Development and optimisation of relaxants for use in the Australian abalone industry.

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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text

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Thesis summary

Handling abalone is often difficult because they adhere strongly to available substrates and are difficult to remove. Relaxants, products that decrease muscle tone and the ability of the abalone to grip the substrate, are used in abalone aquaculture to facilitate handling, transport, grading stock and to minimise injury, immune suppression and mortality associated with handling. The principle aim of this study was to identify and evaluate relaxants for reliable, safe relaxation of greenlip abalone (*Haliotis laevigata*) in the Australian aquaculture industry. Safe relaxation implies that the relaxant has no acute or chronic detrimental effects on abalone, that it can be applied safely, that meat from treated abalone can be consumed without risks to human health and that there are no unduly hazardous environmental effects of use or release of the product.

The efficacy of 7 potential relaxants: propylene phenoxetol; magnesium sulphate ($MgSO_4$); magnesium chloride ($MgCl_2$); MS-222; clove oil; AQUI-S™ and 2-phenoxyethanol were screened using benzocaine, the only permitted relaxant for abalone in Australia (PER 14638) (APVMA 2016c) as a comparative baseline. Benzocaine has adverse effects when used on farmed abalone, including causing mortality in treated stock. Three relaxants ($MgCl_2$, 2-phenoxyethanol and propylene phenoxetol) met all response, behavioural, growth and water quality criteria for use.

Doses of 2-phenoxyethanol, $MgCl_2$ and propylene phenoxetol were optimised for small (shell length 18-28 mm) and large (70-90 mm) abalone at 14, 18 and 22 °C. Successful relaxation occurred in <5 minutes and small abalone tolerated 10 minutes exposure and large abalone tolerated 20 minutes exposure. All abalone remained relaxed for at least 10 minutes after cessation of treatment but successfully recovered in <20 minutes. Propylene phenoxetol did not, however, successfully relax large abalone. Abalone are subject to frequent handling for grading and density management throughout grow-out and these procedures occur in all

seasons, so dose-size-temperature relationships were compiled to show optimised doses under different conditions.

Host safety was assessed for small and large abalone for overdose and overexposure to 2-phenoxyethanol or $MgCl_2$ to assess the lowest doses and shortest durations of exposure at which mortality occur and assess potential negative effects on health or growth. The margins of safety for 2-phenoxyethanol or $MgCl_2$ demonstrate that abalone can be safely relaxed using optimised dose and exposure recommendations with adequate flexibility to avoid acute effects and without long term effects on growth.

Magnesium residues were analysed to determine $[Mg^{2+}]$ in the muscle tissue of large abalone that had been relaxed using 160 g/L $MgCl_2$ for 20 minutes. $[Mg^{2+}]$ depleted to background levels within 3 hours of cessation of treatment and return to seawater. Maximum $[Mg^{2+}]$ in the edible portion of treated tissue is safe for consumption, demonstrating that no withholding period is required and that $MgCl_2$ is a suitable relaxant for use prior to live transport or for rested harvest.

This work successfully identified 2-phenoxyethanol and $MgCl_2$ as suitable for use as relaxants in the Australian abalone industry. Both relaxants meet industry use criteria and are safe for abalone. This work provides a substantial contribution to data required for applications to obtain regulatory authorities for use of these products as veterinary medicines and provides recommendations for work to further refine and optimise practical application in aquaculture.

Chapter 1: General introduction

1.1 Introduction

Global consumption of abalone (*Haliotis* spp.), primarily in Asia, has been greater than supply from wild fisheries since late 1990s (Cook & Gordon 2010). Wild stocks have declined, due to overfishing, disease and environmental change and diminishing world supply of wild caught product has stimulated the growth of global abalone aquaculture (Gordon & Cook 2004, Cook & Gordon 2010).

Abalone aquaculture began in Australia in the 1980s in South Australia and Tasmania. From the mid-1990s specialised diets and improved tank technology allowed the production cycle to be shortened with market size (70 mm shell length) being attained in ~3 years (Burke et al. 2001). Abalone farms were then established in Victoria and Western Australia. Australian farmed abalone production was projected to increase by 456 to 965 metric tonnes per year between 2010 and 2015 (FAO 2014) and continues to expand substantially (AAGA unpublished data). The most commonly farmed species in Australia is greenlip abalone (*Haliotis laevigata* Donovan) and its hybrid with blacklip abalone (*Haliotis rubra* Leach), totalling over 70% of all farm production. Pressure to improve farming efficiency has motivated abalone farmers to increase biomass per area but associated stress-induced disease and mortality has limited production increases at some facilities (Stone et al. 2013).

Abalone possess a large, muscular foot, which facilitates adhering tightly to substrates and makes it difficult to remove abalone from aquaculture systems (Aquilina & Roberts 2000, Bilbao et al. 2010). Abalone husbandry practices such as grading, adjusting stocking density, transfer between systems, system maintenance and harvesting, however, require periodic removal of abalone (Ross et al. 2007). The large numbers of individual abalone in a commercial farm make mechanical removal of each abalone logistically impractical and costly. Mechanical dislodgment, moreover, often causes injury or

death (Ross et al. 2007), principally as a consequence slow healing rates and the limited capacity of abalone to coagulate haemolymph (Hooper et al. 2014).

Stress caused by handling in abalone aquaculture stimulates nociceptive reactions, avoidance and withdrawal from harmful stimuli. This is similar to responses observed in vertebrates and can have acute and chronic effects on abalone health including immune suppression, disease and mortality (Malham et al. 2003). As abalone aquaculture production increases, poor outcomes following mechanical removal of abalone from substrates increase. Production increase requires a larger workforce to achieve basic husbandry activities and economic effects will make Australian abalone farming less competitive with low-cost countries. These factors dictate the need for simplified methods to handle abalone and reduce associated stress. The main method for reducing stress is finding methods to minimise handling. These include using movable plates for settlement and once the abalone have depleted the algae, transferring juveniles to grow-out systems by moving the plates (Daume & Ryan 2004), minimising grading and increasing cleaning frequencies by using more flow or pulsed addition of new seawater and the use of immersion practice for chemical muscle relaxation.

Relaxants are products that cause a decrease in muscle tension. Chemical muscle relaxants are external neuromuscular blocking agents that can be added directly to the water. While ‘anaesthetics’ is usually used to describe neuromuscular blocking agents, which also blocks , invertebrate pain is still undescribed, so the term ‘muscle relaxant’ best describes the veterinary medicines used to induce this state for abalone in our study. Handling relaxed abalone is safer, less invasive and more economical than other methods (Burke et al. 2001). For a relaxant to be useful for abalone aquaculture, it must: induce gradual loss of adherence to the substrate in <5 minutes, allow recovery in <20 minutes after cessation of treatment with no mortality or ongoing effects on behaviour, have an adequate margin of safety, be

easy to administer and be inexpensive. If products could be identified that are safe for human consumption at harvest, the product would have the added advantage of being suitable for rested harvest (Hooper et al. 2011).

In Australian abalone aquaculture, only benzocaine (ethyl 4-aminobenzoate CAS 94-09-7) is legally available for use as a relaxant pursuant to an Australian Pesticides and Veterinary Medicines Authority (APVMA), Minor Use Permit (MUP) PER14638 (APVMA 2016c). Literature (Hooper et al. 2011, Hooper et al. 2014) reports indicate that benzocaine use has some adverse effects, such as hyperactivity during induction of relaxation, stimulation of increased mucus production, alteration of product taste and long term mortality. Benzocaine use is also subject to a 500 degree-day withholding period, so it is not suitable for rested harvest (Hooper et al. 2011).

Alternative relaxants such as 2-Phenoxyethanol, are currently used for managing abalone on an *ad hoc* basis in abalone aquaculture in some Australian states under veterinary prescription (AAGA consultation). Prescription of unregistered products places substantial liability on veterinarians and growers and the absence of safety data can cause mortalities if the products are not used judiciously. Differences in veterinary medicine legislation also mean that off-label prescription is not legal in all Australian States and Territories. Obtaining regulatory approvals for more and better relaxants will improve survival, welfare outcomes and farm profitability, particularly where adverse responses to benzocaine have been observed. Improved relaxants will aid industry to improve product quality and the efficiency of Australian farmed abalone production and increase certainty in export markets. Obtaining regulatory approval for a relaxant that can be used for rested harvest would also improve product quality and survival after transport to market for live abalone.

Legitimizing the use of other relaxants for Australian farmed abalone species requires data collation, identification of data gaps against APVMA data requirements (Burke et al.

