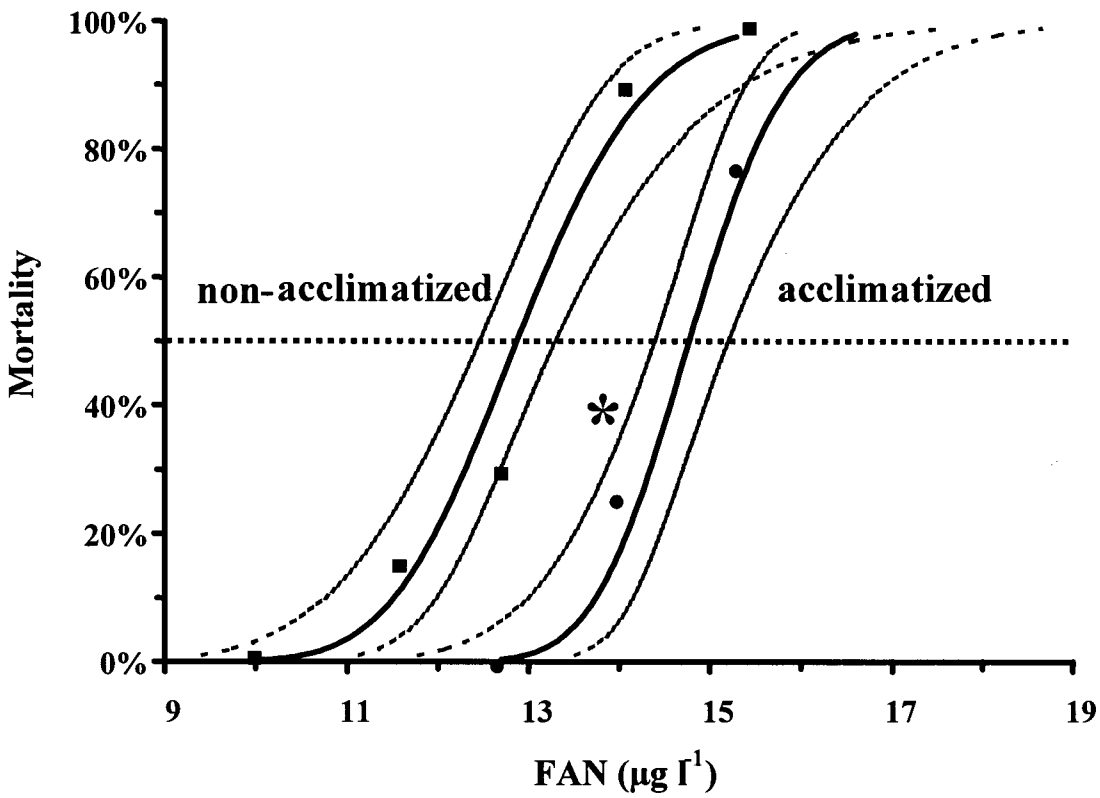

Figure 3.5. Comparison of ammonia tolerance of wild cocktail-size abalone that were acclimatized or not acclimatized to a sub-lethal concentration of ammonia for 48 h prior to experimentation in a 36 h acute toxicity test. Curves were constructed using data from Table 3.3 and Table 3.6 and probit analyses thereof. Faint dotted lines indicate respective 95% confidence limits. Goodness of fit of curves for non-acclimatized abalone: $X^2 = 3.386$, $R^2 = 0.986$ and acclimatized abalone $X^2 = 0.828$, $R^2 = 0.996$. *Curves are significantly different from each other (log rank test, $df = 1$, $p < 0.05$).

3.4.8. Comparison of LC values of ammonia for farmed versus wild cocktail size abalone.

Best-fit curves for farmed and wild cocktail size abalone, constructed from the 48 h probit data, were significantly different, as were the LC values (Log rank test, $p = 0.001$, $df = 1$) (Figure 3.6). Farmed abalone of cocktail size were less sensitive to ammonia than wild cocktail size abalone. There were insufficient data points for the farmed cocktail size abalone to obtain the curve for the 36 h exposure (see Table 3.5).

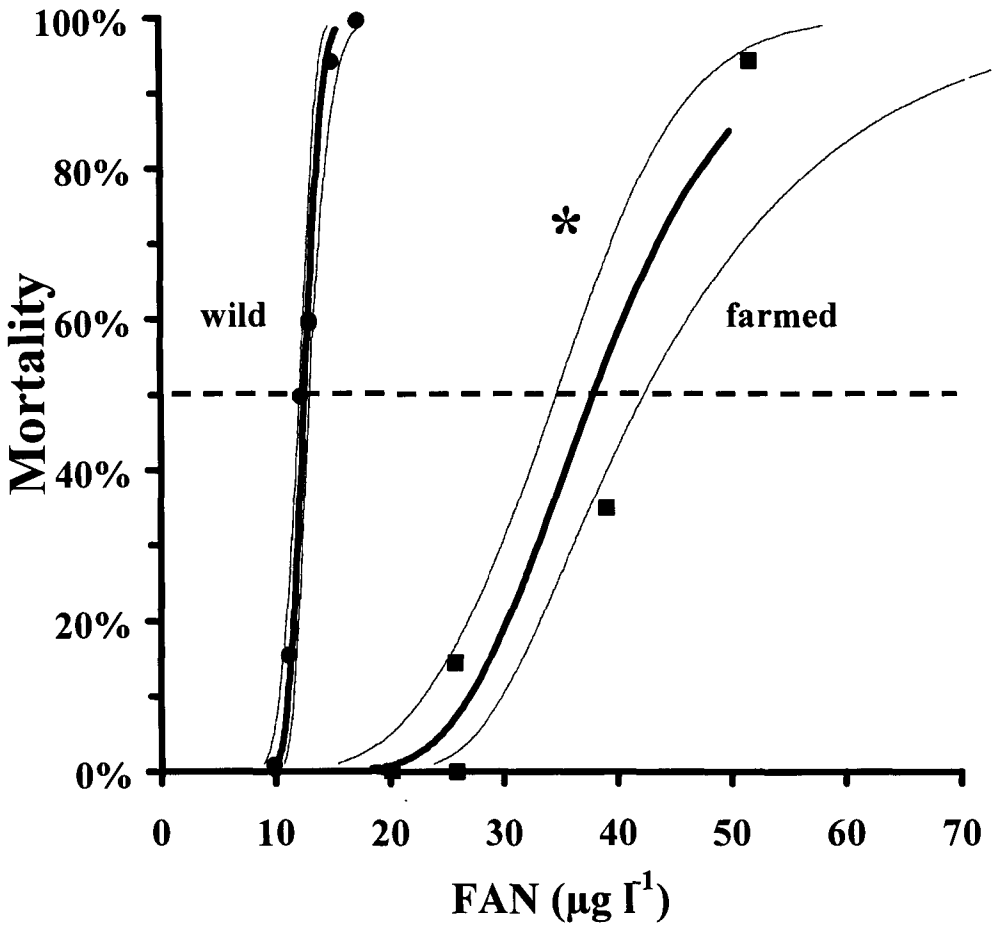

Figure 3.6. Comparison of ammonia tolerance of wild cocktail size abalone versus farmed cocktail size abalone in a 48 h acute toxicity test. Curves were constructed using data from Table 3.3. and Table 3.5. and probit analyses thereof. Faint dotted lines indicate respective 95% confidence limits. Goodness of fit of curves for wild cocktail size abalone $X^2 = 4.544$, $R^2 = 0.978$ and farmed cocktail size abalone $X^2 = 21.768$, $R^2 = 0.919$. *Curves are significantly different from each other (log rank test, $df = 1$, $p<0.001$).

3.5. Discussion

In general, the ability of abalone to withstand stress increases with size (Fallu, 1991). This is consistent with our data on *H. midae* as there is an overall increase in the LC values of ammonia with increasing size of the abalone (Tables 3.2 - 3.4). Smaller and younger abalone are, therefore, the most critical size class because they have low tolerance to elevated ammonia concentrations in the surrounding water. The size-dependence of sensitivity to ammonia is illustrated in the dose-response curves for the three size classes used (Figure 3.4), based on the results of the probit analysis of the 36 h values (see Tables 3.2 - 3.4). The 36 h curve was chosen because 1) it is best supported by the amount of measured data in all size classes and is, therefore, most comparable, and 2) it represents a more acute response than the 48 h curve. The shift of the curves to the right (i.e. towards higher ammonia concentrations) indicates an increased ammonia tolerance (Figure 3.4). Juvenile abalone exhibit faster growth than the other size classes used. They also have a larger surface area relative to body volume. It is not surprising, therefore, that this size class was most sensitive to ammonia in the environment.

Both nitrite and nitrate concentrations in the present study (Table 3.1) were substantially lower than the maximum safe concentrations (nitrite: > 5 mg l^{-1}; nitrate: 100-250 mg l^{-1}) recorded for *H. tuberculata* (Basuyaux & Mathieu, 1999) and the chronic sublethal level (nitrite = 7.8 mg l^{-1}) recorded for the greenlip abalone, *H. laevigata* (Harris et al., 1997). There are limited data available on the toxicity of both nitrites and nitrates to *H. midae*, but we can assume that nitrite and nitrate had negligible influence on the stress of the abalone in the present study.

As has been found previously for fish (EIFAC, 1973), acclimatization of *H. midae* to sub-lethal concentrations of ammonia increased their subsequent resistance. The shift of sensitivity is illustrated in the dose-response curves (Figure 3.5) that were constructed from the results of the probit analysis of the 36 h values (Table 3.3 and Table 3.6) for cocktail size abalone: the shift to the right (i.e. towards higher ammonia concentrations) indicates an increase in ammonia tolerance (Figure 3.5) for acclimatized abalone. Possible mechanisms that lead to the increased tolerance (acclimatization) to ammonia in abalone, *H. midae*, will be discussed in Chapter 5.

Farmed abalone are exposed to much higher concentrations of ammonia compared to wild abalone because of ammonia build-up in tanks resulting from enclosure and high densities. TAN values that had been obtained from seven abalone farms in South Africa ranged from 0.015-0.675 mg l^{-1} while the TAN value of unpolluted seawater rarely exceeded 0.18 mg l^{-1} (Samsukal, 2004). Higher values of TAN on farms have been observed (D. Robertson-Andersson, pers. comm.) but the values have not been recorded or the farmers are not willing to disclose them. During the present study, the seawater (no toxicant added) readings ranged from 0.035 -0.40 mg l^{-1} TAN. Therefore, it is reasonable to hypothesise that wild abalone will be more sensitive to ammonia than farmed abalone of the same size as a result of farmed abalone acclimatizing to higher ammonia levels. The present study supports the above hypothesis. The shift of sensitivity is illustrated in the dose-response curves (Figure 3.6) that were constructed from the results of the probit analysis of the 48 h values (Table 3.3 and Table 3.5) for farmed and wild cocktail size abalone. The LC_{50} values of the farmed abalone (37.9 µg l^{-1} FAN) are approximately three times greater than those for wild abalone (12.7 µg l^{-1} FAN) (Table 3.7), but they are substantially lower than the lethal concentrations for most fish, crustaceans, and echinodermata (Table 3.8). In general fish excrete much higher levels of nitrogen componds (ammonia, nitrites and nitrates) than molluscs, including abalone (*H. diversicolor*) (Qian et al., 2001) and subsequently acclimatize to the higher ammonia levels in the tanks which may explain why abalone are more sensitive to ammonia than fish. A more even distribution of mortalities for farmed cocktail size abalone (as observed for wild cocktail size abalone) (Table 3.3 and Table 3.5) may be achieved if more experiments at higher ammonia concentrations were conducted. Unfortunately the number of available farmed abalone was limited.

Table 3.7. Summary of the LC values for wild and farmed abalone for all three size classes emphasized in the present study.

Size class	*Origin*	*FAN (µg l^{-1})*		
		sublethal	LC_{50}	
			36 hours	48 hours
juvenile	farmed	7.4	9.8	
cocktail	farmed	19.69		37.9
cocktail	wild	10.0	12.9	12.7
brood stock	wild	12.7	16.4	

In conclusion, ammonia can be toxic to the South African abalone, *H. midae*, but this sensitivity decreases with size. Abalone can adapt to sub-lethal concentrations of ammonia, and such acclimatization reduces their ammonia sensitivity. From results of acute ammonia toxicity tests (LC_5 = 8.8 µg l-1 FAN) with juvenile abalone (the most vulnerable size class), we suggest a maximum FAN level for *H. midae* of 7.4 µg l^{-1} (no mortalities). However, this concentration of 7.4 µg l^{-1} FAN has been shown to reduce growth in juvenile abalone, *H. midae*. This is the topic of Chapter 4.

Table 3.8. Acute and chronic effects of ammonia on various aquatic organisms, including *H.midae* (present study). (See abbreviation list on IX for abbreviations used in table)

Source	*Species (common name)*	*N*	*Life stage (size)*	*Acute/Chronic (duration)*	*Applied factors*	*FANμg.l⁻¹*	*TANmg.l⁻¹*
Abalone							
Harris et al. 1998a	*H. laevigata* (greenlip abalone)	953	Juvenile (31.8 ± 0.10 mm)	Chronic (95 days)	EC_5	41	-
					EC_{50}	158	-
Basuyaux &Mathieu 1999	*H. tuberculata* (ormer)	150	Juvenile (36.5 ± 1.90 mm)	Chronic (15 days)	Safe level	45	1
					EC_{50}	452	10
					Slight toxicity	226	5
Huchette et al., 2003	*H. rubra* (blacklip abalone)	1800	Juvenile (15.0 ± 65.0 mm)	Chronic (5 months)	EC_5	4	0.7-1.51
Cheng et al., 2004	*H. diversicolor supertexta* (Taiwan abalone)	50	Juvenile (33.6 ± 9.60 mm)	Acute (96 hour)	EC_{50} (ImR)	122.34	2.55
Present study	*H. midae* (perlemoen) (wild)	150	Cocktail (50 - 80 mm)	Acute (36 hour)	LC_{50}	12.9	0.97
				Acute (48 hours)	LC_{50}	12.7	0.96
		180	Juvenile (10 - 25 mm)	Chronic (90 days)	EC_{50}	7.4	0.56
Present study (Buisson, 2005)	*H. midae* (perlemoen) (farmed)	150	Cocktail (71.32 ± 4.24 mm)	Acute (48 hours)	LC_{50}	37.96	1.81
Echinodermata							
Basuyaux & Mathieu, 1999	*Paracentrotus lividus* (sea urchin)	300	5.67 ± 1.5 g	Chronic (15 days)	Safe level	45	1
Siikavuopio et al., 2004	*Strongylocentrotus droebachiensis* (green sea urchin)	264	Adult (58.2 ± 15.2)	Chronic (42 days)	NOEC	16	-
					LC	32-68	-
Crustaceans							
Chin & Chen, 1987	Penaeus monodon (tiger prawn)	-	postlarvae	Acute (96 hours)	Safe level	10	-
				Acute (72 hours)	LC_{50}	1540	17.05
				Acute (96 hours)	LC_{50}	1040	11.51
Meade & Watts, 1995	*Cerax quadricarinatus* (Australian crayfish)	-	Juveniles (9 - 13 mm)	Acute (24 hours)	LC_{50}	2020	94.3
				Acute(48 hours)	LC_{50}	1630	76.3
				Acute (96 hours)	LC_{50}	980	45.9
Kir et al., 2004	*Penaeus semisulcatus* (penaeid shrimps)	-	Juveniles (1.6 ± 0.2 g)	Acute(96 hours)	LC_{50} (at 14°C)	1920	55.84
					LC_{50} (at 22°C)	1800	26.72
Fish							
Knepp & Arkin, 1973	*Ictalarus punctatus* (channel catfish)	80		~7 days	LC_{50}	-	37.5
				~8 days	LC_{100}	-	45.7
Thurston et al., 1981	*Salmo gairdneri* (Rainbow trout)	-	Fingerlings (4 - 6 months)	Acute(96 hours)	LC_{50}	320-810	-
Ruyet et al., 1995	*Dicentrarchus labrax* (seabass)	-	Juveniles (6-163 g)	Acute(96 hours)	LC_{50}	1700	40
	Sparus aurata (seabream)	-	Juveniles (6-163 g)	Acute(96 hours)	LC_{50}	2500-2600	57-59
	Scophthalmus maximus (turbot)	-	Juveniles (6-163 g)	Acute(96 hours)	LC_{50}	2500-2600	57-59

Chapter 4

Chronic effects of ammonia on the growth of juvenile abalone, *Haliotis midae*.

Abstract

Ammonia has been shown to retard growth in a number of abalone species. The long term impacts of sub-lethal ammonia concentrations were investigated for growth of farmed juveniles (1–2.4 cm shell length). It was hypothesised (Hypothesis 4) that elevated but sublethal ammonia concentrations would have a chronic effect on *H. midae* by retarding growth. Growth was inhibited during chronic exposure to sub-lethal FAN (7.4 $\mu g\ l^{-1}$). The mean ± s.d. specific growth rates (0.10 ± 0.03 % d^{-1}) were reduced (ANOVA: $F = 3.102$, $df = 114$, $p < 0.05$) to 59 % of that in a control group (no added ammonia) (0.24 ± 0.06 % g d^{-1}).