2001) then obtaining information that addresses those gaps. An application to the APVMA for regulatory approval for a new relaxant requires exhaustive documentation of chemistry and manufacture, toxicology, metabolism and kinetics, residues, occupational health and safety, trade and any other data needed to demonstrate that efficacy and safety requirements and environmental safeguards are met and that human health and export restrictions are addressed (APVMA 2016a). The data need to demonstrate that the proposed patterns of use of the relaxant is effective while also being safe for people and the environment, will not have negative health effects or affect trade by exceeding safe residue limits. Generating the data to support a regulatory authority application for a veterinary product is costly, which dictates that careful product screening is required before detailed studies are commenced and aquaculture businesses have strong economic drivers to support only the development of products that are necessary and needed by industry.

1.2 Study objectives

This project arose from needs identified by the Australian Abalone Growers' Association (AAGA) and consultation with industry. The two main objectives of this study were to find suitable relaxant products(s) as alternatives to benzocaine for mass immersion relaxation for the Australian abalone industry and to obtain sufficient information on the identified candidate relaxants to generate applications to the APVMA to obtain Minor Use Permits. The objectives of this work were:

1. To identify candidate relaxants for use on Australian abalone from literature and identify target species data gaps for approval by APVMA.
2. To compare the efficacy of current and candidate relaxants for use in Australian abalone.
3. To optimise relaxant dose of candidate relaxants for use on Australian abalone.
4. To assess host impact for the candidate relaxants.
5. To analyse the fate of administered relaxants and their residues.
6. To assess potential of candidate products for rested harvest for direct sale of relaxed animals from farms to human consumption markets.

1.3 Thesis outline

This thesis is presented in 7 chapters, a general introduction, a literature review, four experimental studies and a general discussion. Chapters 2 - 6 are currently in review or preparation for submission to peer-reviewed journals.

Within chapter 2 is a description of the relaxant pattern of use in abalone aquaculture and selected candidate relaxants with potential for use on Australian abalone and identification of data gaps for approval by APVMA. The need for relaxants to enhance animal welfare, decrease stress and improve husbandry procedures to increase commercial

efficiency is described. Literature on relaxant use in molluscs is reviewed and current and potential use is identified. The process for obtaining regulatory approval for veterinary medicines through the APVMA is also summarised.

In chapter 3, the screening of candidate relaxants for abalone, for behaviour and dose efficacy and effects on growth is investigated. Seven potential relaxants identified in chapter 2 were investigated for use on Australian abalone. A range of doses were derived from literature from other mollusc species and adapted for use on one year old abalone. The products were assessed against industry requirements for practical, good relaxant use, including effects on behaviour, growth and survival. Negative behavioural reactions to the relaxants were used as proxies for stress when observing responses to the relaxant. These trials were designed to provide comparisons of current and candidate relaxants for abalone, to identify relaxants with the least detrimental effects on abalone. Three relaxants were identified for further investigation.

In chapter 4, the effect of dose, temperature and abalone size on efficacy of three prioritised candidate products is investigated. Doses were optimised for two size classes of *H. laevigata* at three different temperatures. Relaxants were assessed against industry requirements. Two candidate relaxants were prioritised for further investigation.

Chapter 5 includes dose and duration safety studies for immersion treatment of abalone using the two relaxants identified as suitable for further work in chapter 4. Optimal dose and exposure time for these relaxants were exceeded and assessed for mortality. Margin of safety (MOS), LT_{50} and LC_{50} values were obtained to guide safe use and to prevent mortality under the proposed pattern of use.

In chapter 6, residues of one of the prioritised relaxants in the edible portion of abalone following exposure using inductively coupled plasma mass spectrometry (ICP-MS) is investigated. The product assessed was determined to be safe for rested harvest.

Chapter 7 is the general discussion, where all major research outcomes are compiled and summarised. Final product recommendations to the abalone aquaculture industry are provided to improve husbandry and to ensure effective, safe patterns of use. Recommendations to extend this research are provided.

1.4 Publications

Chapter 3, 4, 5 and 6 are presented in stand-alone manuscript format for the *Journal of Shellfish Research*. As a result, there is some repetition between chapters, particularly in methods and background. I wrote all chapters, but each chapter is co-authored due to major contributions from other people. Each chapter is co-authored by my supervisors Associate Professor James Harris and Dr Marty Deveney, who provided major contributions to experimental design and the preparation of manuscripts.

1.5 Trials

Greenlip abalone, (*Haliotis laevigata* Donovan) were used in this study. This species is endemic to southern Australia. All abalone used for this project were obtained from available aquaculture stock. Small abalone were 18-28 mm shell length and one-year-old and large abalone were 70-90 mm SL and two and a half years old. The large abalone were used to test effects of relaxation on harvest size abalone. Experimental observations included careful monitoring of temperature, relevant water quality parameters and abalone feeding and behaviour. Existing data was compiled from published literature and data gaps were addressed through experimental and laboratory analyses. All studies were preceded and followed by extensive industry consultation. All studies were conducted in accordance with the OECD Principles of Good Laboratory Practice (GLP) (WHO. 2016). All studies were undertaken at GLP-compliant facilities. Tank studies were conducted at the South Australian Research and Development Institute (SARDI), South Australian Aquatic Sciences Centre, West Beach and Flinders University, Bedford Park, South Australia.

Chapter 2: Mollusc relaxants and their use in the Australian Abalone Industry.

Mercer G. J., J. O. Harris & M. R. Deveney. 2016. Mollusc relaxants and their use in the Australian Abalone Industry. J. Shellfish. Res. (in preparation)

2.1 Abstract

Demand for molluscs as food creates market forces that drives expanding aquaculture industries. The substantial taxonomic differences between molluscs and other, often vertebrate, aquaculture species and the low knowledge base about molluscs mean that there are numerous areas where improving production techniques requires research. One area of invertebrate biology that is poorly understood is stress. Human interaction during production techniques is a serious cause of stress in aquaculture. Farmed molluscs exhibit nociceptive reactions to noxious stimuli similar to the responses of vertebrates and stress associated with routine husbandry procedures such as size-sorting, tank splitting and harvest can have acute and chronic effects on growth or cause mortality. Relaxant products are needed in abalone aquaculture because they reduce responses to noxious stimuli and relax the foot, facilitating easy removal of abalone from the substrate for husbandry procedures and may reduce or eliminate stress induced limits on production.

This chapter presents an overview of invertebrate nociception, its relationship with stress and health and how relaxation can decrease nociception, stress and associated negative effects on health. Mollusc relaxation is reviewed and patterns of relaxant use, how these are influenced by current knowledge and the target species is described, with a focus on abalone aquaculture.

2.2 Introduction

Molluscs are farmed worldwide as a food source and for jewellery. Global demand for abalone (Mollusca: Haliotidae: *Haliotis* spp.), primarily from Asia, has exceeded supply from wild fisheries since the late 1990s (Cook & Gordon 2010). Overfishing, disease and habitat loss have caused declines in wild abalone stocks (Cook & Gordon 2010). Market forces and diminishing wild supply have stimulated the growth of abalone aquaculture globally (Gordon & Cook 2004, Cook & Gordon 2010). While there is up to 100 species of abalone worldwide, only about 15 are grown with the use of aquaculture for human consumption (Gordon & Cook 2004, Cook & Gordon 2010). Sustainably farmed abalone can increase world abalone supply without increasing pressure on wild abalone stocks (Burke et al. 2001). While the total of species of abalone worldwide ranges (source dependent) from approximately 56 to 100, aquaculture production only centres around roughly 15 species, subspecies, and hybrids of Haliotis spp. (Geiger 2000) (Allsopp et al. 2011). China is the leading producer of abalone in the world, producing nearly 115 400 tonnes in 2014 and the Republic of Korea has become the second largest worldwide producer at over 9 000 tonnes in 2014 (FAO 2014). Farmed mainly in Asia, Japanese abalone (*H. discus hannai*) totals to about 97% of worldwide production (Allsopp et al. 2011).

Worldwide, abalone farming comprises of three phases: hatchery, juvenile, and grow out. During hatchery and juvenile phases, abalone are grown in land-based raceway tanks and/or holding tanks. During the grow out phase, abalone can be grown using a variety of production systems, including on land raceway tanks, tethered cages or other enclosures at sea and unenclosed sea ranches (Cook & Gordon 2010). Land based production systems may use any combination of single-pass, flow-through tanks. Primarily in Asia, tethered cages or other enclosures are used for grow out stages at sea. While some forms of sea ranching still

take place, sea ranching is not as common as it once was and new, more sustainable approaches for sea ranching are being developed (Allsopp et al. 2011).

While tethered cages or other enclosures at sea are primarily used in Asia for grow out production systems, land-based systems are by far the most popular system used in Australia (MESA 2014) (Government of South Australia 2015), with over 880 metric tons (MT) out of 945 MT of production coming from land-based farms in 2014/2015 (pers. comm., Australia Abalone Growers Association).

Australian farmed abalone production was predicted to increase by 456 metric tonnes to an estimated 965 metric tonnes per year between 2010 and 2015 (FAO 2014). The Australian abalone aquaculture industry shows considerable potential, with the availability of suitable species such as greenlip abalone (*Haliotis laevigata* Donovan), a clean environment, a local base of skilled, experienced workers, availability of high quality abalone feeds, access to advanced processing, quality marketing and a range of locally developed grow out systems (Burke et al. 2001, Gordon & Cook 2004). In Australia and worldwide, however, the continued pressure to increase production caused increased cumulative stress on farm stock and prevented further increases in productivity (Gordon & Cook 2004).

Respect for food animals needs to be considered in the context of an ethical template that gives respect to the principles of wellbeing, autonomy and fairness to farm animals, consumers, farmers and the living environment (Webster 2001). The welfare of a farm animal depends on its ability to sustain fitness and avoid suffering (Chandroo et al. 2004a, Chandroo et al. 2004b, Braithwaite & Boulcott 2007). Finfish are often assumed to feel pain (Rose 2002, Sneddon 2002, Sneddon et al. 2003, Sneddon 2004, Sneddon 2009) and a wide range of anaesthetic and relaxant products have been tested and developed for finfish aquaculture use (Neiffer & Stamper 2009). It is unknown but contentious if invertebrates experience pain and stress comparable to that encountered by vertebrates (Robyn & Edgar 2001, Rose 2002,

Elwood 2011). In invertebrates, veterinary relaxant agents induce physiological states variously described as “relaxants”, “anaesthetics” and “narcotics” (Burke et al. 2001, Robyn & Edgar 2001, Cooper 2011). Invertebrate pain is still undescribed, however, so the term ‘muscle relaxant’ best describes the veterinary medicines used to induce this state. It is safer to operate assuming that welfare needs must be addressed for all farmed species (Rose 2002, Ross & Ross 2008). Improved animal welfare also has economic benefits for industry (Ross & Ross 2008).

In molluscs the adductor muscle clamps the shells shut (in bivalves) or holds the shell tightly to substrates (in univalves) (Aquilina & Roberts 2000). Abalone possess a large, muscular foot, which facilitates the animal adhering tightly to substrates and it is extremely difficult to remove abalone from aquaculture systems (Aquilina & Roberts 2000, Bilbao et al. 2010). Abalone husbandry procedures such as grading, adjusting stocking density, transfer between systems, system maintenance and harvesting, however, require periodic removal of abalone from their holding tanks. In some procedures, however, including grading, re-attachment to equipment is problematic (Fleming & Hone 1996, Burke et al. 2001). The numbers and density of abalone in a commercial farm make removal of each abalone logistically impractical and the stress caused by handling is linked to morbidity, mortality, reduced growth, immunosuppression and increased susceptibility to disease (Hooper et al. 2007, Hooper et al. 2011).

Removal following relaxation offers a safer, less invasive and more economic large-scale alternative to individual removal (White et al. 1996, Ross & Ross 2008). Relaxants are products that induce a state where muscle tone and tension decrease and contraction is prevented. A good relaxant for use in abalone aquaculture induces a gradual loss of adherence from the substrate with no mortality or negative effects on growth or behaviour (Ross & Ross 2008). Use of good relaxants facilitates removal without force (Aquilina &

Roberts 2000) and reduces nociception and stress caused by handling (Aquilina & Roberts 2000, Burke et al. 2001).

Providing the Australian abalone industry with a choice of approved legal relaxants will improve outcomes of husbandry procedures for *Haliotis* spp. There is a relaxant available for Australian abalone aquaculture; benzocaine (PER 14638) (APVMA 2016c). Benzocaine has anecdotally caused adverse effects when used on abalone, including behaviours indicative of irritation or stress and mortality. Benzocaine cannot be used for rested harvest because PER 14638 prescribes a 500 degree-day withholding period (WHP) (PER 14638) (APVMA 2016c). The ideal aquaculture relaxant, including for abalone aquaculture, would have negligible residues or be safe for human consumption immediately following use, so that it requires no WHP and can be used for rested harvest. Rested harvest can benefit the abalone industry by decreasing shell and muscle damage during removal from the substrate for harvest, reducing mortality during transport and enhancing product quality (Wilkinson et al. 2008). While the immediate and short-term benefits of using relaxant products for removal are apparent, little is known about the long-term effects of relaxation and handling following relaxation on the growth and health of abalone.

Veterinary medicines used in Australia are regulated by the Australian Pesticides and Veterinary Medicines Authority (APVMA). Legitimising the use of relaxant products for Australian cultured abalone species requires data collation across mollusc species, identification of target species data gaps and obtaining information from studies that address these gaps (White et al. 1996, Elwood 2011, Lewbart & Mosley 2012). Applications to the APVMA for regulatory authorities to use relaxant products require documentation to understand product chemistry and manufacture, toxicology, metabolism and kinetics, residues, occupational health and safety, trade and any additional data so that efficacy, safety and environmental safeguards can be met and export restrictions addressed (APVMA 2016a).

This review aims to examine invertebrate stress, focusing on gastropods, nociception and its induction of stress and how relaxant products can potentially mitigate these effects. Relaxants and patterns of use in gastropod and bivalve aquaculture and associated farming systems are reviewed. Understanding why other mollusc aquaculture industries require relaxants and how they have addressed this need provides a guide for relaxant development for abalone. The need to relax abalone is required during certain stages of the season and farmers would be the best to judge the health and endrogenous and exogenous factors that affect their stock. Site specific studies will need to be undertaken to assess such factors and will be conducted by individual farms to finalise this study. This review informs an understanding about why relaxant products are beneficial for the Australian abalone industry and why candidate relaxant products need further investigation.

2.3 Stress

2.3.1 *Pain, stress and invertebrates*

Invertebrates are a paraphyletic group (Barnes et al. 1968) comprising more than 95 % of animal species, unified by the lack of a vertebral column, or “backbone” (Ottaviani & Franceschi 1996, Geiger 2000, Allsopp et al. 2011). Molluscs are invertebrates and among the most diverse phyla, with over 103,402 extant species (Brusca & Brusca 2003). The Gastropoda, which includes slugs and snails, is among the diverse groups in the Mollusca, with approximately 93,195 extant species (Brusca & Brusca 1990).

Gastropods have a muscular foot which is used for "creeping" locomotion in most species but which can be modified for swimming or burrowing (Harrison et al. 1994). Gastropods have diverse and specialized sensory systems because most are active, motile foragers (Marois & Carew 1990, Chase 2002). Molluscs do not have a central nervous system, but gastropods possess the basic molluscan nervous system. A pair of cerebral

ganglia (masses of nerve cell bodies) innervate the head, mouth and associated sense organs. From the dorsal cerebral ganglion, two pairs of longitudinal nerve cords arise: a pair of lateral (pleural) nerve cords, often forming pleural ganglia (which innervate the mantle) and a ventral pair of pedal nerve cords, often forming pedal ganglia which innervate the foot (Bullock & Horridge 1966, Robyn & Edgar 2001, Benjamin et al. 2005). The number of cells and their specialization is expanded in gastropods (Benjamin et al. 2005) and some gastropods possess large neuronal somata, which facilitate studying effects of neuronal structure on behaviour (Moffett 1995, Chase 2002, Marois & Carew 2004). Molluscs have proven to be invaluable models for basic neuroscience research, yielding fundamental insights into a range of biological processes involved in action potential generation, synaptic transmission, learning, memory and more recently, biology of nociception (Bullock & Horridge 1966, Robyn & Edgar 2001, Benjamin et al. 2005). Nociception, the detection of stimuli that are injurious or would be if sustained or repeated, has clear adaptive advantages because it triggers withdrawal and escape during injury or in the face of impending injury and withdrawal from stress situations (Bullock & Horridge 1966). Pain in invertebrates is poorly understood and contentious (Rose 2002), but molluscs possess nociceptor cells with opioid receptor systems and these systems have been shown to control pain in vertebrates (Robyn & Edgar 2001, Tobin & Bargmann 2004). Molluscs display nociception and detrimental effects of associated stress (Robyn & Edgar 2001, Tobin & Bargmann 2004) and nociception, avoidance behaviour and the effects of stress on invertebrate physiology, learning and memory, is understood from studies of gastropods (Bullock & Horridge 1966, Robyn & Edgar 2001, Hooper et al. 2007, Hooper et al. 2011). Elwood (2011) reviewed literature on pain in invertebrates and concluded that while physiological changes such as signs of irritation and colour changes may be useful indicators of pain, the study of behaviour in invertebrates is limited and contributes little towards understanding any pain experience.