4.1. Introduction

Abalone is one of the most valuable seafood species in the world. Its demand, especially in Asia, outstrips supply by far (Gordon & Cook, 2004; Stanford, 2004). This has ensured high prices but also a decline in natural resources of abalone by over-fishing, disease, habitat loss and poaching (Gordon & Cook, 2004). As a result of these developments, mariculture has become a viable supplier, despite the slow growth and resulting long culture periods of abalone. It is important to understand the factors that affect abalone growth in order to develop optimal conditions for maximising production (Huchette et al., 2003). There are numerous factors that affect the growth of juvenile abalone, such as stocking density (Huchette et al., 2003, Koike et al., 1979), parasite infestation (Ruck & Cook, 1998), depth (Liu and Chen, 1999), food quality and quantity (Britz, 1996, Day and Flemming, 1992) and water quality. Water quality is the single most important factor affecting an abalone (Fallu, 1991). Water is not only a source of essential substances but acts as a waste disposal system. Hence, poor water quality resulting from toxic substances (such as ammonia and nitrites) or insufficient quantities of essential substances (such as dissolved oxygen) may retard growth and may cause death in abalone (Fallu, 1991). Low dissolved oxygen has been shown to affect growth in juvenile Australian greenlip abalone, *H. laevigata* (Harris et al., 1999).

Little information is available from long-term toxicity tests with ammonia and aquatic organisms, and fewer data are available from partial chronic or chronic studies (Rand & Petrocelli, 1985). Ammonia is the principal nitrogenous compound excreted by aquatic animals (Colt & Armstrong, 1981) and is known to have chronic effects on the growth of a number of marine organisms, namely, fish (Foss et al., 2004; Lenmarie et al., 2004, Lukenbach et al., 2003), sea urchins (Siikavuopia et al., 2004); crustaceans (Allan et al., 1990; Koo et al., 2005) and abalone (Harris et al., 1998a, b; Huchette et al., 2003). Chronic exposure to ammonia may result in the deterioration of several physiological functions, any one of which may be the ultimate cause of death (Russo, 1985). Ammonia has been shown to affect the immune response of Taiwan abalone, *H. diversicolor*

supertexta (Cheng et al., 2004), and kidney structure in greenlip abalone, *H. laevigata* (Harris et al., 1998a), which will ultimately influence growth.

The influence of ammonia on the survival and growth of abalone has been investigated mainly for juvenile Australian species (Harris et al., 1998b; Hindrum et al., 2001; Huchette et al., 2003). In these studies ammonia is shown to significantly affect growth in abalone, except in the study by Hindrum et al. (2001) (see discussion). There is limited information available to South African abalone farmers on the influence of ammonia on growth of the local species, *H. midae*, but it is expected it will retard growth, as has been found for the Australian species. The present study aimed to test this by investigating the influence of high sub-lethal levels of ammonia on the growth of juvenile *H. midae* (1-2.5cm in length, ~ 1 year old). Juvenile abalone were selected for the growth experiments because they are the most vulnerable size class (Fallu, 1991), exhibit fast growth (Shepherd et al., 1995), are the least costly size class and require least logistical effort (i.e. water volume) because of their small size. Information on sublethal ammonia concentration for juveniles was drawn from results of the toxicity tests (see Chapter 3).

4.2. Materials and Methods

4.2.1. Experimental animals

A total of 180 juvenile abalone (1.0 - 2.5cm shell length; ~1 year old) was provided by a commercial abalone farm (Aquafarms) in Hermanus on the southwest coast of South Africa. The study was conducted in the Zoology Department at the University of Cape Town, South Africa. One month prior to experiments, juveniles were fed on a daily diet of fresh kelp, *Ecklonia maxima*, their predominant food in their natural habitat (Branch et al., 1994). The abalone were held in holding tanks with a closed-circulation system that used sand-filtered seawater. The system contained aerated and continuously flowing natural seawater at 14 – 16 °C. This temperature range is well within the optimal range of 12 – 20 °C for this species (Britz et al., 1997; Sales & Britz, 2000).

4.2.2. Experimental design and protocol

The abalone were transferred to their experimental glass tanks (21.5x21.5x21.5cm) a week prior to the experiments on 7 July 1999, in order to minimise handling stress. The juvenile abalone were easily removed by sliding them off the glass without the use of a spatula/plastic card (Harris et al., 1999) or anaesthetics (Hindrum et al., 2001). Abalone were gently scrubbed with a small, soft nail brush (Lu et al., 1999) to remove any organisms and foreign particles that could cause changes in ammonia levels. All juveniles were individually labelled with numbered plastic 'Dymo' tags attached to their shells with quick-setting epoxy glue (Britz, 1996).

Twenty abalone were placed in each of six glass tanks. Two additional abalone were placed in each tank to replace any injured or dead abalone. Water levels in each tank were kept to a maximum and mesh lids were tied down to prevent 'crawl-outs' observed in another study on *H. midae* (Britz et al., 1997). Each tank was individually aerated using air stones. Mean (± SD) dissolved oxygen (7.90 ± 0.07 ppm; range:7.68 – 8.20 ppm) was measured using a dissolved oxygen meter (YSI DO200, ± 0.01) (Figure 3.2). Temperature (15 °C) and mean pH (7.8 ± 0.04; range 7.78 – 7.85) were measured using a pH meter (YSI pH100, temperature ± 0.1 °C and pH ± 0.01) and calibrated with Crison buffers. The pH 7.8 was maintained using reagent grade HCL and NaOH pellets. Salinity (34.6 ± 0.1 ppt) was measured with a salinity meter (YSI EC300, ± 0.1 ppt). The baseline readings of the seawater used (T = 15 °C, pH = ~7.8, salinity = ~35 ppt) are listed in Table 3.1 (Chapter 3). There were no measurable differences in the above parameters between the three control tanks and the three experimental tanks.

Reagent grade ammonium chloride was used as the toxicant (Thurston et al., 1981; Barimo & Walsh, 2005). In three of the six tanks, ammonia concentrations were adjusted to a sub-lethal level (FAN: 7.4 $\mu g\ l^{-1}$; TAN: 0.56 $mg\ l^{-1}$) by adding appropriate amounts of the toxicant (experimental tanks) whereas no ammonium chloride was added to the seawater in the three control tanks. Dissolved ammonia concentrations were maintained by periodically (every second day) replacing 50% of the water in the tanks (Schmitt &

Uglow, 1996). TAN was measured using the Nesslers method with the Hach DR/2000 spectrophotometer (see Chapter 2, section 2.2.3.1) and FAN was calculated (see Chapter 2, section 2.2.3.3). The weight (wet whole weight), shell length and shell width of each abalone was measured once every two weeks over a three month period. The abalone were weighed to the nearest 0.01g and their length measured with vernier callipers to 0.1mm. Equal amounts of kelp were weighed and placed in each tank during the night as *H. midae*, like other abalone, is a nocturnal grazer (Barkai & Griffiths, 1987). This ensured that starvation did not act as an additional stressor (Greenberg et al., 1992).

4.2.3. Calculation of specific growth rates.

Specific growth rates for weight (SGRW), shell length (SGRL) and shell width (SRWSW) (% d^{-1}) were calculated according to Hindrum et al. (2001) as follows:

$$\text{SGRW } (\% \text{ d}^{-1}) = \frac{[\ln(\text{final weight}) - \ln(\text{initial weight})] \times 100}{\text{number of days}}$$

$$\text{SGRL } (\% \text{ d}^{-1}) = \frac{[\ln(\text{final length}) - \ln(\text{initial length})] \times 100}{\text{number of days}}$$

$$\text{SGRSW } (\% \text{ d}^{-1}) = \frac{[\ln(\text{final width}) - \ln(\text{initial width})] \times 100}{\text{number of days}}$$

4.2.4. Statistical analysis

Differences in initial body sizes of animals assigned to the experimental groups of the growth experiment were tested by one-way ANOVA after testing homogeneity of variances by Levene's test. Specific growth rates of groups were tested by ANOVA followed by post-hoc Bonferroni tests. Combined changes for each treatment were analysed by repeated measures ANOVA followed by a post-hoc Bonferroni test.

4.3. Results

4.3.1. Influence of sub-lethal ammonia concentrations on the growth of juvenile abalone.

Initial values for weight, shell length and shell width of each of the six experimental groups were not significantly different (ANOVA, $p<0.05$, $df = 5$ for wet weights, shell width and shell length, and $F= 1.881$, 0.462 and 0.576, respectively.). Data for the full 90 days of experimental treatment were used to calculate the specific growth rates (SGR) for all experimental groups. The SGRs for weight (Fig.4.1A), shell length (Fig.4.1B) and shell width (Fig.4.1C) were significantly lower in abalone that were kept in sub-lethal ammonia levels compared with abalone from the control groups. In total, the SGR of ammonia-exposed abalone was 59 % (weight), 51 % (shell length) and 58 % (shell width) of those of control abalone. The results from animals in groups that were exposed to the same treatment (i.e. ammonia or control) were not significantly different (ANOVA, $p<0.05$, for ammonia treatment groups: $df = 2$ for wet weights, shell width and shell length, and $F = 0.592$, 2.678 and 2.485, respectively; for control groups: $df = 2$ for wet weights, shell width and shell length, and $F = 0.670$, 2.718 and 1.665, respectively) and were combined for further analysis.

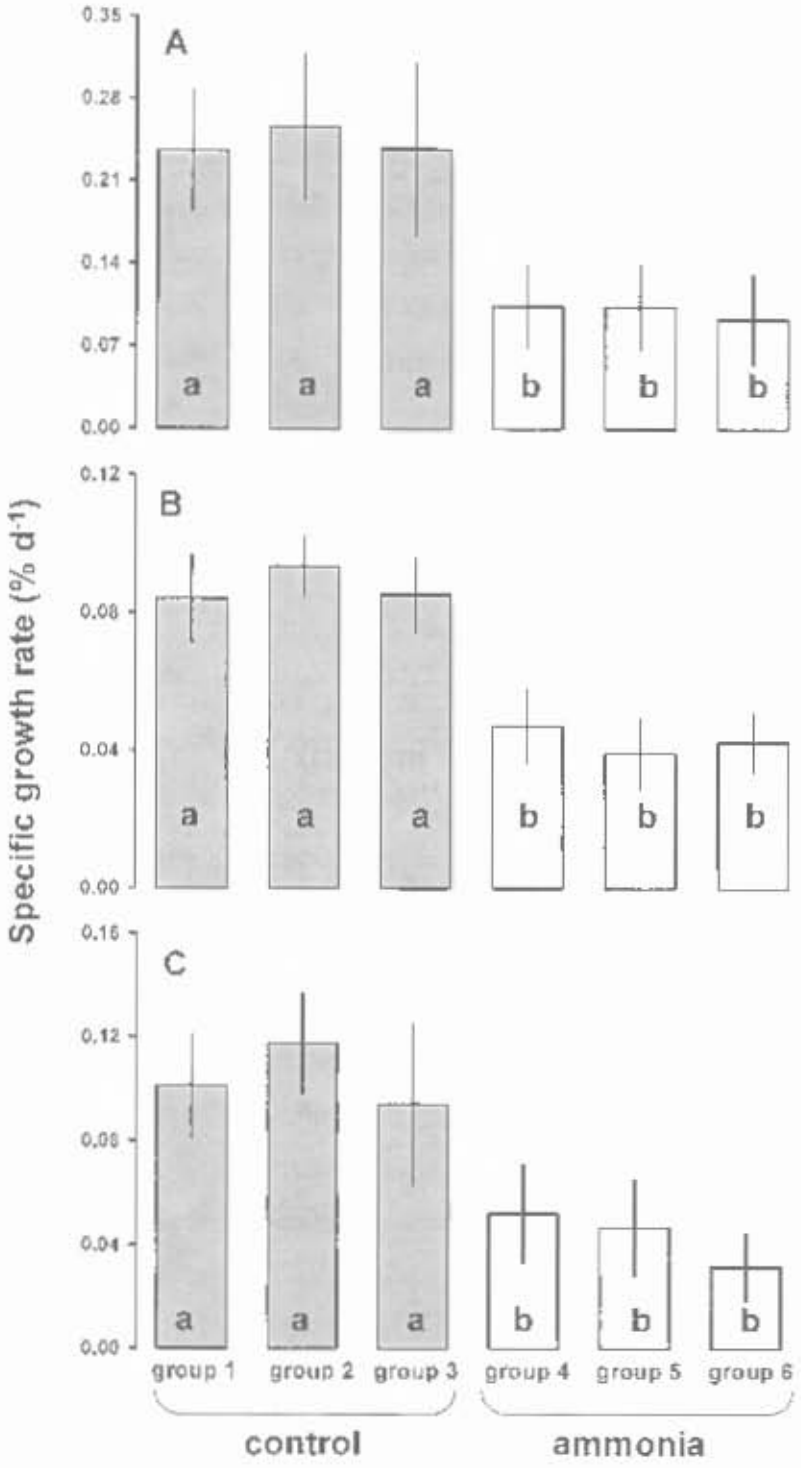

Figure 4.1. Specific growth rates for weight (A), shell length (B) and shell width (C) of six groups of juvenile abalone that were reared for 90 days in either ammonia-free seawater (grey bars) or seawater that contained 7.4 µg l^{-1} FAN (white bars). Data are presented as means ± SD, n = 20 for each group. Significance of difference between groups ($p<0.05$) was calculated using ANOVA (post-hoc Bonferroni test: df = 114 for weight, shell length and shell width and F = 3.102, F = 1.589 and 3.228, respectively). Identical lower-case letters indicate no statistical difference.

The mean (+ s.d.) changes in size at each time step (Figure 4.2 A – C) were significantly suppressed after only 14 days of exposure to sub-lethal ammonia concentrations when compared with data from animals of the control (no added ammonia).

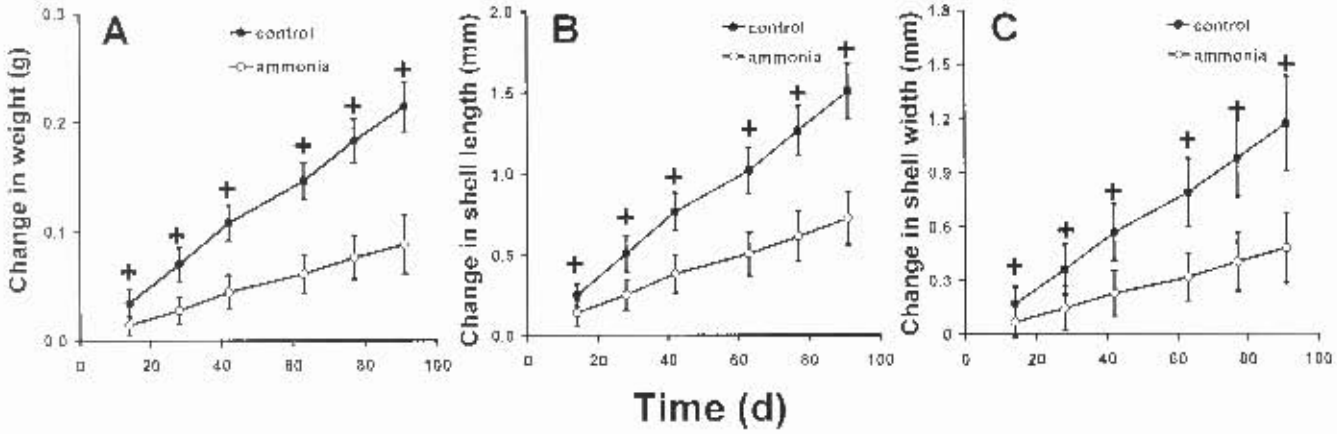

Figure 4.2. Influence of ammonia level on growth of juvenile abalone (n=60): change in fresh weight (A), shell length (B) and shell width (C) compared with initial values in abalone reared in ammonia-free seawater (control) or seawater containing 7.4 $\mu g\ l^{-1}$ FAN. Significance of growth differences (+) ($p<0.05$) between treatments was tested using repeated measures ANOVA (post-hoc Bonferroni test: df = 113 for width, shell length and shell width and F = 153.7, 109.1 and F = 51.43, respectively).

4.4. Discussion

A good understanding of a species response to environmental factors is a prerequisite in achieving high revenue in a high-density land-based culture system (Siikavuopio et al., 2004). This chapter presents results on the chronic effects of ammonia on juvenile *H. midae* and supplements the acute toxicity tests performed on juvenile *H. midae* (see Chapter 3). Specific growth rates are used to indicate relative changes in growth rates (Figure 4.1), especially because the experimental abalone grew slower than is found on farms. However, to illustrate the patterns of growth over time, absolute growth increments were also presented (Figure 4.2). Although abalone appear to be tolerant to sub-lethal ammonia concentrations, this seems to be a costly effort (for the animal as well as for the farmer). During their long stay under such sub-lethal ammonia levels, no fatalities occurred in the experiments, but growth rates were substantially reduced (by more than 50 %). It is therefore important to not only maintain ammonia concentrations below toxic levels in the long term, but to keep them as low as possible to ensure optimal growth.

Colt and Armstrong (1981) predicted significant growth reductions in most aquatic animals between 0.05-0.2 mg FAN l^{-1}. Basuyaux & Mathieu (1999) measured differences in shell length and weight for five ammonia concentrations over a 15 day period and suggested that the safe level of ammonia for abalone, *H. tuberculata,* is 1 mg TAN l^{-1} (0.045 mg FAN l^{-1}) with slight toxicity at 5 mg TAN l^{-1} (0.226 mg FAN l^{-1}). Harris et al. (1998b) found that growth depression by ammonia was greater in the Australian greenlip abalone than in some other aquatic animals and significant reduction in length and weight occurred at 0.054 mg FAN l^{-1} and 0.110 mg FAN l^{-1}, respectively. These values were all much higher than the sub-lethal value of 0.0074 mg FAN l^{-1} used in the present study and may be due to the larger juveniles used in these studies (see Table 3.8).

In growth studies EC_x values are used which refer to the effective concentration causing x percent growth retardation. A comparison of the chronic effects of ammonia on abalone growth (EC_x values) in the above mentioned studies and in the present study are

summarized in Table 3.8 (see Chapter 3). Huchette et al. (2003) showed that in the Australian blacklip abalone (1-2 year old), growth was significantly reduced by ammonia and that the EC_5 (the effective concentration causing 5% growth retardation) value was 10-fold lower than the one found by Harris et al. (1998b), where the EC_5 = 0.041 mg FAN l^{-1} for 3 year old abalone. EC_5 values of 0.004-0.006 mg FAN l^{-1} are suggested by Huchette et al. (2003).

In the present study, the level of chronic ammonia exposure causing specific growth rates for weight, shell length and shell width respectively of 59, 51 and 58 % of controls, was at 0.0074 mg FAN l^{-1}, consistent with the findings of Huchette et al. (2003). The EC_5 values might be slightly lower, however, for *H. midae* than their Australian counterparts. This could be due to a higher sensitivity of *H. midae* to ammonia. A more likely reason is that ammonia levels were better controlled in the present study compared with naturally-produced ammonia in the experiments of Huchette et al. (2003). The EC values reported by Huchette et al. (2003) were tenfold lower compared with those of Harris et al. (1998b) and Basuyaux & Mathieu (1999). The authors attributed this to species sensitivity and, to a larger extent, to the small sample size and short term duration of experiments carried out by the latter research groups. Day & Fleming (1992) suggested that the effect of chronic exposure is more likely observed in long-term experiments, especially when the main biological parameter is growth. Since abalone are known to exhibit slow and variable growth, the duration of the growth studies should be long to observe any significant effects (Huchette et al., 2003).

Despite the differences in the EC values of ammonia, all of the above studies showed that high sub-lethal levels of ammonia retard growth. However, one particular study by Hindrum et al. (2001) found that in the Australian blacklip abalone, ammonia exposure (ranging from 25 - 188 µg FAN l^{-1}) did not produce a significant reduction in growth. The authors suggested that this was due to the pulse and challenge exposures in their experiments for only 8 hours (compared to the other studies of continuous exposure). In addition, the result could have been further impacted by the fact that the experiments

were conducted during daytime (inactive phase) when gill activity might have slowed down causing reduction in the transfer of ammonia across the gills.

Both nitrite and nitrate levels in the present study were substantially lower than the safe levels (nitrite: > 5 $mg.L^{-1}$; nitrate: 100-250 $mg.L^{-1}$) recorded for *H. tuberculata* (Basuyaux & Mathieu, 1999) and the chronic sublethal level (nitrite = 7.8 mg. $L^{-1)}$ recorded for the greenlip abalone, *H. laevigata* (Harris et al., 1997). As there are limited data available on the toxicity of both nitrites and nitrates for *H. midae*, we can assume that nitrite and nitrate had no influence on the stress of the abalone in the present study.

In conclusion, sub-lethal levels of ammonia caused a substantial reduction in growth of juvenile *H. midae*. The results showed that it is essential for animal health and viability of abalone farming that ammonia levels are kept at the lowest possible level.

Chapter 5

Stress proteins and P-glycoprotein – indicators of ammonia stress in *Haliotis midae*.

Abstract

Two possible protein induction indicators of stress have been identified in aquatic organisms: heat shock proteins (HSPs; stress proteins) and the P- glycoprotein (P-gp) mediating multixenobiotic resistance (MXR). HSPs are known to confer tolerance by folding and refolding of partially denatured proteins while P-gp reduces toxins in the system by actively pumping them out of the cell. Neither of these responses has yet been demonstrated in ammonia-stressed abalone.

It was hypothesised (Hypothesis 5) that increase of HSPs and/or P-gp mediated MXR are responsible for the observed increased tolerance to ammonia in *H. midae*. Four HSPs (-90, -70, -60 and -27) and the P-gp were investigated in the foot and gills of *H. midae* using immunological assays (using stress protein-specific antibodies). Induction of HSPs and P-gp by heat shock, a well known stressor of HSPs but not P-gp, was also investigated. It was demonstrated for *H. midae* that heat shock had the greatest impact in the foot (increase in HSP 90 and -70, $P < 0.05$) within the first hour, with no significant changes in the gills. In contrast, ammonia exposure had the greatest impact on the gills after 24 hours (increase in HSP 70 and -60, $P < 0.05$ and $P< 0.001$ respectively) and after 36 hours ($P < 0.01$ and $P < 0.001$ respectively). There were no significant differences of HSP 70 and -60 levels in the foot during ammonia exposure, but there appeared to be an observed increase during longer exposure for HSP 70 (after 24 and 36 hours) and HSP 60 (36 hours). HSP 90 was not present in the foot and gills of *H. midae* under normal (control) conditions and was only present in the foot after heat shock and in the gills after ammonia shock. HSP 27 was not present in the foot and gills under normal conditions as well as during ammonia exposure but was present in the foot and gills after 24 hour heat exposure.

The presence and increase of a ~257 kDa protein (cross reacting with the C219 antibody) in the gills after ammonia exposure but not after heat shock, indicates that the P-gp might be present and play a role in ammonia tolerance in *H. midae*. It appears that HSPs 90, -70

and -60 are involved in conferring temperature as well as ammonia tolerance, whereas HSP -27 is solely up-regulated during heat stress and P-gp during ammonia exposure.

5.1. Introduction

Ammonia has been shown to negatively affect *H. midae* (see Chapters 3 & 4). This chapter examines two possible responses/indicators of ammonia stress in *H. midae*: the so-called stress proteins or heat shock proteins (HSPs) and the P-glycoprotein (P-gp) mediating multixenobiotic resistance (MXR). HSPs are part of a general cellular response, protecting and repairing partly denatured proteins (Nover & Scharf, 1997) while the P-gp mediated MXR helps pump out toxins from the cells (Feder & Hoffmann, 1999; Smital & Kurelec, 1998). It has been suggested that most organisms when exposed to elevated temperatures, respond by synthesising a group of highly conserved proteins (HSPs) (Lindquist, 1981; Sanders, 1988). HSPs are found in organisms as diverse as bacteria, molluscs and humans (Burdon, 1982; Sanders, 1993). HSP induction by heat is found in almost all organisms except in the freshwater *Hydra oligactis* (Bosch et al., 1988). The cellular stress response to severe heat shock was first reported in *Drosophila* in 1962 by Ritossa (Ritossa, 1962; Sanders, 1993), hence the term heat shock response. HSPs act as molecular chaperones, promoting the initial folding of other proteins at the ribosome and the refolding of unfolded proteins when they are partially denatured (Nover & Scharf, 1997). HSPs are also present in cells under non-stress (normal) conditions. The HSP response is involved in protecting organisms from damage resulting from a wide variety of stressors (Sanders, 1993). HSPs are activated not only by heat but also by other physiological stresses. Ammonium chloride is an example of an agent that activates HSP genes (Ananthan et al., 1986). One of the HSP family, HSP 70 (described below) is induced by ammonia in the advanced early life stages of the brown trout (*Salmon trutta* f. *fairo* L.) (Luckenbach et al., 2003). In Chapters 3 and 4, ammonium chloride (NH_4CL) was the toxicant used.

The HSPs are designated into families according to their molecular weight and sequence homology (Nover & Scharf, 1997). Many organisms produce multiple classes of stress proteins but there are five general families of HSPs: HSP 110 (100-110 kDa), HSP 90 (83-90 kDa), HSP 70 (68-72 kDa), HSP 60 (60 kDa, also referred to as GroEL or chaperonin) , and a low molecular weight series of HSPs (ubiquitin-7kDa; 15-40 kDa).

The major characterised stress proteins are HSP 90, 70 and 60. Below are important findings summarized for each HSP family, highlighting characteristic features and functions:-

The HSP 110 family includes proteins with molecular weights ranging from 100 to110 kDa. HSP 110 is located in the nucleus and nucleolus in vertebrates (Nover, 1991) and has only been characterized in detail in mammalian cells (Lindquist & Craig, 1988). It is perhaps the least characterized family of HSPs (Black & Subjeck, 1991).

The HSP 90 family includes molecular weights ranging from 83 to 90 kDa. HSP 90 is a very highly conserved HSP with 50% amino-acid identity in the most distantly related eukaryotes, all having greater than 40% identity with the *E.coli* protein (Lindquist & Craig, 1988). This family is found mostly in the cytoplasm with one group (94) located in the endoplasmic reticulum (Black & Subjeck, 1991). Under normal conditions, HSP90 controls many cellular activities by binding to target proteins (such as enzymes, hormone receptors, and cytoskeleton components), creating an inactive or unassembled complex (Gething & Sambrook, 1992). Under stress conditions, HSP 90 may redirect cellular metabolism to enhance tolerance (Sanders, 1993). The carboxyl terminal of these proteins is the most divergent, whereas the terminal four amino acids of HSP 90, EEVD, are more conserved, which is also true for HSP 70 (Lindquist & Craig, 1988).

The HSP 70 family includes molecular weights ranging from 68 to 72 kDa. The 70 kilodalton HSPs are the most highly conserved proteins known with greater than 50 % sequence homology between *Drosophila*, bacteria, yeast and mouse (Sanders, 1988). They are localized in the cytosol, mitochondria and endoplasmic reticulum (www.hytest.fi/high_lights9.php). The HSP 70 family are also synthesized in non-stressed cells and have diverse functions such as folding, assembly, intracellular localization, secretion, regulation and protein degradation (Feder & Hoffmann, 1999). Under adverse stress, HSP 70 prevents the formation of insoluble aggregates that may otherwise damage cells and HSP 70 can also break up these aggregates and aid in the refolding of damaged proteins to obtain their original biological activity (Sanders, 1993).

Identical amino acid sequences TVPAYFNDS*QRQATKDA were found in the HSP 70 gene from organisms as diverse as humans, *Drosophila* and yeast (Dunlap & Matsumura, 1997). HSP 70 is a prominent protein in so-called HeLa cells particularly during heat shock (Lindquist, 1981) and heat shocked HeLa cell lysate (LYC-HL101) was therefore, used as positive control in the protein analysis.

The HSP 60 chaperonin family has a molecular weight of 60 kDa. Members of this family are mitochondrial HSPs and have homology to the dominant heat shock-induced bacterial groEL protein (Black & Subjeck, 1991; Nover, 1991) and therefore HSP 60 is also referred to as GroEL. HSP 60 is also found in the chloroplasts of eukaryotes (Ellis, 1990). Members of this family in eukaryotes are required for normal mitochondrial functions in terms of importing proteins and folding them into proper functional conformations (Ryan et al., 1997). Under normal conditions, HSP 60 binds incompletely folded proteins, directing them to the correct conformation and also preventing aggregations of incompletely folded proteins (Sanders, 1993). Under adverse stress, the HSP 60 binds to damaged proteins helping them to attain their original conformation but unlike HSP 70, they are unable to break up aggregates (Sanders, 1993).

The low molecular weight (LMW) HSPs range from 15-40 kDa in molecular weight. All eukaryotes seem to have at least one HSP with a molecular weight between 15 and 40 kDa, however, the number of these proteins vary greatly between organisms (Lindquist & Craig, 1988) making them the most divergent of the HSPs with little homology between species (Black & Subjeck, 1991). The low molecular weight HSPs are of interest as they could indicate to be inter-species differences. Despite differences in size, the small HSPs are clearly related to one another in that they all have a conserved sequence near the C-terminus in addition to conserved amino acids at various positions (Nagao et al., 1996). These LMW HSPs are related in that one can identify small regions of identical amino acid sequences such as the X-X-G-X-L-X-X-X-X-P-X found near the carboxy terminus (Lindquist & Craig, 1988). There is some evidence to suggest that these LMW proteins are involved in thermotolerance but heat is not the only inducer of LMW proteins (Black

and Subjeck, 1991). Members belonging to the third subfamily with molecular weights of 21-24 kDa are localized in the chloroplasts (Nover, 1991).

Extensive work has been conducted on HSPs in aquatic organisms, namely, in fish (Sanders et al., 1994), sea urchins (Nemer et al., 1991; Roccheri et al., 1981; Sanders & Martin., 1994) and crayfish (Rochelle et al., 1991; Xue & Grossfeld, 1993). Most work on HSPs in molluscs has been conducted using mussels (Sanders, 1988; Sanders et al., 1994; Veldhuizen-Tsoerkan et al., 1991), chitons (Schill et al., 2002) and limpets (Sanders et al., 1991). Recently, work has been conducted by Drew et al. (2001) on the identification of expressed HSP 70 in the Australian blacklip abalone, *Haliotis rubra*, during heat (increased temperature by 2°C/day until 25°C) and salinity stresses. Drew et al. (2001) were unable to show the expression of HSP 70 following hypo-salinity shock, but found increased levels of HSP 70 in the foot after heat stress. Snyder et al. (2001) looked at HSP 70 and HSP 90 in the Californian abalone, *Haliotis rufescens*, in response to thermal and chemical (pentachlorophenol-environmental toxins - a problem experienced in Californian waters) stress. They found that HSP 70 in the foot was not affected significantly by chemical exposure, except one day after return to control conditions, but HSP 90 was significantly higher following chemical exposure. Snyder et al. (2001) also found that HSP 70 and 90 were not significantly affected by heat shock (one hour at 37 °C), but HSP 74 increased significantly two hours after return to control conditions. In addition to assessing whether *H. midae* responded in a similar way to other *Haliotis* species by increasing HSP 70 and HSP 90 concentrations during stress, this study also aimed to investigate HSP 60 and HSP 27 during sub lethal ammonia exposure, as suggested by Sanders (1993).

Investigation by several groups demonstrated that HSPs may not be the only indicators of xenobiotic stress. The multixenobiotic resistance (MXR) found in many aquatic organisms acts as an initial defence system against xenobiotics (Epel, 1998; Smital et al., 2000). The definition of xenobiotic is "chemical substances that are foreign to the biological system, including naturally occurring compounds, drugs, environmental agents, carcinogens, insecticides, etc" (www.medical.webends.com). A more detailed

definition of xenobiotic is "ammonia, metabolic waste, drugs, alcohol, and chemicals including enterotoxins (potentially toxic chemicals endogenously generated by gut bacteria), exotoxins (ingested, inhaled and absorbed chemicals) and endobiotics (intermediate/end products of normal metabolism/enzymolysis etc)" (www.liverdoctor.com). Generally, xenobiotics are regarded as being toxic to the organism.

Many aquatic organisms can thrive and reproduce in polluted waters (Smital & Kurelec, 1998) suggesting that they possess a well equipped defence mechanism against xenobiotics. One particular response in aquatic organisms to xenobiotic exposure, is to reduce the toxin in the system by the multixenobiotic resistance (MXR) mechanism (Eufemia & Epel, 2000) which is similar to the MDR (multidrug resistance) mechanism. The MDR mechanism was first reported by Dano in 1973 whereby resistant tumor cell lines actively pumped out daunomycin while the sensitive parent cell line failed to pump out the drug (Eufemia & Epel, 2000), hence MDR. Kurelec (1992) named this protection in aquatic organisms as MXR. Further studies showed that the presence of an ATP-dependent membrane P-glycoprotein (P-gp) (170 kDa) is responsible for this MXR phenomenon (Eufemia & Epel, 2000, Smital & Kurelec, 1998), by pumping xenobiotics out of the cell (Kurelec et al., 1996). Other molecular weights have been reported for P-gp and this has been addressed in detail in the discussion. This protein and members of its family (ABC-proteins) have been found in almost all organisms investigated (Higgins, 1992). A remarkable feature of the P-gp is its broad substrate recognition (Smital et al., 2004) and therefore possibly recognises ammonia as a substrate. The potential of this mechanism to prevent nuclear damage in aquatic organisms emphasises its importance (Waldmann et al., 1995). The first report of the P-glycoprotein-mediated line of defence against a toxin in aquatic animals was in the freshwater mussel, *Anodonta cygnea* (Kurelec & Pivcevic, 1989). The MXR mechanism have subsequently been demonstrated in a number of marine and freshwater organisms, for example in fish (Doi et al., 2001, Luckenbach et al, 2003), several mussel species (Eufemia & Epel, 2000; Hamer et al, 2004; Minier et al., 2000; Smital et al, 2000), limpets (Smital et al, 2000), clams (Archard

et al., 2004; Legeay et al, 2005) and oysters (Minier et al., 1993) but not in abalone (see Table 5.1).

Table 5.1. Summary of studies using antibodies to detect HSPs and/or P-gp in aquatic organisms.

Marine organism	Stress	HSP 90	HSP 70	HSP 60	HSP 27	P-gp C219	Positive Control	Authors
Vertebrates								
1: channel catfish, *Ictalurus punctatus*	dietary contaminants/ xenobiotics					murine monoclonal C.2 ug/ml no ref Signet Lab. Dedham, MA		Doi, et al., 2001
2: Brown Trout (*Salmo trutta f. fario*) brackish, freshwater & seawater	xenobiotics ammonia (NH4Cl)(25ug.l-1) PCP & PAH		mouse & human Hsp70 Dianova GmbH, Germany				none	Luckenbach, et al., 2003
Molluscs- General								
1: mussel, *M.californianus* (gill tissue)	p-gp substrates- pesticides PCP & chlorthal non-substrates- DDE & sodium arsenite					monoclonal antibody Signet Lab, Dedham, MA		Eufemia & Epel, 2000
	heat shock 20°C & 25°C		HSP Ab 7.10 Affinity Bioreagents					
2: mussel *M. galloprovincialis*	temperature					anti-hamster Pgp C219 monoclonal ab (Centocor Diagnostics, Malvern,PA) unavailable	gill protein (authors)	Minier, et al., 2000
			bovine brain Hsp70 (Sigma H-5147)				clam protein (authors)	
3: mussel *M. galloprovincialis* gill tissue	urban and industrial pollution Salinity= no. sig. diff.		bovine brain Hsp70 (Sigma H-5147)					Hamer, et al., 2004
4: Asiatic clam *Corbicula fluminea* freshwater gills	metal pollution (Cd & Zn)					mouse monoclonal Signet Lab, Dedham, MA		Achard, et al., 2004
5: Asiatic clam *Corbicula fluminea* freshwater gills	cadmium contamination					monoclonal antibody Signet Lab, Dedham, MA		Tregoy, et al., 2005
Molluscs- Abalone								
1: *H.rubra* (Australian)	temperature 20°C salinity		goat α-human Hsp70 polyclonal ab(Santa Cruz)				none, MWM only	Drew, et al., 2001
2: *H. rufescens* (California) plus *M. Galloprovincialis* - mussel	temperature 30°C Pentachlorophenol (PCP)	monoclonal α-Hsp 90 SPA-830 (StressGen)	monoclonal α-Hsp 70 SPA-822 (StressGen)	polyclonal α-Hsp 60 SPA-805 (StressGen)			human Hs60, 70 & 90 (StressGen)	Snyder, et al., 2001
3: *H.midae* (South African)	temperature 25°C ammonia	monoclonal α-Hsp 90 SPA-830 (StressGen)	monoclonal α-Hsp 70 SPA-822 (StressGen)	polyclonal α-Hsp 60 SPA-805 (StressGen)	monoclonal α-Hsp 27 SPA-800 (StressGen)	monoclonal antibody Signet Lab, Dedham, MA	Hella Cell Lysate (LYC HL101) (StressGen)	Present study

Surprisingly, the MXR mechanism, believed to be induced by toxic substances, was found also to be induced by heat shock in gills of the mussel, *Mytilus californianus* (Eufemia & Epel, 2000). However, Minier et al. (2000) were not able to establish a direct role for temperature on MXR protein induction. They suggested that parameters such as temperature influence on micro-organisms, or phytoplankton growth, may influence the presence of natural toxins that will induce MXR proteins.