Irrespective of understanding these phenomena, farmed and research invertebrates should still be provided respect and care because nociceptive responses have direct negative impacts on invertebrate health (Robyn & Edgar 2001, Elwood 2011). Stress is a symptom resulting from exposure of an organism to a hostile environment or noxious stimulus that disrupts homeostasis (Moberg & Mench 2000). Invertebrates display behavioural signs of stress following nociception which can comprise inappetance, behavioural disruption, suppression of social behaviours and adoption of unusual behaviours including repetitive or stereotyped behaviours (Iwama et al. 2011). Physiologically stressed invertebrates display respiratory and cardiovascular changes, inflammation, release of stress hormones and immune suppression (Hooper et al. 2007, Hooper et al. 2011, Iwama et al. 2011)

With pressure to increase production and accompanying increases in animal density in farms, humane care and the elimination and/or reduction of stress has significant benefits which has economic advantages (Ross & Ross 2008). Determining the health impacts of stress in molluscs and understanding immune responses remains problematic (Hooper et al. 2007). For aquaculture to be economically viable, however it is essential to minimize stress and monitor health, but the diversity of taxa in aquaculture makes informed understanding and developing methods for monitoring complex (Wells & Baldwin 1995, Malham et al. 2003, Cheng et al. 2004a, Cheng et al. 2004b, Cheng et al. 2004c, Hooper et al. 2007, Wang et al. 2008, Hooper et al. 2011). The lack of consensus about invertebrate experiences of pain does not deny that stress responses occur or negate their impacts in aquaculture. Husbandry procedures need to be managed; to improve production, minimise health impacts and negate welfare concerns (Hooper et al. 2007). Relaxation can improve welfare outcomes and decrease stress in invertebrate culture because it removes stress from procedures impacting nociception (Burke et al. 2001).

2.3.2 *Abalone aquaculture and stress*

Abalone (Haliotidae: Haliotis) are a monogeneric group of marine gastropods (Boxshall et al. 2014). There are 130 (Cox 1962) to 30 (Dauphin et al. 1989) valid, extant species which are widely harvested as a high value delicacy Gordon & Cook 2004). Overfishing, disease and environmental change have reduced wild populations to such an extent that farmed abalone now supplies most of the abalone meat consumed (Gordon & Cook 2004). Abalone farming began in the late 1950s and early 1960s in Japan and China (Gordon & Cook 2004), but since the mid-1990s, commercial abalone farming for food production has emerged in several countries and expanded (Flemming & Hone 1996). Abalone aquaculture is a high value sector (Gordon & Cook 2004) and the need to develop improved culture techniques has prompted studies on stress and immune responses to reduce mortality and sublethal effects of aquaculture husbandry practices (Baldwin 1992, Malham et al. 2003, Hooper et al. 2007, Fabling 2008). In *H. tuberculata* Linnaeus, stress decreases circulating haemocytes counts and haemocyte migratory activity and reduces phagocytic capacity and respiratory burst reactions of haemocytes during exposure to noxious stimuli (Bilbao et al. 2010). Environmental and water quality stressors including air exposure and hypoxia (Tjeerdema et al. 1991, Wells & Baldwin 1995, Cheng et al. 2004a), temperature and salinity changes (Wang et al. 2008) and chemical exposure (Malham et al. 2003, Cheng et al. 2004b, Cheng et al. 2004c) also negatively influencing immune responses and survival in *Haliotis* spp. (Hooper et al. 2007). Compounded stressors reduce appetite, which leads to diminished growth and, in extreme cases, causes starvation (Malham et al. 2003, Wang et al. 2008). Managing stress in abalone farms is key to improved health and productivity.

Husbandry procedures often induce stress and cause sublethal effects on health and growth and increase mortality in commercial aquaculture (Burke et al. 2001, Hooper et al. 2007, Bilbao et al. 2010). Procedures such as grading, adjusting stocking density, transfer

between systems, system maintenance and harvesting, require periodic handling and removal of abalone from the aquaculture system (Ross et al. 2007). Abalone have a powerful, muscular foot, that adheres tightly to substrates and they cannot be removed simply by hand (White et al. 1996). Abalone use suction methods to adhere to the walls of raceways and tanks by the use of their adductor muscle or foot. Mucus released by the abalone can also act as a viscoelastic adhesive to substrates (White et al. 1996). Mechanical removal, using a blunt blade or scraper to lever the animal from the surface to which they are adhering, is commonly used. Mechanical removal, however, is suboptimal because it often leads to soft tissue injuries (White et al. 1996, Chacon et al. 2003). Inexpert mechanical removal of abalone <15 mm shell length can tear the shell from the adductor muscle and kill the animal (unpublished observations). Less severe injuries are likely to induce substantial stress (Hahn 1989, Burke et al. 2001, Chacon et al. 2003) and often cause injury or death (Ross et al. 2007), principally as a consequence of slow healing rates and limited capacity to coagulate haemolymph (Hooper et al. 2007, Hooper et al. 2011). The limited capacity to coagulate haemolymph contributes to a net loss of fluids (Hahn 1989) and increases the probability of bacterial infection (Sharma et al. 2003), which often also leads to mortality (Prince & Ford 1985, Hahn 1989, White et al. 1996). Mechanical removal induces strong contractions of the abalones muscular foot, depleting oxygen resources in the muscle and accelerating post- harvest autolytic spoilage (Personal communication AAGA, Hatake et al. 1995, Hooper et al. 2011). Mechanical harvest causes cortisol release and physically stresses the tissues, further reducing product quality by decreasing consistency, texture and potentially negatively affecting taste (Hatake et al. 1995). The large numbers of individual abalone in a commercial farm, furthermore, make mechanical removal of each abalone logistically impractical, time consuming and costly, unless labour costs are very low. As aquaculture production increases, poor outcomes following mechanical removal of abalone from substrates and the increased

need for labour dictate the need for methods to simplify removal and reduce stress, immune suppression and mortality associated with handling.

2.4 Relaxants

2.4.1 *Pearl seeding and relaxant use: a case study*

The cultured pearl industry has benefited from the introduction and routine use of relaxants. A cultured pearl is a nacreous pearl produced in a pearl oyster in response to human interference (Hildemann et al. 1974, Alagarswami 1987, Ruiz-Rubio et al. 2006). Although recipient pearl oysters do not require relaxation for pearl production (Norton et al. 1996, Norton et al. 2000, Acosta-Salmon & Davis 2007) use of effective relaxants reduces stress during the operative processes of pearl seeding (Acosta-Salmon et al. 2005, Kishore 2011) and mortality following pearl seeding (Mamangkey et al. 2009). Pearl seeding procedures have been altered to maximise benefits of relaxants. Oysters need to be forced open with a blunt metal spatula prior to seeding, which can damage the adductor muscle, shell and mantle (O'Connor & Lawler 2002, Mamangkey et al. 2009). Opening the oyster shell and holding it open is also stressful for the oyster (Norton et al. 2000). Oysters that can be opened have a wooden wedge inserted in between the lips of their shells to maintain the opening and are placed in a holding tank. When the oysters are removed from the holding tank they clamp down on the wedges and the resultant pressure can damage the mantle, the adductor muscle or the hinge ligament or the shell can crack (Heasman et al. 1995, Norton et al. 1996, Mamangkey et al. 2009). Relaxants decrease tone and tension of the adductor muscle and enables the oyster to be held open with less force, reducing the risk of damage, stress and subsequent mortalities (Mamangkey et al. 2009).