There are various approaches to analysing stress proteins, such as immunological assay (electrophoresis, immunoblotting, and antibody detection), mass spectrophotometry, centrifugation, chromatography, and protein sequencing (www.rci.rutgers.edu/molbiol). The immunological assay (using stress protein-specific antibodies) of both HSPs and P-gp was the method of choice in the present study as it is robust, relatively simple and appears to be most commonly used (Clayton et al., 2000, Hamer et al., 2004; Minier et al., 2000). This method requires that the desired protein be semi-purified. Visualization of the protein bands after immunoblotting (see section 5.2.3.4) can be achieved in many ways, including colorimetric detection (the addition of a substrate that reacts with the enzyme conjugated to the secondary antibody resulting in a coloured compound - see section 5.2.3.4), chemiluminescence, radioactive detection and fluorescent detection (http://en.wikipedia.org/wiki/Western_blot). Colorimetric detection was used in the present study as it is relatively simple, frequently used (Clayton et al., 2000, Hamer et al., 2004; Minier et al., 2000) and the results are reproducible.

This chapter investigates HSP and P-gp induction in both foot and gills of *H. midae* during both heat shock (most commonly known physical stressor) and ammonia exposure. It was assumed that the organs most likely to show high levels of HSPs and/or P-gp during ammonia exposure are the gills, where water exchange takes place (Fallu, 1991) and where ammonia is most likely to enter. P-gp in gills has been widely documented in a number of studies on molluscs including the Californian mussel *Mytilus californianus* (Eufemia & Epel, 2000; Smital et al., 2004), the mussel *Mytilus galloprovincialis* (Kurelec et al., 1996) and the clam, *Corbicula fluminea* (Legeay, 2005). Once ammonia enters the abalone via the gills, it could affect the rest of the organs, but it

was assumed that ammonia (determined by the presence of HSPs) would have a lesser/delayed effect in the foot of abalone. For both organs (foot and gills), the effect of temperature was also investigated.

It has been shown that certain chemicals differ in their ability to induce stress proteins among species. This may result from differences in the physiology of the species such as differences in absorption of the contaminant or the ability to detoxify the contaminant (Nover, 1991). The time course/duration experiments for both heat shock and ammonia exposure were based on a number of assumptions. The induction and accumulation of stress proteins by chemical contaminants (such as ammonia) are assumed to be slower than induction by heat shock (Nover, 1991; Sanders, 1993). Damage to proteins caused by heat is assumed to be faster than damage by chemical contaminants which are dependent on biological availability, absorption and mechanisms of toxicity (Sanders, 1988). The time course, therefore, is expected to differ for heat and ammonia exposure, and abalone were heat shocked for only 24 hours whereas they were exposed to ammonia for 36 hours.

5.2. Material and Methods

A total of 180 cocktail abalone (5.0 – 8.0 cm shell length) was donated by a commercial abalone farm (Irvin and Johnson Ltd) at Danger Point in Gansbaai on the southwest coast of South Africa. Cocktail size abalone were selected as this is commercially one of the most important size classes (Cook, 1988). The present study was conducted at the Zoology Department at the University of Cape Town and the immunological analysis of tissue samples was conducted at the Division of Immunology, University of Cape Town. The farmed abalone had been reared on the farms with a mixture of diets which included *Ecklonia maxima*, seaweeds (*Gracillaria* and *Ulva*) and Abfeed (a commercial abalone feed) (L. Schoonbee, I & J Farms, pers. comm. 2005). One month prior to experiments, the farmed abalone were fed exclusively on *E. maxima*, their predominant food in their natural habitat (Branch et al., 1994). All abalone were held in holding tanks with a

closed-circulation system that used sand-filtered seawater. The system contained aerated and continuously flowing natural seawater at 14 – 16 °C. The latter temperature range is well within the optimal range of 12 – 20 °C for this species (Britz et al., 1997; Sales & Britz, 2000). The experiments were carried out in a constant environment (CE) room (humidity = 50% and ambient temperature = 15 °C). Abalone were gently scrubbed with a small, soft nail brush (Lu et al., 1999) to remove any organisms and foreign particles that could cause changes in ammonia levels. Buckets (20 Litres) were used as experimental tanks and each bucket was individually aerated using air stones. Water levels in each bucket were kept to a maximum and mesh lids (Figure 3.2) were used to prevent 'crawl-outs' observed in another study on *H. midae* (Britz et al., 1997).

Dissolved oxygen (7.90 ± 0.07 ppm; range:7.68 – 8.20 ppm) was measured using a dissolved oxygen meter (YSI DO200, ± 0.01). Temperature (15 °C) and pH (7.8 ± 0.04; range 7.78 – 7.85) were measured using a pH meter (YSI pH100, temperature ± 0.1 °C and pH ± 0.01) and the meter was calibrated with Crison buffers. The pH was maintained at 7.8 using reagent grade HCL and NaOH pellets. Salinity (34.6 ± 0.1 ppt) was measured with a salinity meter (YSI EC300, ± 0.1 ppt). Each animal was used only once and was not included in any further experimentation.

5.2.1. Stress induction by heat- Alterations in stress protein levels were measured after 1, 2, 3, and 24 hours during a 24-hour heat shock experiment. These time intervals were shorter than those used in the ammonia exposure experiments as induction and accumulation of stress proteins by chemical contaminants appears to be slower than induction by heat shock (Nover, 1991; Sanders, 1993). Only a few time intervals could be used, because there was limited availability of antibody, and the shorter intervals were deemed most likely to yield an increase in HSPs and/or P-gp. Small sample sizes (n=3) were used because of limited availability of animals.

A total of 12 abalone (four abalone per bucket = three experimental buckets) was heat shocked at 25°C. The temperature range of 12 – 20 °C is optimal for farmed *H. midae*

(Britz et al., 1997) with a critical thermal maximum at 27.9 °C (Hecht, 1994). Heat shock at 25°C was therefore chosen to avoid mortalities. The temperature was attained and maintained using a completely submersible automatic aquarium heater (Heater, Y978-B, 300 watts). Another twelve abalone (control) were placed in seawater at 15°C (4 abalone per 20 litre bucket= three control buckets). Three abalone were removed (one from each bucket) at each time interval, namely 1, 2, 3 and 24 hours.

5.2.2. Stress induction by ammonia- Alterations in stress protein levels were measured after 12, 24 and 36 hours during a 36-hour ammonia exposure experiment. These time intervals were longer than those used in the heat shock experiments.

A total of 12 abalone (four abalone per bucket = three experimental buckets) were exposed to sublethal levels of ammonia of 19.69 μg l^{-1} FAN (1.03 mg l^{-1} TAN) (determined previously; see Chapter 3, Table 3.5). Ammonia concentrations were obtained by adding appropriate amounts of ammonium chloride (Thurston et al., 1981, Barimo & Walsh, 2005) to the seawater. Dissolved ammonia concentrations were maintained by periodically (every day) replacing 50% of the water in the buckets (Schmitt & Uglow, 1996) and TAN was measured using a Hach DR/2000 spectrophotometer (see chapter 2, section 2.3.2.1) and FAN was calculated (see Chapter 2, section 2.3.2.1). Another 12 abalone (control) were placed in seawater with no added ammonium chloride (four abalone per bucket = three control buckets). Three abalone were removed (one from each bucket) at each time interval namely, 12, 24 and 36 hours.

5.2.3. Biochemical assays

5.2.3.1. Tissue extraction

The abalone were killed humanely by snap freezing in liquid nitrogen. The abalone were thawed, dissected and foot tissue (5 g) and gills (1 g) removed. Excess mucus from the gills was removed with forceps after swirling the gills in filtered seawater (Eufemia & Epel, 2000). Epithelial tissue from the foot was removed by scrubbing (Pitcher et al.,

2001). The dissected foot and gills were finely chopped and homogenized in tissue buffer (see Appendix 5a for buffer composition). The samples were placed in a sonicator for 45 s (Archard et al., 2004; Dyer et al.,1991), centrifuged (4300 rpm for 30 minutes), the pellet discarded and the supernatant stored at -80°C (Kurelec et al., 1996), until further analysis. Between the above procedures, the samples were kept on ice.

5.2.3.2. Total protein determinations in the samples

The protein extracted from the tissue was quantified using the BCA™ (Bicinchoninic acid) protein Assay Kit (Pierce) with the microplate procedure (Figure 5.1). The chemical reaction involved in this process is the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction). The purple-coloured complex formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}) is detected colourmetrically. This method is highly sensitive, with strong absorbance at 540 nm that is linear with increasing protein concentrations over a broad working range (20-2000 $\mu g ml^{-1}$ - Pierce Instruction sheet). The protein concentrations in the samples are determined with reference to standards of a common protein such as bovine serum albumin (BSA). A dilution series of known concentrations of BSA forms the standard curve (bottom row of microplate - Figure 5.1) which is assayed alongside the unknown concentrations of the sample. The protocol for this assay is provided with the kit (Pierce Instruction sheet).

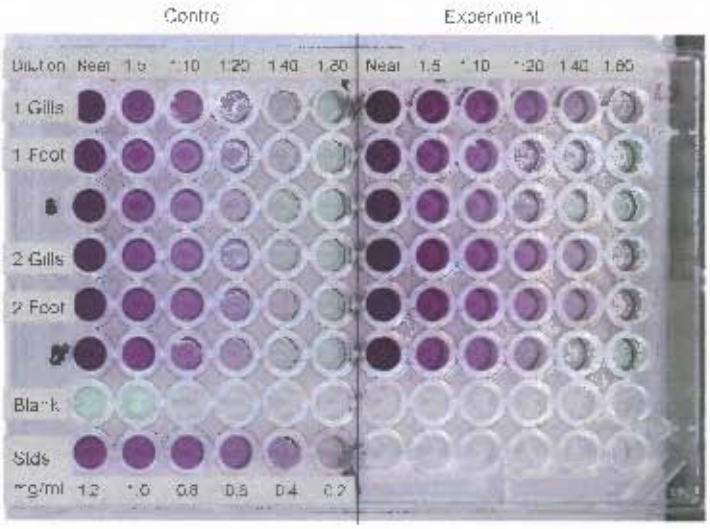

Figure 5.1. Dilutions of proteins in the foot and gills of 2 abalone (1&2) using BCA™ protein Assay Kit. It is clearly visible that both the foot and gills of two experimental abalone have more total protein than the control abalone by comparing the intensity of the purple colour complex formed at the 1:40 and 1:80 dilutions. Stds = standards of bovine serum albumin.

5.2.3.3. One-dimensional Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) analysis

One-dimensional SDS-PAGE separates the proteins according to their molecular weights (size). The sample extracts are heated under denaturing conditions. The proteins become unfolded and coated with SDS detergent in the loading buffer (see Appendix 5b for composition) attaining a high net negative charge proportional to the length of the polypeptide chain (Westermeier, 1993; QIAGEN Bench Guide). Bromophenol blue, a small anionic dye molecule, is added to the loading/sample buffer to enable visualization of the protein migration in the gel. The samples are loaded onto the gel matrix and subjected to an electric field. The negatively charged protein molecules migrate towards the positively charged electrode, separating the proteins according to their molecular weights (QIAGEN Bench Guide). Visualization of the protein bands is obtained by using a protein-specific staining method; the gels are placed in Coomassie stain and then destain (see Appendix 5c for both stain compositions). The protein size can then be estimated by comparing migration distance with that of a standard of known molecular

weight (molecular marker). The molecular marker used in the present study was Precision Plus Protein™ Standards (Bio-Rad). Gradient gels are obtained by continuously changing the acrylamide concentration (5-16 %) in the polymerization solution resulting in a pore gradient (Westermeier, 1993), and were used for the initial visualization of the protein bands and for densitometric analysis (see 5.2.3.5). Minigels are smaller gels with a constant pore size (12 % acrylamide concentration was used), and these were used for immunoblotting (see 5.2.3.4) because much lower volumes of antibodies are required compared to the larger gradient gels. Standard protocols for preparation and running of gels is provided in the QIAGEN Bench Guide using the appropriate buffers and staining solutions (see appendix 5b and 5c, respectively).

5.2.3.4. Immunoblotting (also referred to as Western blotting)

After electrophoresis, the proteins were transferred to a nitrocellulose membrane in a buffer-tank-blotting apparatus (see QIAGEN bench guide for protocol). This apparatus was chosen as the transfer is more efficient compared to semi-dry electroblotting (QIAGEN bench guide). The appropriate blotting buffers are provided in Appendix 5e. Once the proteins are transferred onto the membrane, the remaining protein-free sites are blocked using non-fat dry milk to prevent the antibodies from binding directly onto the membrane. The proteins of interest are visualized with appropriate antibodies (Figure 5.2) and the general procedure is as follows:

After blocking, the primary antibody is added which binds to the protein of interest (HSP or P-gp) (Figure 5.2, step 1). The membrane is then washed to remove nonspecifically-bound antibody. The secondary labelled antibody is added to detect the bound primary antibody (Figure 5.2, step 2). The membrane is washed further, the location of the secondary antibody (and therefore the primary antibody and the protein of interest) is determined by the addition of a substrate that reacts with the enzyme conjugated to the secondary antibody (Figure 5.2, step 3) resulting in a coloured compound (see blots). The substrate used was NBT/BCIP (nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) stock solution which detects alkaline phosphatase (AP) (Roche, Germany) (Appendix 5e).

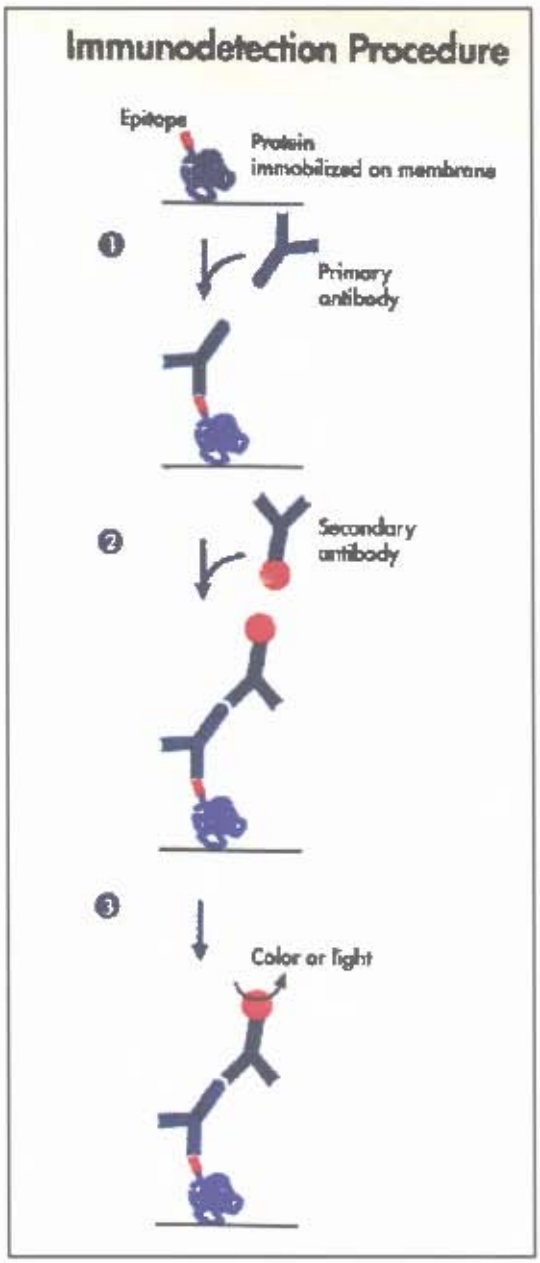

Figure 5.2. Immunodetection of the protein (HSP or P-gp) immobilized on a membrane (Adapted from QIAGEN Bench Guide).

The primary antibodies used to detect HSPs in *H. midae* were monoclonal (mouse) anti-HSP 90 (SPA-830), anti-HSP 70 (SPA-822) and anti-HSP 27 (SPA-800) and polyclonal (rabbit) anti-HSP 60 (SPA-805), all purchased from StressGen (Canada). The secondary antibody used to detect the monoclonal primary antibodies, anti-HSP 90, anti-HSP 70 and

anti-HSP 27 was goat anti-mouse IgG (H+L), labelled with alkaline phosphotase (Southern Biotech, USA). The secondary antibody used to detect the polyclonal primary antibody anti-HSP 60 was goat anti-rabbit IgG (H+L), labelled with alkaline phosphotase (Southern Biotech, USA). All of the above antibodies were diluted 1:1000. A positive control for HSP 90, HSP 70, HSP 60 and HSP 27 was HeLa Cell lysate (LYC-HL101) (StressGen, Canada). The antibodies used to detect the above HSPs (except HSP27) have been used in a number of studies (including abalone) (see Table 5.1). The mouse anti-HSP27 monoclonal antibody from StressGen (Canada) is the most appropriate antibody for this study and because HSPs are highly conservative, it is likely that this antibody will react with abalone HSP27.