Pearl seeds are made from a donor oyster; small amounts of shell, plastic or metal balls are covered with mantle tissue excised from a donor oyster to prevent the recipient

oyster from rejecting the pearl seed (Heasman et al. 1995). Seeding relaxed recipient oysters reduces the time taken to open the oysters and increases seeding efficiency by reducing the processing time per oyster (Hildemann et al. 1974, Norton et al. 1996). Oysters that cannot be opened are returned to the farm to attempt to re-used them during the next seeding operation. Removal of mantle tissue from relaxed donor pearl oysters limits subsequent mortality, allowing donor mantle tissue to be provided by multiple oysters rather than a smaller number of animals that are euthanized to obtain the tissue (Acosta-Salmon et al. 2004). High quality donor oysters can, furthermore, be used for seeding more than once (Norton et al. 1996, Acosta-Salmon et al. 2004, Mamangkey et al. 2009, Acosta-Salmon et al. 2004, Mamangkey et al. 2009). Relaxants reduce stress during the removal of donor mantle tissue and the oysters return to a normal physiological state more quickly (Norton et al. 1996). Relaxed recipient oysters have higher seed retention rates and produce pearls of superior quality to oysters that have not been relaxed (Mamangkey et al. 2009). Pearl seeding is a long and delicate process (Acosta-Salmon et al. 2005) and overdose or prolonged exposure to a relaxant (magnesium sulphate) can cause the oyster to lose rigidity and collapse or produce excess mucus, rendering them unsuitable for seeding (Norton et al. 1996, Acosta-Salmon et al. 2005; Mamangkey et al. 2009). Doses have been optimised by industry (unpublished data) but no published data are available.

The pearl industry has benefited from the use of relaxants and approaches to improve previously wasteful husbandry procedures. The economic benefits of relaxants has improved profitability and efficiency of the pearl industry, much of which is relevant to the abalone aquaculture industry.

2.4.2 *Relaxant use in abalone aquaculture*

Time to harvest in Australian abalone aquaculture is 2-4 years and production costs are high, so relaxants are essential to prevent injury and minimize mortalities (Burke et al. 2001). Relaxants reduce physiological and behavioural responses to handling during husbandry procedures (Aquilina & Roberts 2000). The routine use of relaxants during culture is widely accepted in abalone farming (White et al. 1996) and relaxants are used to improve the efficiency of common aquaculture husbandry procedures (Hahn 1989, Heasman et al. 1995, Aquilina & Roberts 2000, Sharma et al. 2003). Worldwide, relaxant use is poorly regulated by governing authorities and a wide range of relaxants are usually administered *ad-hoc* on farms without Veterinary or governing body approval (Personal communication AAGA). As governing and food regulating authorities' further monitor aquaculture species, relaxants will increasingly come under observation when used during production procedures (Aquilina & Roberts 2000).

In Australia, abalone 3 mm shell length and larger are maintained in large cement slab tanks, 10-20 metres long, 2-3 metres wide and 5-10 cm deep (Burke et al. 2001, Stone et al. 2013) and require periodic removal for size-sorting and tank splitting procedures, often requiring the abalone to be relaxed and placed into a mechanical sorting machine. Handling following appropriate relaxation is safer, less invasive, lessens welfare concerns and is more economical than removing individual abalone (White et al. 1996). The abalone must remain relaxed throughout these processes, however, to ensure that early recovery and re-attachment of the foot to surfaces does not impede processes or jam equipment (AAGA unpublished observations). Although abalone aquaculture relaxants are primarily used to easily remove abalone from substrates and render them immobile and more pliable for handling, relaxants are also used to decrease stress, minimize physical injury, slow metabolism (particularly oxygen consumption and excretion) and reduce mortalities associated with handling (Hahn

1989, Heasman et al. 1995, Aquilina & Roberts 2000, Sharma et al. 2003). Relaxants also have potential to facilitate the development of the abalone pearl sector. Good relaxant properties can aid artificial seed insertion in abalone and can potentially increase seed retention, quality pearls and reduce mortality (Frankboner 1994, Langdon 2002, Ruiz-Rubio et al. 2006, Fabling 2008).

Shipping and marketing live aquatic products increases market value (Olin 2001). Decreased water quality, crowding and handling during transport are significant stressors of live abalone (Burke et al. 2001). Harvest and transport can benefit from the use of relaxants. Without relaxants, high mortality of abalone is often observed during and after transport with survivors displaying reduced immune function and making the abalone susceptible to disease (Hooper et al. 2011). Better harvesting procedures also improve the quality and storage of seafood products (Hooper et al. 2011). In rested harvest, animals are harvested while relaxed, conserving the animal's intra-cellular energy reserves and suppressing stress-related changes that are detrimental to taste and flesh quality, providing improved product quality and consistency (Hooper et al. 2011, Hatake et al. 1995). Rested harvest can only achieve maximum benefits if relaxation does not cause any adverse reactions (White et al. 1996, Aquilina & Roberts 2000) and animals are gathered carefully, without stress (Hooper et al. 2011). Rested harvest also needs a relaxant with no withholding period (WHP) so that the harvested stock can be sent to market with no harmful residues from the relaxant remaining in the edible portions (APVMA 2015b). Rested harvest is routinely used in finfish aquaculture (Ferreira et al. 1984, Southgate & Wall 2001, Coyle et al. 2004, Schnick 2006, Iversen & Eliassen 2009) but the abalone industries have not capitalised on the advantages of rested harvest. The only relaxant with regulatory approval for use in the Australian abalone industry, benzocaine cannot be used for rested harvest because PER 14638 (APVMA 2016c) prescribes a 500 degree-day WHP (APVMA 2016c).

Obtaining regulatory approvals for alternative relaxant product(s) to benzocaine, with fewer negative effects and with potential for rested harvest is an industry research and development priority. Benzocaine use on farms has been observed to result in unwanted effects, including bleeding (AAGA unpublished observations). Consultation with the Australian Abalone Growers Association (AAGA) determined that a useful relaxant must induce gradual loss of adhesion to the substrate in <5 minutes, allow recovery in <20 minutes following cessation of treatment with no mortality or ongoing effects on behaviour, have an adequate margin of safety to be confident about the pattern of use, be easy to administer and be inexpensive. Products that are safe for human consumption at harvest would also have the advantage of being suitable for rested harvest. Few studies have examined abalone relaxants (White et al. 1996, Aquilina & Roberts 2000, Bilbao et al. 2010, Hooper et al. 2011, Hooper et al. 2014) and no data exist to indicate which relaxants are most suitable for use in Australian abalone aquaculture.

2.4.3 *Relaxants and species specific response*

Taxonomic differences in response to medicines are well described in vertebrates (Ross & Ross 2008) but little is known for invertebrates (Kunigelis & Salueddin 1983, Culloty & Mulcahy 1992, Araujo et al. 1995, Heasman et al. 1995, Aquilina & Roberts 2000, Chacon et al. 2003, Mamangkey et al. 2009). Although many products effectively induce relaxation in molluscs, the product and its optimal dose is dependent on administration method, animal size, water temperature and associated effects on metabolism (Culloty & Mulcahy 1992, Araujo et al. 1995, Heasman et al. 1995, Aquilina & Roberts 2000, Chacon et al. 2003, Mamangkey et al. 2009, Hooper et al. 2011, Hooper et al. 2014). Successful use of relaxant products in other molluscs can guide selection of candidate relaxant products for abalone. The utility of this knowledge needs to be assessed but is a useful guide to develop

effective, safe relaxant products for the Australian abalone industry.

2.4.4 *Regulation of veterinary medicine use in Australia*

In Australian abalone aquaculture, only benzocaine has a legal authority for use as an abalone relaxant. Scheduling of benzocaine in the Poisons Standard (TGA 2016) means that legal administration needs to be prescribed by a veterinarian pursuant to APVMA Minor Use Permit PER 14638. No other relaxants have formal legal authorities for use, but in practice, situations arise where unregistered products are required or veterinary products are needed for a use for which they are not registered. In Australia, products can be used for approved purposes specified on the label, or prescribed 'off label' by a veterinarian under a variety of State-based legislation. 'Off label' use and prescription of unregistered products can be expensive for the grower because it requires additional veterinary consultation and may delay use, causing production losses. 'Off-label' prescription also places substantial liability on veterinarians and growers and use of products without established safety margins or known environmental safety is hazardous. Legislation in some States also precludes off-label prescription other than to individual animals, preventing use in production species.

The APVMA has a permits scheme that enables the legal use of products for applications other than those on the product label or for unregistered products. APVMA minor use permits (MUPs) apply to situations involving species other than 'major trade' species and particularly where the economic benefit of use of the product does not have adequate economic benefit to justify an application for full registration (assessment of which alone can cost $200K+) (APVMA 2016a). All farmed seafood species are currently regarded as 'minor trade' species. APVMA issues Emergency Use Permits for outbreaks of diseases or pests and research permits, which allow products to be used in trials to generate data necessary for product permitting or registration. Most products used in Australian aquaculture are administered

pursuant to MUPs or prescribed 'off-label'. Permits are provided on the basis of data on chemistry and manufacture, toxicology, metabolism and kinetics, residues, occupational health and safety, trade and any other data needed to demonstrate that efficacy and safety requirements and environmental safeguards are met and that human health and export restrictions are addressed (APVMA 2016a). These data demonstrate that the product is effective, safe for target animals, people and the environment and will not negatively affect trade (Ross & Ross 2008). There is substantial cost involved in generating the data required to support permitting a veterinary product. Aquaculture businesses are therefore likely to only support obtaining data for products whose use is likely to be economically beneficial and in practice only products for which there is a significant demand are investigated. The scientific standards of the decision process mean permits endorse use of products that are safe and effective (Acosta-Salmon & Davis 2007, Ross & Ross 2008).