The monoclonal antibody (C219; 1:200 dilution) used to investigate P-gp in *H. midae* was donated by Signet Laboratories, Inc. (Dedham, MA) and was the antibody of choice because it is the most commonly used antibody in the literature (see Table 5.1). The C219 antibody is regarded as a 'universal probe for the detection of P-gp" as it is found to recognise a sequence (in bacteria to man) expressed by all MXR genes (Endicott and Ling, 1989). A positive control for the P-gp, mouse liver, was provided by Ms. Natalie Nieuwenhuizen (Division of Immunology, University of Cape Town). P-gp is known to occur naturally in mammalian liver (Bard, 2000; Silverman & Thorgeirsson, 1995).

5.2.3.5. Quantification of stress proteins by densitometric analysis

Both the gel and blot protein bands were quantified by scanning followed by densitometric analyses (Figure 5.3 III) using a public domain NIH Image software package (http://rsb.info.nih.gov/nih-image/) (Doi et al., 2001; Rossi & Snyder, 2001; Snyder et al., 2001). The scanned gels had to be converted into an 8-bit grey scale image (included in the package) to get the best results.

To illustrate how the band intensity of each stress protein was obtained from the gels and immunoblots, the following two examples of HSP induction in the foot and gills of *H.*

midae after heat shock (1 hour) and ammonia exposure (24 hours), respectively, were chosen (Figure 5.3).

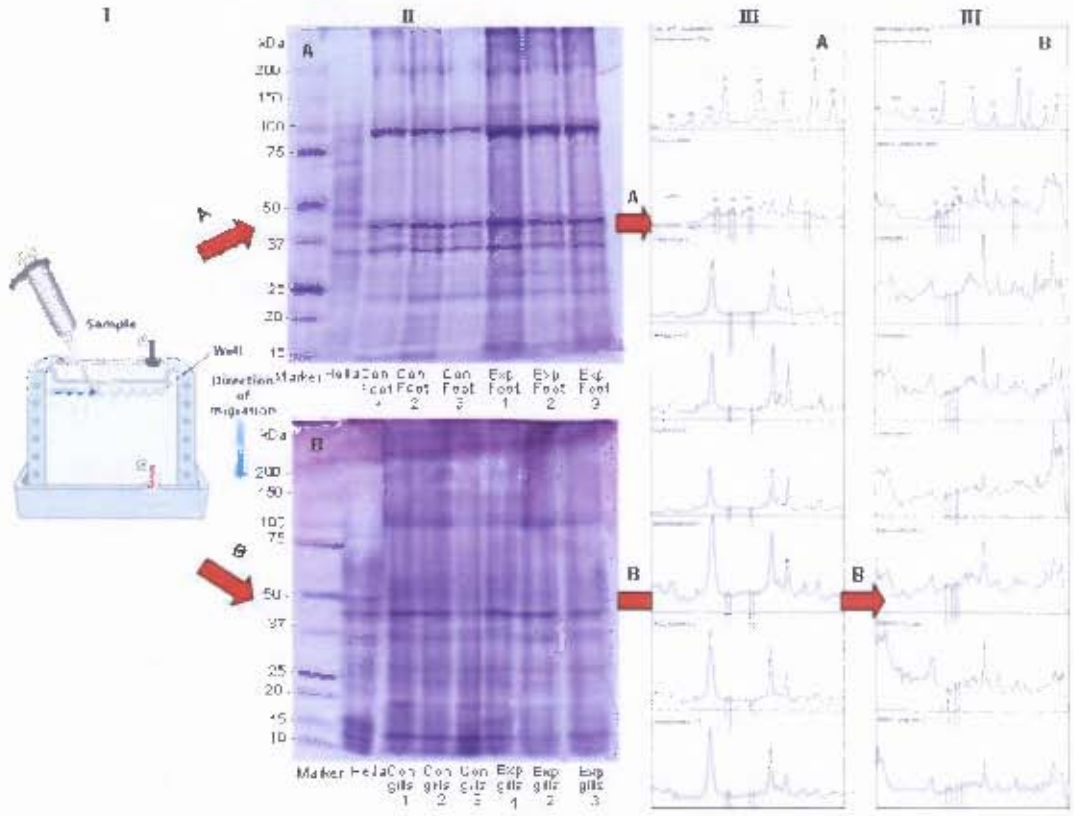

Figure 5.3. The tissue extract (sample) is loaded into the gels (gel electrophoresis) (I). The gels are stained, scanned (II) and the peaks (area=band intensity) of the desired proteins (HSPs) for each lane are obtained using densitometric analysis (III). The amount of each HSP is given as band intensity (area of the peaks)/ ug of total protein in a sample + standard deviation. (A) is the gel and the peak profile for the 1 hour heat shock in the foot and (B) is the gel and its peak profile for the 24 hour ammonia exposure in the gills.

For both ammonia exposure and heat shock in the foot and gills, the band intensities of the HSP 70 and HSP 60 were analyzed using scanned stained PAGE gels (Figure 5.3 II). However, peaks obtained for P-gp, HSP 90 and HSP 27 overlapped with adjacent peaks, making it difficult to obtain accurate measurements. To compare the differences between band intensities for P-gp, HSP 90 and HSP 27 between control and experimental abalone, immunoblots were compared. Since pooled tissue (n=3) was used in all blots (to minimize the amount of antibody used due to its limited availability and the need to use antibody for the necessary detection of HSPs in HeLa), no means of band intensities could be calculated. For immunoblots, 60µg and 70µg of foot and gill tissue was used,

respectively. For the gels, 10μg and 20μg of foot and gill tissue was used, respectively. The protein band intensities obtained from the densitometric analysis are, therefore, standardized and reported as per μg of total protein.

5.2.3.6. Statistical analysis

The data were analyzed by repeated measures ANOVA and post-hoc Tukey test using the statistical software GraphPad Prism Version 4 (statistical analyses for laboratory and clinical research).

5.3. Results

Preliminary analysis of various abalone tissue included stalk, digestive gland and blood. The protein band intensities for the connective stalk of the foot were much less than the foot and since the foot is attached to the stalk, it was omitted. The protein bands obtained for the digestive gland were not clearly defined, most likely as a result of the large amount of digestive enzymes present in this tissue. The blood of abalone demonstrated very weak protein bands. Both the digestive gland and blood were also omitted from the following analyses.

5.3.1. Determination of HSPs in foot and gill tissue during heat and ammonia shock using immunoblots

Two bands (isoforms) of HSP 70 were detected in the foot and gills after 24 hour ammonia exposure and one hour heat shock with approximate molecular weight of 72.4 and 70.8 kDa (Figure 5.4). Both these bands produce a single peak using the densitometric analysis and has been referred to as HSP 70 in the present study.

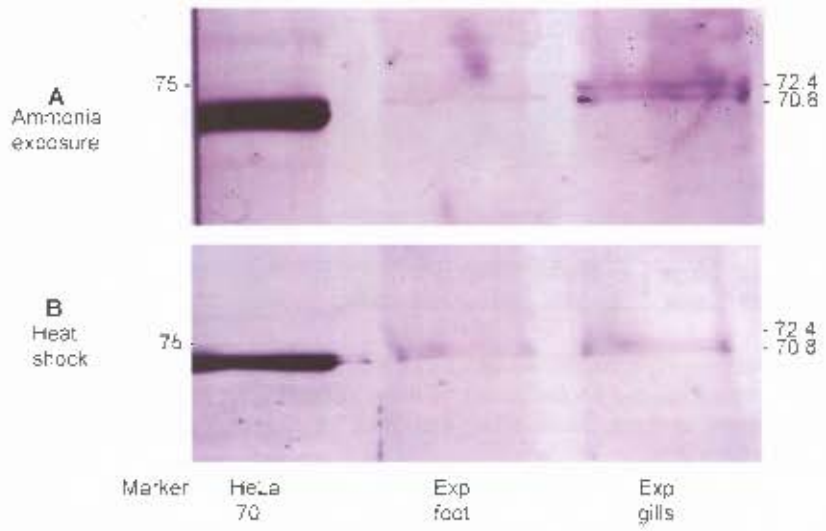

Figure 5.4. Immunoblot of HSP 70 in foot and gill tissue after 24 hour ammonia exposure (A) and one hour heat shock (B) illustrating two bands (isoforms) of HSP 70 with molecular weights of ~72.4 and ~70.8 kDa. Each lane represents the pooled tissue of three abalone. The molecular marker is indicated in kDa. Exp = Experimental abalone. Note: 60µg and 70µg of foot and gill protein extracts were used respectively.

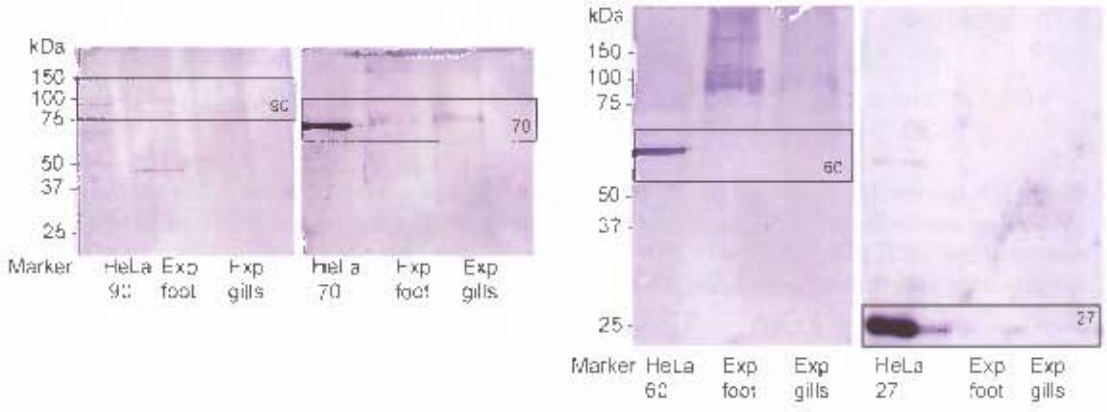

Figure 5.5. Immunoblots for HSP 90, 70, 60and 27 in foot and gills of *H. midae* after 1 hour heat shock. Each lane represents the pooled tissue of three abalone. The molecular marker is indicated in kDa. Exp = Experimental abalone. Note: 60µg and 70µg of foot and gill protein extracts were used respectively.

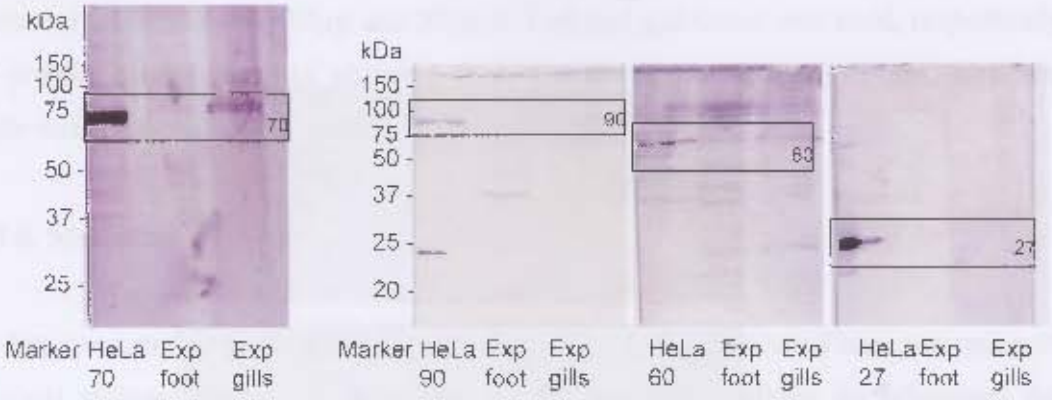

Figure 5.6. Immunoblots for HSP 70, 90, 60 and 27 in foot and gills of *H. midae* after 24 hour ammonia exposure. Each lane represents the pooled tissue of three abalone. The molecular marker is indicated in kDa. Exp = Experimental abalone. Note: 60µg and 70µg of foot and gill protein extracts were used respectively.

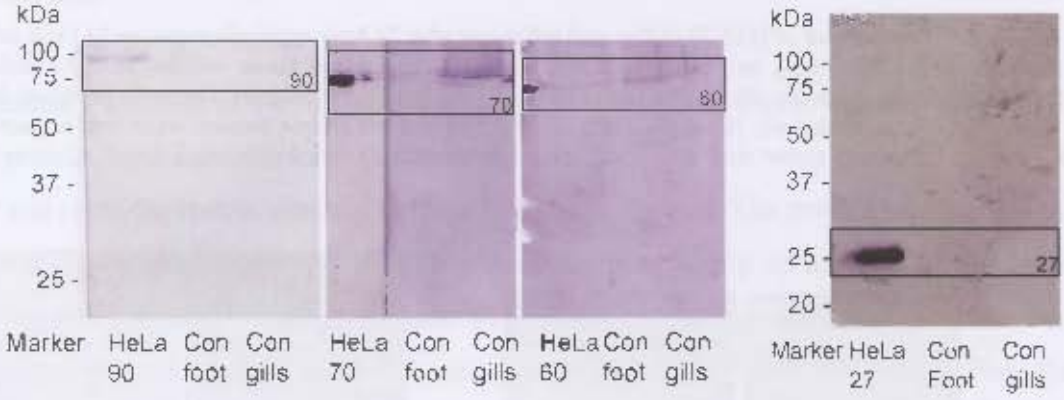

Figure 5.7. Immunoblots for HSP 90, 70, 60 and 27 in foot and gills of *H. midae* under controlled (not heat shocked or ammonia exposed) conditions. Each lane represents the pooled tissue of three abalone. The molecular marker is indicated in kDa. Con = Control abalone. Note: 60µg and 70µg of foot and gill protein extracts were used respectively.

Analysis of the immunoblots (Figures 5.5 – 5.7), Figure 5.8 and Table 5.2, shows that HSP 90 is detectable only in the foot after one hour heat shock and in the gills after 24 hour ammonia exposure. HSP 70 is present in both tissues in the control abalone and appears to increase only in the foot after one hour heat shock and in the gills after 24 hour ammonia exposure, respectively. HSP 60 appears to increase slightly in the foot after one hour heat shock and 24 hour ammonia exposure. There is a substantial increase in HSP 60 in the gills after 24 hour ammonia exposure. HSP 27 is detectable only in the foot and gills after one hour heat shock.

Table 5.2. Band intensities/ µg of total protein for HSP 90, 70, 60 and 27 after 1 hour heat shock and 24 hour ammonia exposure in both control and experimental abalone. Each value represents pooled data of three 3 abalone and obtained using blots.CF – Control foot, CG – Control gills, EF – Experimental foot, EG – Experimental gills, X – not detectable.

			Heat shock		Ammonia	
	CF	CG	EF	EG	EF	EG
HSP90	x	x	2.15	x	x	4.06
HSP70	4.88	5.31	8.20	5.80	3.03	15.11
HSP60	6.22	8.10	8.28	7.59	8.38	12.99
HSP27	x	x	4.01	2.97	x	x

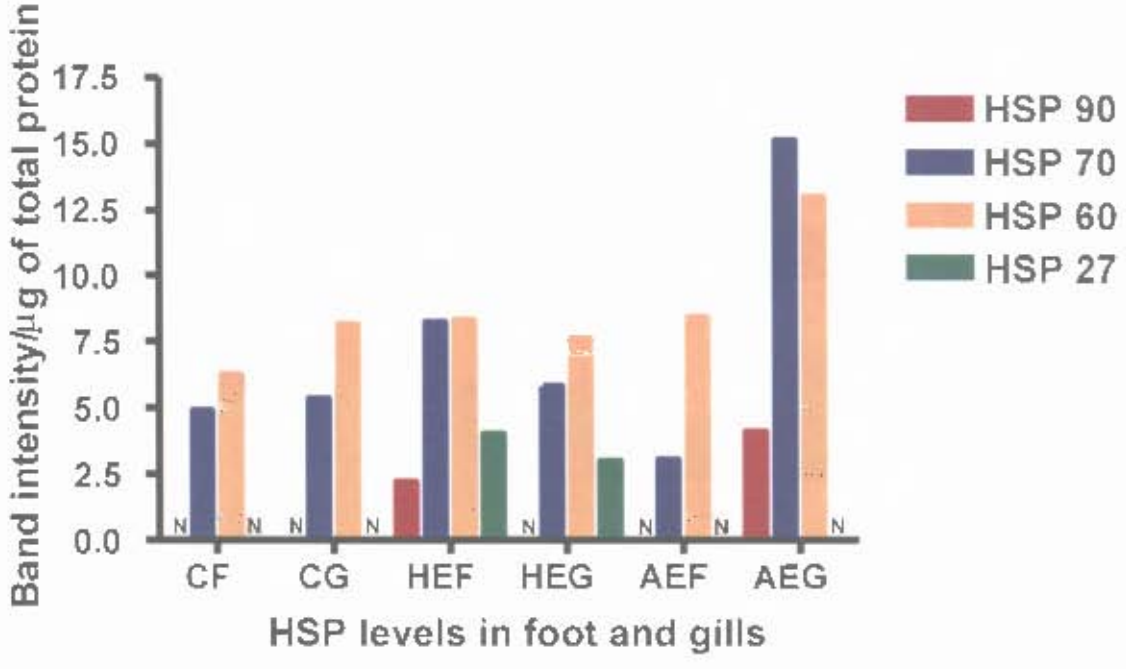

Figure 5.8. HSP-90, -70, -60 and -27 levels in foot and gills after 1 hour heat shock and 24 hour ammonia exposure obtained from immunoblot analysis. Each column represents pooled data of three abalone. CF–Control foot; CG= Control gills; HEF= Heat shocked experimental foot; HEG= Heat shocked experimental gills; AEF Ammonia exposed experimental foot; AEG – Ammonia exposed experimental gills. N – not detectable.