2.4.5 *Choosing relaxants for screening*

In Table 2.1, there is a summary of literature on relaxants for molluscs, including the relaxant products most commonly used in mollusc aquaculture: 2-phenoxyethanol (CAS 122-99-6), AQUI-S™, benzocaine (Ethyl 4-aminobenzoate CAS 94-09-7), clove oil, magnesium chloride ($MgCl_2$) (CAS 7786-30-3), magnesium sulphate ($MgSO_4$) (CAS 13778-97-7), tricaine methanesulfonate (MS-222) (Ethyl 3-aminobenzoate methanesulfonic acid CAS 886-86-2) and propylene phenoxetol (1-(phenoxy) propan-2-ol CAS 770-35-4). These relaxants are available in Australia from chemical suppliers, can be purchased economically in bulk and have potential for regulatory approval. Other products were identified but not investigated, such as pentobarbitone (5-ethyl-5-(1-methylbutyl)-2,4,6(1H,3H,5H)-pyrimidinetrione CAS 76-74-4), chloral hydrate (2,2,2-trichloroethane-1,1-diol CAS 302-17-0), carbon dioxide (CAS 124-38-9) and onion powder, because they are

either subject to strict regulatory controls, very hazardous for users, not readily available in Australia or would be expensive in large quantities. Variation in efficacy of these products is observed between species and animal size (Table 2.1). For abalone species investigations, narcosis is observed as the complete removal from substrate. Fish have various degrees of relaxation and can be monitored and observed for this during experiments. Abalone have no documented 'degrees' of relaxation and simple observation analysis of their relaxation is difficult. Further research can potentially be undertaken to observe heart rate and metabolic function- but this would not be very practical at a farm based level and the potential differences in abalone physiology between farms and sites. If the farmers were interested in attachment tenacity, a method of assessment and measure can be investigated and a separate experiment can be conducted in the future. There are few investigations into relaxant products for abalone but candidate relaxants can be sourced from available literature and studies can be designed from APVMA data guidelines (APVMA 2016a).

Table 2.1 Relaxant use in mollusc species.

Relaxant	Class	Species	Reference	Shell length (mm)	Dose	Relaxation time (minutes)	Recovery time (minutes)
2-phenoxyethanol (mL/L)	Gastropoda	*Haliotis tuberculata coccinea*	Bilbao et al. 2010	42.0-80.0	2.0	4.9±0.9	7.2±3.6
		Haliotis rubra	Burke et al. 2001	40.0	1.0	N/A	N/A
		Haliotis laevigata	Burke et al. 2001	40.0	1.0	N/A	N/A
		Haliotis midae	White et al. 1996	5.0-15.0	0.5	0.8±0.7	2.6±0.8
				20.0-50.0	2.0	1.8±1.1	2.4±1.5
				60.0-90.0	3.0	2.9±2.3	46.0±24.6
		Strombus gigas	Acosta-Salmon & Davis 2007	87.4±1.93	3.0	N/E	N/A
	Bivalvia	*Pinctada maxima*	Mamangkey et al. 2009	128.9±12.5	3.0	13.8±6.4	<30.0
		Pinctada albina	Norton et al. 1996	N/A	3.0-4.0	N/A	>30.0
AQUI-S™ (mL/L)	Gastropoda	*Haliotis rubra*	Burke et al. 2001	40.0	0.05	N/A	N/A
		Haliotis laevigata	Burke et al. 2001	40.0	0.05	N/A	N/A
benzocaine (mL/L)	Gastropoda	*Haliotis iris*	Aquilina & Roberts 2000	70.0-140.0	100.0	45	N/A
		Haliotis rubra	Burke et al. 2001	40.0	100.0	<20.0	N/A
		Haliotis laevigata	Burke et al. 2001	40.0	100.0	N/A	N/A
	Bivalvia	*Pinctada fucata*	Acosta-Salmon & Davis 2007	N/A	250.0	N/A	N/A
					500.0	N/A	N/A
					1200.0	10.27±4.41	N/A
		Pinctada margaritifera	Acosta-Salmon & Davis 2007	N/A	250.0	N/A	N/A
					500.0	11.0±7.3	N/A
					1200.0	9.0±4.0	N/A
		Pteria penguin	Kishore 2011	(3yr old)	500.0	25	15
					1200.0	16	15
		Pinctada maxima	Mamangkey et al. 2009	128.9±12.5	500.0	17.5±8.9	N/A
					1200.0	10.5±7.9	N/A
		Pinctada albina	Norton et al. 1996	large	1000.0	N/E	N/A
					1200.0	all relaxed	N/A

Unavailable data is referred to as not available (N/A). Doses that resulted in no response are referred to as no effect (N/E).

Table 2.1 continued. Relaxant use in mollusc species.

Relaxant	Class	Species	Reference	Shell length (mm)	Dose	Relaxation time (minutes)	Recovery time (minutes)
clove oil (mL/L)	Gastropoda	*Haliotis tuberculata coccinea*	Bilbao et al. 2010	42.0-80.0	0.3	>30.0	2.4±2.2
					0.5	8.9±3.5	2±1.5
					0.7	7.9±2.3	3.1±2.3
		Haliotis rubra	Burke et al. 2001	40	0.5	N/A	N/A
		Haliotis laevigata	Burke et al. 2001	40	1.5	N/A	N/A
	Bivalvia	*Pinctada albina*	Norton et al. 1996	N/A	0.5	N/A	N/A
					1.5	<15.0	<30.0
		Pinctada maxima	Mamangkey et al. 2009	128.9±12.5	1.5	10.6±4.7	N/A
magnesium chloride (g/L)	Bivalvia	*Saccostrea glomerata*	Butt et al. 2008	N/A	50.0	<6 hours	48
		Pinctada albina	Norton et al. 1996	N/A	30.0	N/E	N/A
		Crassostrea gigas	Suquet et al. 2009	large	2.0	>16 hours	N/A
		Ostrea eaulis	Culloty & Mulcahy 1992	(3yr old)	30.0	< 90.0	<90.0
					20.0	44.5±18.9	8.2±4.7
					25.0	12.5±10.6	4.9±1.9
		Pecten fumatus	Heasman et al. 1995	55.0-75.0	30.0	4.8±3.3	4.3±3.9
					35.0	3.5±1.8	2.3±1.5
					50.0	3.6±3.6	4.2±3.2
	Gastropoda	*Strombus gigas*	Acosta-Salmon & Davis 2007	87.4±1.93	30.0	<30.0	N/A
magnesium sulphate (g/L)	Gastropoda	*Haliotis midae*	White et al. 1996	5.0-15.0	40.0	2.0±2.3	9.2±5.0
				20.0-50.0	140.0	5.2±5.1	24.0±10.3
				60.0-90.0	220.0	9.3±4.9	35.2±20.4
	Bivalvia	*Ostrea eaulis*	Culloty & Mulcahy 1992	(3yr old)	200.0	>24 hours	N/A
MS-222 (g/L)	Gastropoda	*Haliotis iris*	Aquilina & Roberts 2000	70.0-140.0	1.0	30.0-60.0	N/A
		Strombus gigas	Acosta-Salmon & Davis 2007	87.4±1.93	1.0	N/E	N/A
	Bivalvia	*Pinctada albina*	Norton et al. 1996	90.0-117.0	0.5	N/E	N/A
					1.0	N/A	>90.0
propylene phenoxetol (mL/L)	Gastropoda	*Haliotis iris*	Aquilina & Roberts 2000	70.0-140.0	2.5	29	N/A
	Bivalvia	*Pinctada margaritifera*	Hildemann et al. 1974	N/A	2.5	15	12.5
		Pteria penguin	Kishore 2011	(3yr old)	3.0	N/A	N/A
		Pinctada maxima	Mamangkey et al. 2009	128.9±12.5	2.5	15±7.1	<2 hours
		Pinctada albina	Norton et al. 1996	large	2.5	15	20
					4.0	10	44
		Pinctada imbricata	O'Connor & Lawler 2002	N/A	2.0	15	N/A
		Pinctada albina	O'Connor & Lawler 2002	N/A	2.0	5.5±0.6	N/A

Unavailable data is referred to as not available (N/A). Doses that resulted in no response are referred to as no effect (N/E).