5.3.2 Determination of stress proteins in foot tissue during heat shock as demonstrated by gel electrophoresis

Both HSP 70 and 60 in the foot of the experimental abalone were significantly higher ($P < 0.001$, repeated measures ANOVA, $F = 0.5112$, $df = 2$, post hoc Tukey test), than in the control abalone after 1 hour heat shock, but there were no differences in the amount of HSP 70 and 60 after 2, 3 and 24 hours (Table 5.3, Figure 5.9).

Table 5.3. Mean ± s.d. (n=3) of the band intensity/µg of total protein calculated for HSP 70 and HSP 60 in the foot at 1, 2, 3 and 24 hours after heat shock. The results are graphically presented below (Figure 5.9). CF Control foot, EF Experimental foot.

	1 hour		2 hours		3 hours		24 hours	
	CF	EF	CF	EF	CF	EF	CF	EE
HSP70	12.67±1.33	39.77±6.51	11.27±4.72	14.13±3.06	12.77±2.96	13.47±2.39	12.87±4.41	12.43±0.95
HSP60	12.27±2.69	38.33±12.47	12.03±1.40	16.13±2.99	14.23±1.21	16.07±3.10	11.87±0.55	12.80±2.07

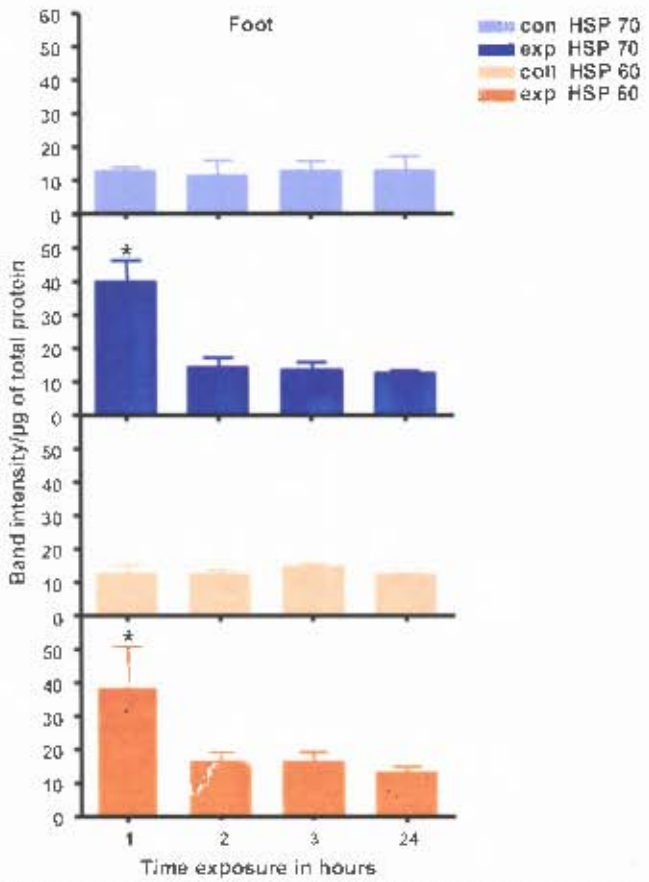

Figure 5.9. HSP 70 and HSP 60 levels in foot at 1-24 hours after heat shock. Each column represents mean band intensity/µg of total protein ± standard deviation of three abalone. Con=Control abalone, exp=Experimental abalone. Results obtained from gel electrophoresis analysis. Significant difference from the control, $P < 0.001$ (*).

5.3.3. Determination of stress proteins in gill tissue during heat shock as demonstrated by gel electrophoresis

There was no significant difference in the amount of HSP 70 and 60 between the experimental and control abalone after 1, 2, 3 and 24 hours of heat shock ($P > 0.05$, repeated measures ANOVA, $F = 1.467$, $df = 2$) (Table 5.4, Figure 5.10).

Table 5.4. Mean ± standard deviations (n=3) of the band intensity/µg of total protein calculated for HSP 70 and HSP 60 in the gills at 1, 2, 3 and 24 hour heat shock. The results are graphically presented below (Figure 5.10). CG – Control gills, EG – Experimental gills.

	1 hour		2 hours		3 hours		24 hours	
	CG	EG	CG	EG	CG	EG	CG	EG
HSP70	21.93±0.75	21.60±0.82	21.53±0.55	21.23±1.67	20.67±2.63	21.27±1.62	21.90±2.43	21.57±1.83
HSP60	12.83±2.37	12.73±1.65	13.57±1.42	14.83±0.31	13.23±2.61	13.77±1.00	13.90±1.59	11.43±0.70

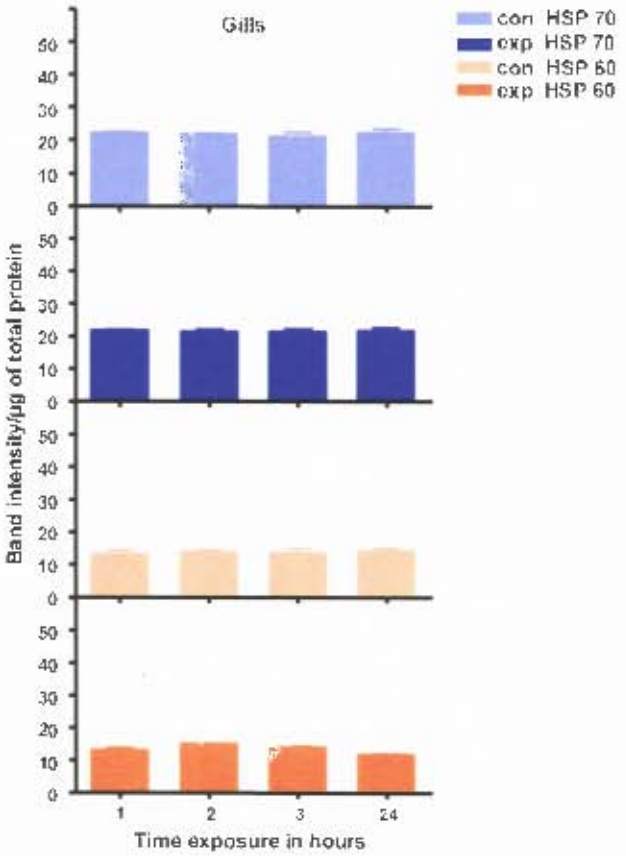

Figure 5.10. HSP 70 and HSP 60 levels in gills at 1-24 hour heat shock. Each column represents mean band intensity/µg of total protein ± standard deviation of three 3 abalone. Con=Control abalone, exp=Experimental abalone. Results obtained from gels.

5.3.5 Determination of stress proteins in gill tissue during ammonia exposure as demonstrated by gel electrophoresis

There was no significant difference in the amount of HSP 70 and 60 in the gills of abalone after 12 hours ammonia exposure between the control and experimental abalone (Table 5.6. Figure 5.12). The amount of HSP 70 in the gills was significantly higher in the experimental compared to the control abalone after 24 hours ($P < 0.05$) and 36 hours ($P < 0.01$) (Table 5.6. Figure 5.12). The amount of HSP 60 in the gills was significantly higher in the experimental compared to the control abalone after 24 hours ($P < 0.001$) and 36 hours ($P < 0.001$) (Table 5.6, Figure 5.12). P-values were obtained using repeated measures ANOVA ($F = 8.018$, $df = 2$) post hoc Tukey test.

Table 5.6. Mean ± standard deviations (n = 3) of the band intensity/µg of total protein calculated for HSP 70 and HSP 60 in the gills at 12, 24 and 36 hour ammonia exposure. The results are graphically presented below (Figure 5.12). CG – Control gills. EG – Experimental gills.

	12 hour		24 hours		36 hours	
	CG	EG	CG	EG	CG	EG
HSP70	23.97±2.42	26.00±3.72	23.07±4.10	32.87±5.03	23.60±3.90	34.57±3.32
HSP60	15.50±2.31	16.73±0.90	13.70±2.55	29.07±1.67	13.00±1.68	28.77±2.59

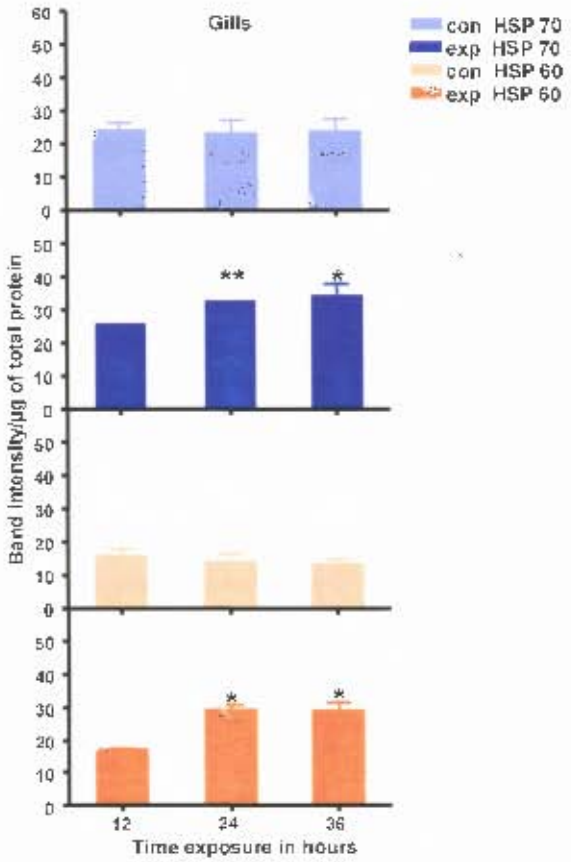

Figure 5.12. HSP 70 and HSP 60 levels in gills at 12-36 hour ammonia exposure. Each column represents mean band intensity/µg of total protein ± standard deviation of three abalone. Con=Control abalone, exp Experimental abalone. Results obtained from gels. Significant difference from the control at $P < 0.001$ (*) and $P < 0.01$ (**).

5.3.6. Determination of P-glycoprotein in gill tissue during ammonia exposure as demonstrated by immunoblots

When reducing gels were used to separate the MXR proteins, it was observed that the antibody cross reacted with a ~70.8 kDa protein in mice liver (Figure 5.13A). A non-reducing gel (removal of DDT from sample buffer and no boiling prior to loading) therefore, resulted in the detection of a ~257 kDa and a ~70.8 kDa protein (Figure 5.13, B) in mice liver.

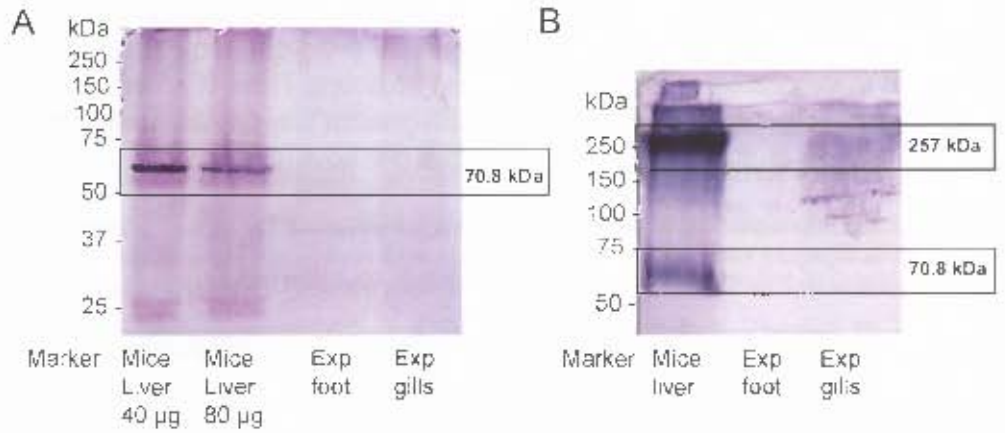

Figure 5.13. Immunoblot A (using reducing gel) and immunoblot B (using non-reducing gel) illustrating P-gp induction in mice liver (positive control) and in foot and gills of *H. midae* after 24 hour ammonia exposure. Each lane represents the pooled tissue of three abalone. The molecular marker is indicated in kDa. Exp = Experimental abalone.

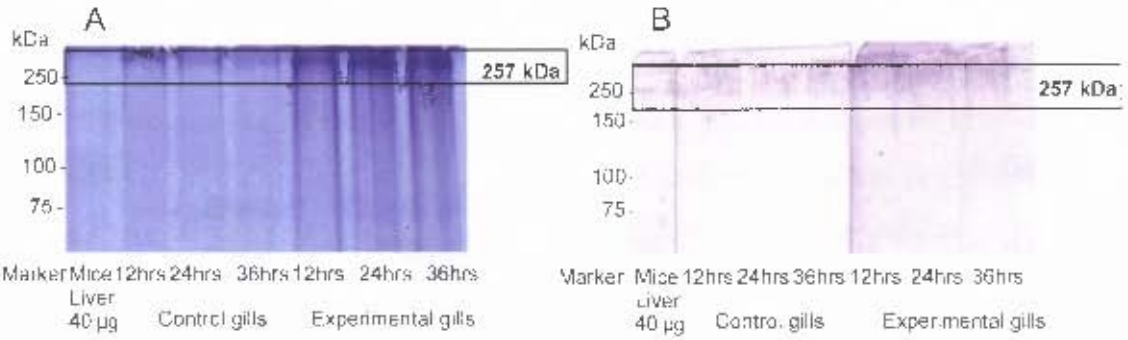

Figure 5.14. Gel electrophoresis A (using non-reducing gel) and immunoblot B illustrating P-gp induction in mice liver (+ control) and in gills of *H. midae* after 12, 24 and 36 hour ammonia exposure. Each lane represents the pooled tissue of three abalone. The molecular marker is indicated in kDa. Con = Control abalone, Exp = Experimental abalone.

Figure 5.15. Immunoblots (using non-reducing gel) illustrating P-gp induction in gills and foot of *H. midae* after one hour heat shock. Each lane represents the pooled tissue of 3 abalone. The molecular marker is indicated in kDa. Con = Control abalone. Exp = Experimental abalone.

Results of the immunoblots (Figures 5.13 – 5.15), Figure 5.16 and Table 5.7 show that a protein of ~257 kDa (most likely P-gp) is increased (~2-fold) in the experimental gills (compared to the control gills) after 12, 24 and 36 hours. There is no increase in this protein in the gills after 1 hour heat shock. This protein was not detected in the foot after 24 hour ammonia exposure (Figure 5.13 B) and one hour heat shock (Figure 5.15).

Table 5.7. Band intensities/ µg of total protein + standard deviation for P-gp in gills after 12, 24 and 36 hour ammonia exposure and after 1 hour heat shock in both control and experimental abalone. Each value represents pooled data of three abalone and obtained using blots. CG = control gills, EG = experimental gills.

P-gp- ammonia						P-gp- Heat shock	
12 hours		24 hours		36 hours		1 hour	
CG	EG	CG	EG	CG	EG	CG	EG
26.01	43.00	20.24	59.94	23.26	44.91	19.55	17.32

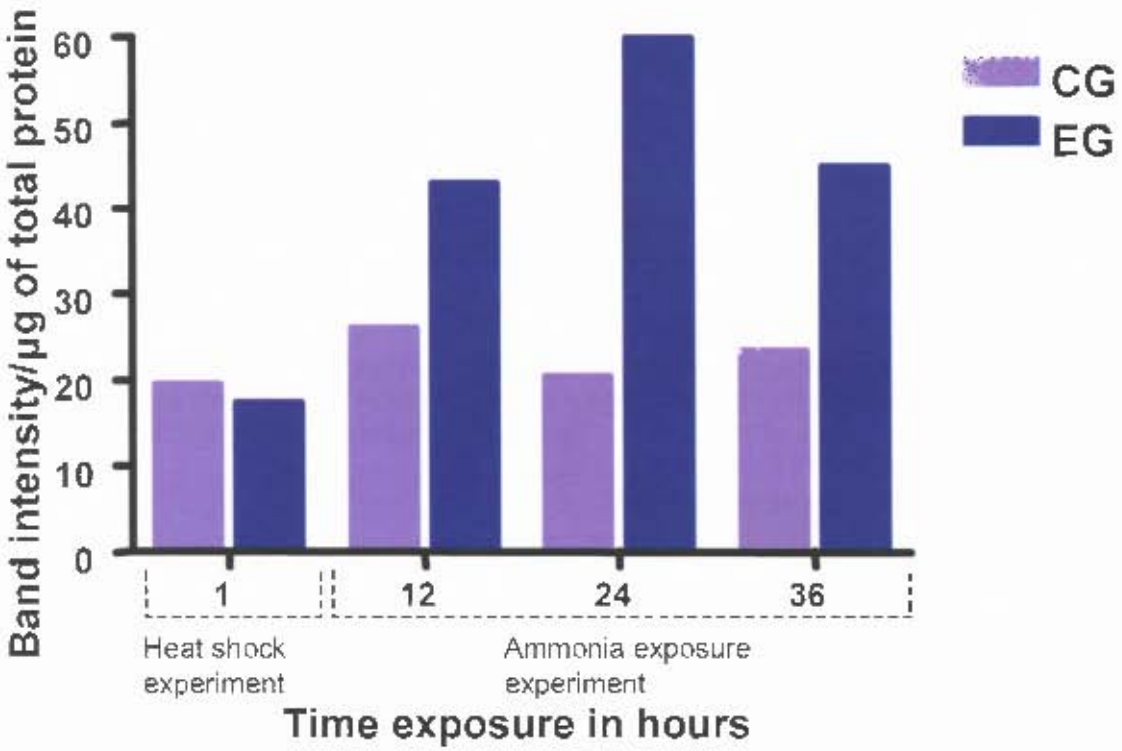

Figure 5.16. P-gp (~257 kDa) levels in gills after 1 hour heat shock and 12, 24 and 36 hour ammonia exposure obtained from immunoblots. Each column represents pooled data of three abalone. CG – Control gills; EG = Experimental gills.