AAGA consulted with members to identify the characteristics of suitable relaxant product(s) as alternatives to benzocaine for mass immersion relaxation. Candidate relaxants need to be screened to assess their suitability and identify any negative effects of use. Good relaxants for further investigation should reduce nociceptive reactions and reduce stress caused by handling (Aquilina & Roberts 2000, Burke et al. 2001).

Legitimizing the use of other relaxants for Australian farmed abalone species requires data collation, identification areas where APVMA data requirements are not already addressed (Burke et al. 2001) and then obtaining information that addresses those gaps.

2.5 Conclusion

Relaxants address three areas of industry interest; improved husbandry techniques, reduced stress and animal welfare concerns. Investigating relaxants that provide alternatives to benzocaine can aid the Australian abalone industry to obtain a variety of products for husbandry and rested harvest. In abalone aquaculture, using good relaxants can prevent injuries and consequent mortality, decrease stress, improve farming efficiency and production value and lower production costs. The Australian abalone industry has not utilised relaxants to their full potential, although the pearling industry has been improving efficacy using relaxants for many decades, providing improvements in procedures, ease of handling, value and stock health. Relaxants that are candidates for use in the abalone industry need to induce safe, gradual loss of adherence from the substrate with no mortality or ongoing negative effects. Identifying relaxants with no withholding period for rested harvest is also a priority. Data collation for local species is needed to address regulatory APVMA requirements to obtain legal authorities to use candidate products on farms. Beyond basic studies, investigations should be directed at bringing the practice of shellfish relaxation to the level of familiarity and competence equivalent to that seen in finfish aquaculture.

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Chapter 3: Screening abalone relaxants for efficacy and effects on growth.

Mercer G. J., J. O. Harris & M. R. Deveney. 2016. Screening abalone relaxants for efficacy and effects on growth. J. Shellfish. Res. (in preparation)

3.1 Abstract

Relaxants are used in abalone aquaculture to facilitate handling, transport and grading stock, with minimal injury, stress, immune suppression and mortality. In Australia, benzocaine has regulatory approval for use as an abalone relaxant but negative behavioural responses to exposure have been observed and it has a 500 degree-day withholding period precluding its use for rested harvest, so alternatives are needed. We screened 7 candidate relaxants (propylene phenoxetol, magnesium sulphate, magnesium chloride, tricaine methanesulfonate (MS-222), clove oil, AQUI-S™ and 2-phenoxyethanol) and benzocaine on small (one year old, 18-28 mm shell length) greenlip abalone (*Haliotis laevigata* Donovan) (abalone). We determined the effect of a range of doses of each product on abalone and assessed relaxation and recovery times and behavioural responses to exposure to the relaxants. The effect of exposure to these products on abalone growth was evaluated over 90 days subsequent to immersion treatment. 2-Phenoxyethanol, propylene phenoxetol and $MgCl_2$ met industry criteria for use and are candidates for further investigation for use as relaxants for abalone aquaculture.

3.2 Introduction

Global demand for abalone (*Haliotis* spp.), primarily from Asia, has been greater than supply from wild fisheries since the late 1990s (Cook & Gordon 2010). Wild stocks have declined due to overfishing, disease and environmental change and diminishing supply of wild caught product has stimulated the growth of global abalone aquaculture (Gordon & Cook 2004, Cook & Gordon 2010). Production has shifted from wild caught to farmed with over 95 percent of abalone now coming from aquaculture and the world aquaculture production has grown to over 135 000 tonnes in 2014 (FAO 2014)**.** Australian farmed abalone production was projected to increase by 456 to 965 metric tonnes per year between

2010 and 2015 (FAO 2014). Pressure to improve farming efficiency has motivated the abalone industry to increase biomass per area but this has increased the incidence of stress-induced disease and mortality which have limited production gains at some facilities (Stone et al. 2013).

Abalone possess a large, muscular foot, which facilitates the animal adhering tightly to the substrate, and makes it difficult to remove abalone from the aquaculture system (Aquilina & Roberts 2000, Bilbao et al. 2010). Abalone husbandry procedures such as grading, adjusting stocking density, transfer between systems, system maintenance and harvesting, require periodic removal of abalone from the aquaculture system (Ross et al. 2007). The large numbers of individual abalone in a commercial farm make mechanical removal of each abalone logistically impractical and costly and increased production compounds this issue. Mechanical dislodgment, moreover, often causes injury or death (Ross et al. 2007), principally as a consequence of slow healing rates, limited capacity to coagulate haemolymph and increased stress and opportunistic infections following handling (Hooper et al. 2007, Hooper et al. 2011). As production increases, poor outcomes following mechanical removal of abalone from substrates and the increased need for labour dictate the need for relaxant products to simplify handling and reduce associated stress. On farms, shock dose is applied. Juvenile abalone grown on plate systems are dipped into a separate tank already with relaxant. The vertical nature of the plates allows for the juvenile abalone when relaxed, to fall from the plates and onto the bottom of the tank- reducing handing stress. Horizontal relaxation for the larger raceway-bound abalone does still require handling for removal. The aim for the large abalone is to use the relaxant to help in ease of removal of the strong adhesive foot and for stress reduction during the removal process. Relaxation for the large abalone is successful when there is a reduction/elimination of the clamp down reaction,

allowing the large abalone in raceway slab tanks to be easily removed by hand. This time effect will be observed during the investigation.

Handling following relaxation is safer, less invasive and more economical than mechanical removal of individual abalone and the routine use of relaxants in abalone aquaculture is widely accepted (White et al. 1996).

Benzocaine is the only relaxant with a regulatory approval for use in Australian abalone aquaculture (PER 14638) (APVMA 2016c). Exposing abalone to benzocaine, however, can cause irritation, sudden muscle contraction, intense torsion, excess mucus production, behaviours that appear to be an attempt to evade exposure to the product and colour changes that can negatively affect marketability (Runham et al. 1965, Kaplan 1969, Heasman et al. 1995, Hooper et al. 2011, Hooper et al. 2014). Benzocaine use is also subject to a 500 degree-day withholding period, so it is not suitable for rested harvest Hooper et al. 2011). Obtaining regulatory approvals for alternative relaxants with fewer negative effects and with potential for rested harvest was therefore an industry research and development priority. For a relaxant to be useful for abalone aquaculture, it must induce gradual loss of adherence to the substrate in <5 minutes, allow recovery in <20 minutes following cessation of treatment with no mortality or ongoing effects on behaviour, be easy to administer and be safe and effective at low concentrations. Observed stress responses exhibited by the abalone during exposure can be useful signs of adverse reaction to the relaxant and a good descriptor to screening candidate relaxants. If products could be identified that are safe for human consumption at harvest, the product would have the added advantage of being suitable for rested harvest. Few studies have examined abalone relaxants (White et al. 1996, Aquilina & Roberts 2000, Bilbao et al. 2010, Hooper et al. 2011, Hooper et al. 2014) and no comprehensive data exists to indicate which relaxants are most suitable for use in Australian abalone aquaculture (Burke et al. 2001).

We aimed to identify effective relaxants that were suitable for dose optimization and development of data for regulatory assessment for industry use. Seven candidate products: 2-phenoxyethanol (2-PE), propylene phenoxetol (PPE), AQUI-S™, tricaine methanesulfonate (MS-222), magnesium sulfate ($MgSO_4$), magnesium chloride ($MgCl_2$) and clove oil were screened to assess their potential for use as relaxants and to identify if there are negative stress or physiological effects associated with exposure. Benzocaine was used as a comparison. This approach provides a framework for investigating and developing further relaxants for use in the abalone aquaculture industry.

3.3 Materials and methods

3.3.1 *Abalone*

Two thousand small (18-28 mm shell length SL) one-year-old farmed greenlip abalone *H. laevigata* were obtained from the Southseas Abalone Group, Kangaroo Island Abalone (KIAB), Kangaroo Island, Smith Bay, South Australia. Abalone were maintained in a 10 000 L fibreglass holding tank containing flow through ambient (18±1.0 °C, 102 % O_2 saturation and 35 PSU salinity) seawater at the South Australian Research and Development Institute (SARDI), West Beach, South Australia, Australia. Seawater was sand filtered to 50 µm. Abalone were acclimated to trial conditions for 60 days. During acclimation abalone were fed 3 mm ECO-feed chip (EP Aquafeeds, Lonsdale, South Australia, Australia), daily, in excess of the industry standard of 3 % body weight per day (Stone et al. 2013). Holding tanks were cleaned and maintained daily.