5.4. Discussion

The results for the different HSPs obtained from the blots are preferred to those from the gels. Immunoblots use antibodies, which are specific for different HSPs, and are therefore a direct measure of the amount of HSP present. In contrast the bands obtained from the gel are identified by molecular weight and could therefore include proteins other than HSPs. The results obtained from the blots (Table 5.2) and those from the gels (Tables 5.3 – 5.6) cannot be compared directly, but the overall patterns of changes in the experimental tissue were similar for both methods, indicating that the increase in the amount of protein after shock treatment represents stress proteins. Two bands (isoforms) of HSP 70 isolated from the foot and gills after 24 hour ammonia exposure and one hour heat shock of approximately 70.8 and 72.4 kDa were recognized with the HSP 70 antibody (Figure 5.4). This was also observed in the HeLa control. Both these bands produce a single peak using the densitometric analysis and have therefore been referred to as HSP 70 in the present study. Snyder et al. (2001) also showed that the HSP 70 antibody recognized three protein bands of 67, 70 and 74 kDa in the Californian abalone, *H. rufescens*, during heat shock and xenobiotic (chemicals used in wood-bleaching operations along the coast) exposure. It therefore seems that two higher molecular weight HSP 70 are inducible by ammonia and heat in *H. midae*. Drew et al. (2001), in contrast, showed that the HSP 70 antibody recognized a single band of 70 kDa in *H. rubra*.

Temperature shock had the greatest impact in the foot tissue (statistically significant increase in both HSP 70 and 60 ($P < 0.001$) within the first hour and these levels returned back to normal after one hour (Figure 5.9). Rocherri et al. (1981) showed that in sea urchin embryos, *Paracentrotus lividus*, HSP synthesis occurs in a burst shortly after heating, and then, after about 4 hours, the pattern of protein synthesis starts to revert back to normal even if the high temperature is maintained. In abalone, in the present study, no significant changes in HSP 90 and 70 were observed in the gills after temperature shock (Figure 5.10). Ammonia exposure had the greatest impact on the gills (but not within the first 12 hours and only after 24 hours). In mussels, P-gp has been found in the gills which are an important route of entry for toxic compounds (Minier et al., 2000). Both HSP 70

and 60 in the gills were statistically higher than the control after 24 hours ($P < 0.01$; P, 0.001, respectively) and after 36 hours ($P < 0.001$; $P < 0.01$, respectively) ammonia exposure (Figure 5.12). Although there were no significant differences in HSP 70 and 60 in the foot during ammonia exposure (throughout the duration of the experiment) (Figure 5.11), there did appear to be a slight trend towards an increase in the amount of HSP 70 (after 24 and 36 hours) and HSP 60 after (36 hours). The large standard deviations obtained in this experiment may have resulted from differences in individual absorption and accumulation rates of the toxin (ammonia) from the serum and the effect on the foot. The induction of the stress response by chemical contaminants appears to be slower than by heat shock (Sanders, 1993). The results of the time exposure experiments for both temperature and ammonia (discussed above) support this view as increase of HSP 70 and 60 in the foot occurred within the first hour of heat shock and in the gills after 24 hours ammonia exposure.

HSP 70 and 60 occur at minimum levels in the foot and gills of abalone living under natural (control) conditions (Table 5.3 -5.6). However, HSP 90 does not appear to be present in the foot and gills of *H. midae* under natural (control) conditions (Table 5.2 and Figure 5.8). HSP 90 is only present in the foot after heat shock and in the gills after ammonia shock (Table 5.2). HSP 90 appears to confer tolerance to organs most affected by a stressor (temperature and ammonia). However the data provided in Table 5.2 is the pooled data of three abalone at one hour heat shock or 24 hour ammonia exposure (one time frame representing minimum time required for a desired effect of the stressor was chosen and pooled tissue was used to minimize the amount and cost of the antibodies used). HSP 27 also does not appear to be present in the foot and gills under normal conditions as well as during ammonia exposure, but was present in the foot and gills after one hour heat exposure (Table 5.2). Therefore, it is possible that HSP 90 and 27 could be present in the foot and gills after 36 hours ammonia exposure due to the increased accumulation of ammonia with time. HSP 27 may therefore prove to be a useful tool in assessing heat stress in *H. midae* while HSP 90 could be used for both heat and ammonia stress.

P-gp appears to be the 'first line of defense' against xenobiotics in most aquatic animals (Epel, 1998; Kurelec, 1992). From results of the present study, it appears that this is, also true for *H. midae* in the present study as the amount of P-gp increases about two fold in the experimental compared to control gills from 12 hours through 36 hours (Table 5.7, Figure 5.16). The stress proteins, however, appear later i.e. changes in the HSP 70 and 60 in the experimental compared to control gills only occurred after 24 hours of ammonia exposure (Figure 5.12).

The presence of P-gp can be measured directly by immunoblotting (Table 5.7), but also by analysis of MXR-related genes (Luedeking and Koehler, 2004) and indirectly by measuring cellular accumulation of radiolabelled or fluorescent labelled substrates (Eufemia and Epel, 2000). Separation with a non-reducing gel resulted in the detection of a ~257 kDa and a ~70.8 kDa protein in mice liver (Figure 5.13, B). It is therefore reasonable to assume that the ~257 kDa is a dimmer of the 170 kDa and the ~70.8 kDa its fragment. However in both experimental and control gills, C219 antibody reacted only with the ~257 kDa protein (Figures 5.14 and 5.15). Antibodies (anti C and C219) are generally used to detect P-gp (Smital et al., 2000), however the antibodies were found to react with proteins of molecular weights other than the 170 kDa. Smital et al. (2000) found that the anti C antibody reacted with a ~ 140 kDa protein in membrane vesicles of gills of the mussels, *Mytillus galloprovincialis* and the marine snail, *Monodonta turbinata*. The C219 antibody reacted with a protein band of 135 kDa in the gills of the clam, *Corbicula fluminea* (Archard, 2004; Legeay et al., 2005). Doi et al. (2001), also found that in catfish intestine the C219 antibody reacted with a ~80 kDa but alterations in the sample preparation (similar to the present study) prior to loading resulted in the detection of both ~170 kDa and ~80 kDa in catfish intestine. Minier et al. (2000) found, in the gills of the mussel, *Mytilus galloprovincilais*, that the C219 antibody reacted with several bands; the main band was ~130 kDa and three other bands of molecular weights 230, 70 and 50 kDa. The authors suggested that if the two shorter bands (50 and 70 kDa) are degradative products, then it is more likely that the larger bands (130 and 230 kDa) are the MXR proteins which are known to have high molecular weights. Minier et al. (1993) found that the C219 antibody reacted with two proteins (220 and 24 kDa) in the

mussel, *Mytilus edulis*. The presence and increase of this ~257 kDa protein in the present study, and its binding to the C219 antibody, makes it likely that P-gp is present and plays a role in ammonia tolerance in *H. midae*. Furthermore, an increase in P-gp levels compared to the control was not observed in gills after temperature shock (Figure 5.15, 5.16 and Table 5.7).

In aquatic organisms there are at least four families of stress proteins involved in acquired tolerance, namely HSP104, HSP70, chaperonin and the LMW HSPs (Sanders, 1993). Under stress conditions, HSP 90 may redirect cellular metabolism to enhance tolerance (Sanders, 1993). In the present study, HSP 90, HSP 70, HSP 60 and HSP 27 appear to be involved in conferring temperature tolerance in *H. midae* (Table 5.2), while P-gp, HSP 90, HSP 70 and HSP 60 seem to play a role in ammonia tolerance (Table 5.2 and 5.7). There is some evidence to suggest that LMW proteins are involved in thermotolerance but heat is not the only inducer of LMW proteins (Black and Subjeck, 1991). The impact of each stress protein on conferring both temperature and ammonia tolerance in *H. midae* varied in the foot and gills, as well as after different time exposures. The outcome of the present study supports the hypothesis that both HSPs and MXR (P-gp) plays a protective role against ammonia exposure in the abalone, *H. midae*.

In Chapter 3, it was demonstrated that when wild cocktail size *H. midae* were pre-acclimatized to sub-lethal ammonia (0.76 mg TAN l^{-1}; 10.03 µg FAN l^{-1}) they increased their tolerance (acclimatized) to higher levels of ammonia. In this chapter, it was demonstrated that when farmed cocktail size *H. midae* were exposed to sublethal ammonia (19.69 µg l^{-1} FAN; 1.03 mg l^{-1} TAN), they produced both heat shock proteins and P-gp. It is reasonable to speculate that the mechanism of acclimatization might be the same mechanism that leads to the production of stress proteins. This speculation is, however, not completely substantiated by the results of this chapter, and to do so would require additional protein work that is outside the scope of the present study. Only very recently has the first HSP 70 cDNA of the Pacific abalone, *Haliotis discus hannai*, been fully sequenced (Cheng et al., 2006), demonstrating high homology to other HSP 70 genes, thereby confirming the presence of HSP 70 in abalone.

The present study is the first to observe, in aquatic invertebrates, the expression of HSP 90, 70 and 60 during increased ammonia levels, which is a major problem in aquaculture. The present study is also the first to report the presence of P-gp, in abalone.

5.1. Appendix

Table 5a. Tissue buffer for protein extraction

Solution	*Composition of solution*	*Source*	*Per 500 ml*
Tissue buffer	0.1 M Tris	Hamer et al., 2004	101.65 mg
	1 mM Magnesium chloride	Dunlap & Matsumura, 1997	6.057 g
	0.5 M Sodium chloride	Hamer et al., 2004	14.61 g
	pH 7.5	Adjust with HCl	
	Add 1% Nonidet	Minier et al., 2000	5 ml
Add seperately	40 mM PMSF (diluted with acetone)	Hamer et al., 2004	*

*For every 100 mg wet weight of tissue, use 0.5 ml tissue buffer + 10 µl PMSF (phenylmethylsulphonylfluoride).

Table 5b. SDS-PAGE Buffers and solutions for protein analysis

Buffers/Solutions	*Composition*	*Components*	
Buffers			**per liter**
2.5x separating gel buffer	1.875 M Tris-Cl	Tris base	227.1 g
	0.25% SDS	SDS	2.5 g
	pH 8.9	Adjust with HCl	
5x stacking gel buffer	0.3 M Tris-phosphate	Tris base	36.3 g
	0.5% SDS	SDS	5 g
	pH 6.7	Adjust with phosphoric acid	
5x electrophoresis buffer	0.5 M Tris base	Tris base	60.6 g
	1.92 M glycine	Glycine	144.1 g
	0.5% SDS	SDS	5 g
	pH 8.8	without adjustment	
			per 10 ml
5x loading/sample buffer	0.225 M tris.Cl, pH 6.8	1 M Tris.Cl, pH 6.8	2.25 ml
	50% glycerol	Glycerol	5 ml
	5 % SDS	SDS	0.5 g
	0.05% bromophenol blue	Bromophenol blue	5 mg
	0.25 M dithiothreitol (DDT)	1 M DDT	2.5 ml
Solutions		**Source**	
30% Acrylamide	30% Acrylamide	Sigma Aldrich (Germany)	
TEMED	N,N,N,N'-tetramethylenediamine	Promega (USA)	
APS	Ammonium persulphate	Bio-Rad (California)	

Table 5c. Coomassie Staining solutions

Solution	*Compositions*	*Components*	
			per 100 ml
Coomassie staining solution	0.05% (w/v) Coomassie Brillant Blue R-250	Coomassie Brillant Blue R-250*	50 mg
	40% (v/v) ethanol	Dissolve * in ethanol, then add:	40 ml
	10 % (v/v) glacial acetic acid	Glacial acetic acid	10 ml
	50% (v/v) water	Water	50 ml
		Filter before use	
Destaining solution	40% (v/v) ethanol	Ethanol	40 ml
	10 % (v/v) glacial acetic acid	Glacial acetic acid	10 ml
	50% (v/v) water	water	50 ml

* purchased from BDH, Biochemical, England

Table 5d. Western Blotting Transfer Buffer

Buffers	*Compositions*	*Components*	
			per liter
Transfer buffer	25 mM Tris base	Tris base	3.0 g
	150 mM glycine	Glycine	11.3 g
	20% (v/v) methanol	Methanol	200 ml
	pH 8.3	without adjustment	

Table 5e. Immunodetection buffers

Buffers	*Compositions*	*Components*	
			per liter
TBS buffer	10 mM Tris.Cl	Tris base	8.8 g
	150 mM NaCl	NaCl	1.2 g
	pH 7.5	adjust with HCl	
TBS-Tween buffer	0.5% Tween	Tween 20	5 ml
	TBS buffer	TBS buffer	1 liter
			per 10 ml
Primary and secondary antibody dilution buffer	5%(w/v) nonfat dried milk powder	Nonfat dried milk powder	0.5 g
	TBS buffer	TBS buffer	10 ml
Substrate (NBT/BCIP)* Stock solution	18.75 mg/ml NBT + 9.4 mg/ml BCIP in 67% (v/v) DMSO	NBT/BCIP stock solution	200 µl
	0.1 MTris-HCl	Tris base	12.114 g
	0.1 M NaCl	NaCl	5.844 g

*Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate

Chapter 6

Synthesis –
Implications of ammonia stress for abalone farming

Abstract

The findings of the present study was synthesised to demonstrate their applicability to the management of ammonia stress in farmed *H. midae*. Firstly, the potential consequences of chronic effects of ammonia on farm production were assessed using two growth models. Model 1 was applied to *H. midae* from 0.550 years to ~0.772 years (~1.89 cm; 0.730 grams), and used specific growth rates (for weights, SGRW) from the present study to calculate changes in mass over time. A purely empirical curve (model 2) which calculated growth as a function of mass, was fitted to a combination model 1 and a field-based growth model, and was applied to abalone from age 0.772 years onwards. The mass of abalone at age 4.5 years (when farmed *H. midae* are typically harvested) was calculated for different periods of exposure to sub-lethal FAN concentrations. Mass decreased with increasing periods of stress, with yield potentially reduced to 20.5% of the normal yield (no ammonia stress) for sustained chronic exposure. Monitoring of water quality can contribute to preventing ammonia stress, but these indicators of animal stress are indirect. Direct monitoring of the animals can be done using biomarkers. Both HSPs and P-gp could be used as biomarkers of ammonia stress and poor health in *H. midae*, although further research and improved (cheaper) technology would be needed to make this feasible.

6.1. Introduction

The successful farming of abalone depends on the selection of the most suitable species for a given culture environment. In South Africa, *Haliotis midae* is the abalone species of commercial importance because of its successful spawning in captivity (Genade et al., 1988), its adequate rate of growth, and its good food conversion efficiencies, all of which are essential for farming (Cook, 1998). The successful growth and health of the species in turn is influenced by the quality of its environment. For *H. midae* (as for all aquaculture animals) this environment is water, and ammonia concentrations can rapidly influence the quality of water.

The overall aim of this chapter was to provide a synthesis of the findings of the present study and to demonstrate their applicability to the management of ammonia stress in farmed *H. midae*. This was achieved by illustrating the effect of increasing ammonia exposure on abalone growth, and by investigating the use of both heat shock proteins (HSPs) and P- glycoproteins (P-gp) as biomarkers for detecting ammonia stress and poor health in *H. midae*.

6.2. Acute and chronic effects of ammonia

Farms routinely use one of two chemical kits to measure TAN, namely the traditional Nesslers method test or the more recent Palintest. The present study (Chapter 2) found that both these tests underestimate ammonia concentrations, but that the Nesslers method was more accurate than the Palintest. This needs to be confirmed by additional studies. Considering that minute changes ($\mu g.l^{-1}$) of FAN (Chapter 3) can be detrimental to the abalone and reduce growth rates substantially, correct TAN measurements are important; abalone farmers should use these tests with caution. Similarly, correct FAN calculations are important.

Regular monitoring of all parameters (TAN, pH, temperature and salinity) that affect the amount of FAN present is necessary (see Chapter 2). The calculation of FAN from TAN

using different tables provided by Bower & Bidwell (1978), Emerson et al. (1975), Huguenin & Colt (1989) and Trussell (1972) lead to discrepancy in the results. The present study (Chapter 2) found that the tables provided by Bower & Bidwell (1978) were the most appropriate for seawater analysis because the effect of salinity on FAN values has been thoroughly investigated and incorporated into the tables.

The present study (Chapter 3) found that the acute effect of ammonia varied among different size classes and the sensitivity to ammonia decreased with increasing size but is higher in wild compared to farmed abalone. The sub lethal levels of ammonia for farmed juveniles, farmed cocktail, wild cocktail and wild brood were 7.4 $\mu g\ l^{-1}$ FAN, 19.69 $\mu g\ l^{-1}$ FAN, 10.0 $\mu g\ l^{-1}$ FAN and 11.4 $\mu g\ l^{-1}$, respectively. The sublethal level of 7.4 $\mu g\ l^{-1}$ FAN (Chapter 3) for juvenile abalone was used to determine safe levels of FAN for *H. midae*, as this is the most sensitive size class. The findings of the present study suggest a safe FAN level for *H. midae* of below 7.4 $\mu g\ l^{-1}$. However, this concentration was subsequently demonstrated to reduce growth by more than 50% in juvenile *H. midae*, though no mortalities occurred at this concentration (Chapter 4). The implications of the results of the chronic ammonia exposure growth experiments (Chapter 4) were investigated by modeling the effect of extended ammonia exposure on the yield of abalone after 4.5 years (age when farmed abalone are harvested).

Because there were no growth data for farmed cocktail *H. midae*, data for their growth parameters were obtained from Tarr (1995) for wild abalone. Growth data for farmed juveniles were obtained from Chapter 4. Two models of abalone growth were developed, applicable to the smallest sizes (model 1) and the full size range (model 2).

Model 1 was applied to *H. midae* from 0.550 years to ~0.772 years (~1.89 cm; 0.730 grams), and used specific growth rates (for weights, SGRW) from Chapter 4 to calculate changes in mass over time:

$$\text{Mass}(t + 1) = \text{Mass}(t) + \text{SGRW} * \text{Mass}(t) \qquad (6.1)$$

where t = time in days and SGRW = 0.00243 d^{-1} under normal growth and 0.001 d^{-1} under ammonia stress. Growth depends on abalone mass in model 1.

For model 2, growth parameters of *H. midae* were obtained from Tarr (1995) for wild abalone from Betty's Bay, such that

$$\text{Shell length (mm)} = (L\infty\ (1-e^{-K \times \text{age}})) \qquad (6.2)$$

where L∞ is the maximum length = 172.76 (mm) and *K* is the rate of growth = 0.186 y^{-1}.

Using equations from Tarr (1993), shell lengths were converted to shell breadths (mm)

$$\text{Shell breadth (mm)} = (0.913 \times \text{length (mm)}) - 11.59 \text{ mm} \qquad (6.3)$$

and shell breadths were converted to abalone mass (g):

$$\text{Whole wet mass (g)} = 0.002 * (\text{Breadth (mm)})^{2.614} \qquad (6.4)$$

These equations allow the calculation of *H. midae* growth as a function of abalone age. The age-dependent mass from model 1 under normal growth (assuming a 0.6 g animal is 0.55 years) was linked to that predicted by Tarr's (1995) growth equations at 0.772 years, when the mass obtained using model 1 corresponded to the mass obtained using Tarr's (1995) model i.e. at 0.73 g. The mass-age relationships from Tarr's (1995) model and model 1 are illustrated in Figure 6.1. A purely empirical curve (model 2) that calculated growth as a function of mass was fitted to these two combined data series but was applied to abalone from age 0.772 years onwards. Model 2 therefore uses mass and not age as the predictive variable for growth increments:

$$\text{Mass}(t+1) = \text{Mass}(t) + a\left(1 + \left(\frac{\text{Mass(t)}}{b}\right)^{c}\right) \qquad 6.5$$

where a, b and c are arbitrary parameters with fitted values of a = 0.001802; b = 0.000841 and c = 0.490600.

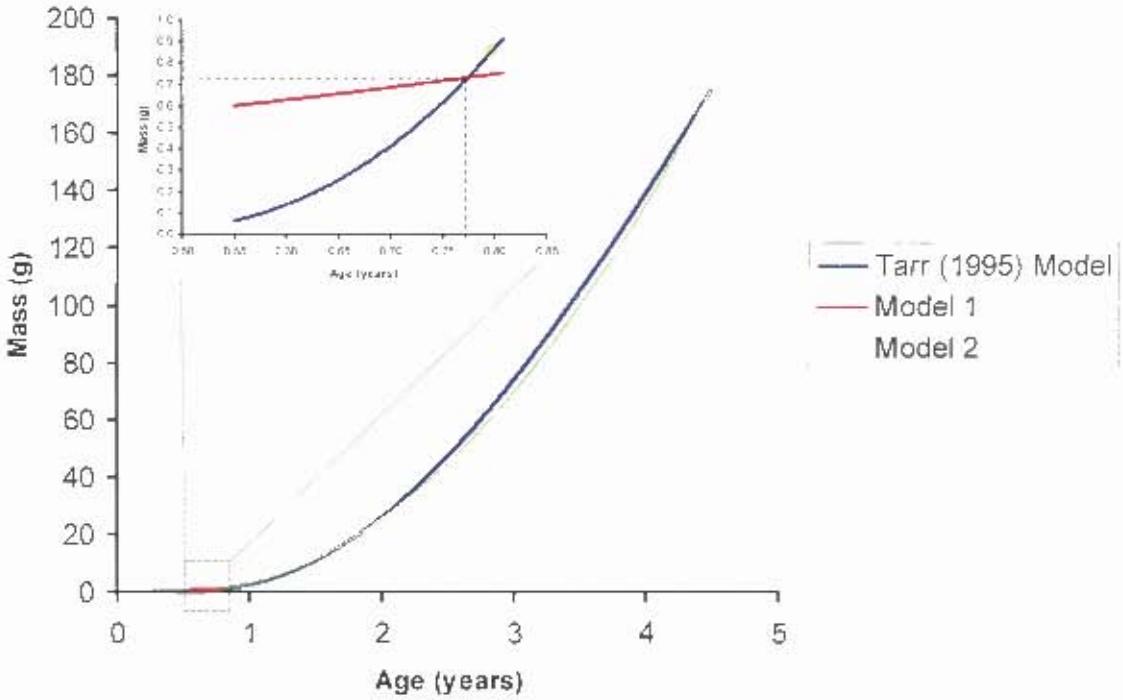

Figure 6.1 Illustration of the mass-age relationships for the different models.

Using the two models of abalone production (in terms of mass), hypothetical *H. midae* were subjected to different periods of ammonia exposure, during which time their growth rates were reduced to 59% of normal, and the final mass at 4.5 years was compared to the "normal" mass (with no ammonia stress) (Figure 6.2). Three trials, which differed in the ages at which exposure started, were used to assess the variability introduced by using models 1 and 2 for different ages. In all three trials ammonia exposure lasted 80 days. In trial 1, ammonia exposure was applied to abalone from age 0.550 to 0.769 years (full age range corresponding to model 1). In trial 2, ammonia exposure was applied from age 0.775 – 0.994 years (first 80 days of model 2). In trial 3, exposure was applied from age 0.660 – 0.879 years (starts at 40 days prior to the end of model 1 and ends at 40 days after the start of model 2). All three trials showed a decrease in the percentage of final mass after 4.5 years (Table 6.1) varying from 93.61% to 99.56%. The calculations are therefore only moderately sensitive to the models used.

Table 6.1 Percentage of final mass at 4.5 years after 80 days of ammonia exposure.

Trial	Percentage of final mass
1	99.34
2	93.61
3	99.56

A fourth trial was then carried out, in which ammonia stress was applied to the youngest *H. midae*, with exposure periods ranging from 10 to 1440 days. Final mass at age 4.5 years decreased with increasing periods of stress (Figure 6.2), with potential yield reduced to 20% of what might be expected, if there is chronic ammonia stress throughout an animal's farmed life. These calculations are simplistic, and would need to be confirmed by experimental testing. However, they provide a first estimate of the potential effect of chronic exposure to ammonia on production of an abalone farm.

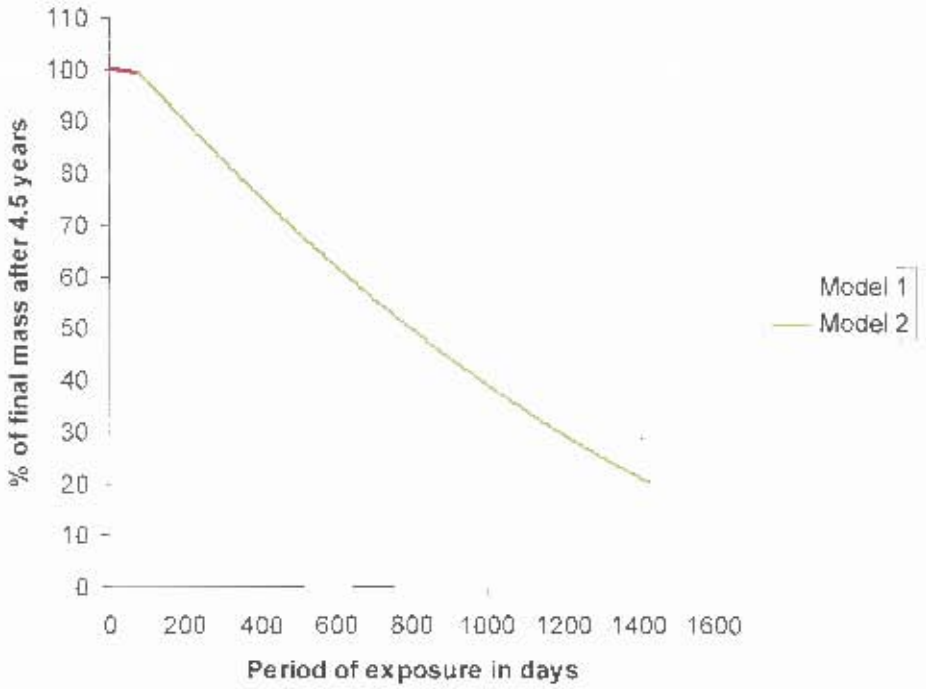

Figure 6.2 The percentage of final mass after 4.5 years for different periods of ammonia exposure.

The results of this chapter demonstrate that significant losses in revenue might occur even for relatively short term ammonia exposures. The impact ammonia will have on the total revenue lost will depend on the exposure time and the total number of abalone that are affected. Routine monitoring of all water quality parameters that affect FAN concentration are needed to maintain low FAN. In addition, various systems can be employed to minimise ammonia levels, although they have had varying degrees of success to date.

Co-culturing of abalone and seaweed (Bredberg, 2003; Robertson-Andresson, 2003) has been introduced on some South African farms on an experimental basis. The main motivation has been due to over-harvesting of kelp (Troell et al., 2006) resulting in a search for a less costly and more reliable feed source. Seaweeds have been shown to have a dual function in the system in that they not only provide feed for abalone but also absorb nutrients (such as ammonia) (Langdon et al., 2004). However, the process of co-culture of abalone and seaweeds is complex. The species of seaweed chosen is important in terms of the amount of ammonia absorbed, as well as in generating the lowest ammonia excretion rates (Bredberg, 2003). Build up of ammonia can occur in intensive culture systems. Abalone fed on a diet of formulated feed, as practiced on farms, excrete higher levels of nitrogenous wastes than abalone fed on kelp (Chalmers, 2003). Some farms utilize co-culture only in the outlet tanks to reduce the nutrient content of the seawater released back into the sea (P. Cook, pers. comm.), thereby minimizing negative impacts on natural ecosystems (Lubchenco, 2003; Naylor et al., 1998; Qian et al., 2001). Currently only two abalone farms in South Africa (located in the Eastern Cape) culture *Gracillaria* and *Ulva* using waste water from the abalone tanks, as they don't have access to fresh supply (Troell et al., 2006). The utilization of seaweed for the removal of ammonia may be a solution for temporary closures of seawater flow-through.

It has been suggested that land-based fish culture systems far from the sea should be operated at low pH, making it possible to operate at high TAN concentrations without exceeding both CO_2 and FAN threshold levels (Eshchar et al., 2006). This would help reduce high seawater pumping costs and/or nitrification biofilter recycling costs

associated with ammonia removal from the system. Most South African abalone farms are based near the coast and operate an open circulation system (with varying degrees of re-circulation). Removal of ammonia is usually not a problem, but during forced closure ammonia can increase and become toxic to the animals. The option of running a system at high TAN and low pH for limited periods should be investigated. More research, however, is required to determine the lethal levels of CO_2 and pH for *H. midae*. Harris et al. (1998a) have reported reductions in growth of abalone at low pH (~7.8). Abalone farmers could then reduce the pH by increasing CO_2 without the addition of chemicals, thereby reducing the toxicity of FAN (EIFAC, 1973).

The problem of elevated ammonia levels is likely to persist in abalone farms. Both chronic and acute effects have been quantified here. Constant monitoring and mitigation measures are both needed to manage the problem. Monitoring of water quality is an indirect measure of stress for farmed animals whereas biomarkers can be direct measures.

6.3 Heat shock proteins (HSPs) and P-glycoprotein (P-gp) as biomarkers of ammonia stress in *H. midae.*

The use of HSPs and P-gp as biomarkers of ammonia stress and poor health in *H. midae* has been proposed. The heat shock or stress response is one of the most highly conserved adaptive responses in nature. HSPs are inducible by a variety of stressors besides temperature and ammonia, and could include other environmental toxins such as PSP and DSP, parasites or bacterial infections or even high stocking density (which leads to high ammonia levels). The complex relationship that exists between the stress response and disease has been implicated in disease outbreaks in many animals, including abalone (Hooper et al., 2006). The central role of stress proteins in the transfer of peptides through the cell (Moseley, 2000) may be responsible for their recently recognised importance in the modulation of the immune system.

Our understanding of the immune system and stress response in abalone is very limited and based mainly on vertebrates; limited work has been conducted on some mollusc

species. Cheng et al. (2004) has demonstrated that even mild stressors resulted in increased susceptibility in abalone to infection with *Vibrio* species, and increased mortality due to suppression of the immune system. The measurement of a single parameter of the immune system, such as fluctuating haemocyte counts or cytokine and hormone levels, does not fully summarise the state of an abalone's immune system. The stress response of abalone, similar to the vertebrate endocrine system, probably originates with corticotrophin releasing hormone (CRH) stimulating the release of adrenocorticotrophic hormone (ACTH), leading to the release of biogenic amines and interleukins, which in turn mediate secondary effects in the molluscs (Hooper et al., 2006; Ottaviani et al., 1995). In molluscs all these molecules of the stress response are located in the haemocyte cells and not distributed in different organs as in vertebrates (Ottaviani & Franceschi, 1997). Among the biogenic amines is noradrenalin, which has been demonstrated to induce expression of HSP 70 in haemocytes of *H. tuberculata* in-vitro (Lacoste et al., 2001). This stimulatory effect could be blocked using various inhibitors, demonstrating a functional link between neuroendocrine signalling and the HSP 70 response in mollusc's cells (Ottaviani et al., 1995; Ottaviani & Franceschi, 1996). Interestingly, the complex interaction of stressors with the immunological stress response has not been studied in detail in abalone, but in other gastropods. Research on identifying which kind of stress or immune parameters accurately predict what is happening in the animals is far from over (Hooper et al. 2006).

The presence of HSPs may indicate that an animal is trying to cope with the immediate effects of a stressor, but it also shows that the animal is stressed. The immune system is weakened by this, allowing other factors such as bacterial infection (Cheng et al., 2004) to affect abalone health. Future research into implementing the use of stress proteins as biomarkers for the detection of ammonia stress in abalone may help with biomonitoring of water quality which in turn can improve abalone health, reducing mortalities and increasing growth rates.

Biomarker responses should be sublethal, quantifiable and reliable for interpretation (Werner, 2004). The results of the present study showed an increase in the induction of

specific HSPs (depending on the type of tissue and stressor) and P-gp in gills of *H. midae* under sublethal ammonia conditions. These results are reproducible and quantifiable using a robust antibody based assay. Immunological detection of both HSPs and P-gp in the present study, although difficult and expensive (Smital et al., 2000), may prove to be useful biomarkers in assessing ammonia and heat stress in aquaculture animals. There is a strong need for multiple biomarker screening, such as for HSPs and P-gp, in biomonitoring of environmental toxins, to assist farming of abalone in South Africa.

The use of HSPs and P-gp as potential biomarkers for environmental studies has been addressed in a number of studies (Bierkens, 2000; Eufemia and Epel, 2000). In molluscs, HSPs have proven to be an important part of a suite of biochemical markers of xenobiotic stress (Snyder et al., 2001). Huchette et al. (2003) found that growth in the Australian blacklip abalone, *Haliotis rubra*, was affected by increased stocking density either directly through competition for space or indirectly by deteriorating water quality (increased FAN levels). Rossi & Snyder (2001) proposed the use of HSP 70 as a tool for evaluating space competition among sessile marine invertebrates. The present study proposes the use of HSPs and MXR as biomarkers for evaluating stress incurred by increased stocking densities (results in increased FAN levels) as ammonia induces both HSPs and MXR. On abalone farms FAN levels can occur in spikes (within a few hours) and frequent incidences of such spikes could impact negatively on abalone growth. Measurements of FAN levels outside these spike occurrences could be misleading and problematic as the FAN levels appear to be normal (due to flushing of the system). Measurements of stress protein levels in the present study were detectable for up to 36 hours, although this was for continuous ammonia exposure. For heat exposure experiments, however, stress protein levels remained elevated for only one hour. Future research should include recovery experiments (return to normal conditions after short term ammonia exposure) to determine how long stress proteins remain elevated. Such experiments have been carried out using heat stressed abalone, *Haliotis dicus hannai*, where the return of HSP 70 to normal conditions occurred only after 96 hours (Cheng et al., 2006). These studies may determine if stress proteins could be used as biomarkers to help monitor ammonia spikes on farms.

6.4. Conclusions

The present study has demonstrated that ammonia impacts negatively on *H. midae*, leading to reduced growth rates and yield. The growth rates obtained in this study for juvenile abalone (model 1) are slower than those observed on commercial farms, which can be attributed to differences in diets; farms include commercial feed in their diets, whereas the experimental abalone were fed kelp for the duration of the experiments. The magnitudes of the potential changes in growth rates might therefore be different in a farm situation. However, the results indicate that a reduction in potential yield of approximately 20 % could be possible if there is chronic ammonia stress throughout the animal's life. Future research into implementing HSPs and P-gp as biomarkers for the detection of ammonia stress in abalone may help reduce these losses. The success of abalone aquaculture in South Africa depends on ongoing research on the farmed species, *H. midae*. "The marine resources are a national asset and part of the heritage of the people of South Africa, present and future, and should be managed and developed for the benefit of the country as a whole, especially those communities whose livelihood depends on these resources" (Martin & Nielsen, 1997).

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