3.3.2 *Tagging*

To facilitate identification of individual abalone, 30 days into acclimation and 30 days prior to the trials commencing, each abalone was tagged with glue-on shellfish tags (Hallprint Fish Tags Pty Ltd, South Australia) using a protocol adjusted for abalone from Henry & Jarne (2007): abalone were mechanically removed from the holding tanks using a soft blunt scraper, the shell was blotted dry and tags were attached with cyanoacrylate gel glue, positioned on the apex of the shell as not to impede shell growth or movement during the duration of the experiment. Abalone were exposed to air for less than 2 minutes. Abalone were then returned to the holding tanks.

3.3.3 *Experimental description*

Abalone were mechanically removed from the holding tanks using a blunt scraper, blotted dry and the mass of each abalone was measured to the nearest 0.01 g. Shell length was measured along the longest axis to the nearest 0.01 mm with Vernier callipers. For each of the 8 relaxants, 120 abalone (18 – 28 mm shell length) were removed from the source population and placed into 12 replicate buckets containing 10 abalone per bucket. All buckets were aerated continuously. Abalone were allowed to settle and re-adhere to the bucket sides. The water was then removed from the buckets and a pre-mixed dose of relaxant in 5 L of holding tank water was added. Doses were derived from literature, with animal size and species taken into consideration shown in Table 3.1 (Hildemann et al. 1974, Culloty & Mulcahy 1992, Norton et al. 1996, White et al. 1996, Aquilina & Roberts 2000, Burke et al. 2001, O'Connor & Lawler 2002, Acosta-Salmon & Davis 2007, Butt et al. 2008, Mamangkey et al 2009, Suquet et al. 2009, Bilbao et al. 2010, Kishore 2011). For each relaxant, three buckets were treated with a dose estimated from literature as suitable for abalone (hereafter optimal), three buckets were treated with 50% of the optimal dose (low dose) and three

buckets were treated with 200% of the optimal dose (high dose). Relaxants were dissolved in seawater from the acclimation system. Three buckets were controls where the seawater was exchanged for fresh seawater when the trial buckets had the treatment added and were returned to the holding tanks after handling.

Benzocaine was dissolved in ethanol to facilitate dissolution; the concentration of ethanol in all benzocaine trials was 1 mL/L. The benzocaine treatments had an additional control containing 1 mL/L ethanol. MS-222 was buffered to pH 8 with tris buffer (Sigma® #21685) following Aquilina & Roberts (2000). Following exposure, for each abalone, relaxation time (REL), defined as the time from initial exposure to when the abalone lost adhesion to the substrate, was recorded.

During exposure to the relaxants, abalone were observed for behavioural and physiological signs of stress such as irregular shell movement, torsion (raising the shell away from the foot while twisting rapidly) or behaviours indicating that the abalone was attempting to avoid contact with the relaxant such as trying to escape the treatment bucket. Mucus production was regarded as excessive if mucus was observed on the surface of the water in the bucket during exposure to the relaxant. Type of relaxation such as gradual loss of adherence to the substrate with a gentle fall to the floor of the bucket, urgent movement with rapid self-removal or clamping without loss of adherence which required subsequent mechanical removal was noted. Tentacle relaxation was also noted as a sign of the relaxant having an effect on the abalone. The abalone foot was observed and any loss of epithelial tissue (cell sloughing) or size decrease that might indicate dehydration was noted. Abalone foot colour was observed during transfer from exposure to recovery and any colour changes, darkening or development of colour irregularity was recorded. All responses to the trialled relaxants were assessed as acceptable (✓) or unacceptable (x) in relation to these criteria.

After each abalone released or was removed from the substrate, it was placed dorsal side down in the holding tanks filled with flow through fresh seawater with continual aeration. The time from cessation of treatment until each inverted abalone could right itself and re-adhere to the substrate was measured (Recovery time (REC)). REL and REC were recorded in seconds. These relaxant descriptors were based on Andrews & Tansey (1981), Messenger et al. (1985), Scimeca & Forsythe (1999), Seol et al. (2007), Sen & Tanrikul (2009), Weber et al. (2009) and Mooney et al. (2010).

The abalone were checked 6, 12 and 24 hours after exposure. Mortalities were recorded at 24 hours and daily for 90 days. After 90 days the abalone were removed using a blunt scraper, measured and weighed. Absolute growth rate (AGR) was determined for both length (L) and weight (W) using the following formulae: AGRL (mm/day) = ((Lf – Li)/t) where Lf = the final mean abalone shell length (mm), Li = the initial mean abalone shell length (mm), ln = natural logarithm and t = grow out trial period (days). AGRW (g/day) = ((Wf – Wi)/t), where Wf = the final mean abalone weight (g), Wi= the initial mean abalone weight (g) and t = grow out trial period (days). Specific growth rate SGRL (% shell growth (mm) per day) and SGRW (% body weight (g) per day) were determined for both length and weight using the following formula: (ln(Lf) - ln(Li))/t x 100 and (ln(Wf) - ln(Wi))/t x 100 respectively. AGRL, AGRW, SGRL and SGRW at 90 days were compared within each relaxant between doses. Growth was assessed as acceptable (✓) or unacceptable (x) in relation to each growth measure.

3.3.4 *Water quality*

Effects of relaxants on water quality were assessed by monitoring temperature (°C) (glass thermometer, Aldrich®), dissolved oxygen (DO (% saturation)) (Oxyguard meter, Handy Polaris H01P, OxyGuard®), pH (IC-MW-102 meter) and salinity (PSU) (RHS-10ATC refractometer, Magnum Media™). Meters were calibrated as per manufacturers recommendations. All water quality parameters were measured in each holding tank and during exposure in each trial bucket (Table 3.3). Water quality for all relaxants was assessed as acceptable (✓) or unacceptable (x) for use in industry.

3.3.5 *Analysis*

For each relaxant, REL and REC between doses were analysed separately. The mean proportion of abalone that re-adhered to the substrate by 60 minutes was calculated (REC_{60}). Survival was expressed as the mean proportion of abalone alive 24 hours and at 90 days post exposure. Survival at 90 days data was taken from surviving abalone after 24 hours as a long term comparison. One-way ANOVA (Zar 1999) was used to determine differences between doses, water quality parameters (salinity, dissolved oxygen and temperature) and growth. All data were tested for normality with the one-sample Kolmogorov-Smirnoff test and for homogeneity of variances with Levene's test (Zar 1999). Data that were non-normal were log transformed. A post-hoc Tukey's test (Zar 1999) was used to determine the homogenous subsets. For all tests statistical significance was judged at the alpha level of 0.05. All statistical analysis was performed using SPSS Software for Windows (SPSS Inc., Chicago, IL, USA). All values are reported as the mean ± standard error. For dose ion concentrations, ICP-MS analysis was conducted with an Agilent 7500 auto sampler (Agilent Technologies™, USA) (RF Power 1370 W, Wavelength 213nm). The calibration solution for Mg analysis was prepared in de-ionized water for a calibration curve analysis for quantification.

3.4 Results

3.4.1 *Ease of administration*

Double dilutions were required for clove oil, benzocaine and AQUI-S™ to achieve target doses. Clove oil at 0.50 and 0.70 mL/L and AQUI-S™ at 0.50 and 0.80 mL/L reached saturation and formed a film on the surface of the treatment water.

3.4.2 *Behaviour and relaxation*

Abalone treated with $MgCl_2$, PPE or 2-PE showed gradual, gentle release, the foot became soft and the adductor muscle displayed moderate extension. Abalone treated with $MgCl_2$, PPE and 2-PE showed no signs of irritation, no excess mucus production and no discernible change in foot colour.

Exposure to AQUI-S™, MS-222, benzocaine or clove oil caused the abalone foot muscle to become rigid and clamp to the substrate, eventual release was achieved due to excess mucus production. $MgSO_4$ exposure elicited the foot muscle rigidity followed by a rapid, violent release from the substrate. Exposure to all doses of clove oil, benzocaine or MS-222 caused tentacle relaxation was observed, but the foot became rigid and the abalone did not release from the substrate, necessitating mechanical removal. AQUI-S™ at 0.80 mL/L, benzocaine at 0.20 mL/L and clove oil at 0.70 mL/L caused the abalone to clamp to the substrate sufficiently tightly that the epithelial tissue on the ventral foot surface usually detached from the foot when the abalone were mechanically removed from the substrate. Abalone exposed to all doses of AQUI-S™, $MgSO_4$, benzocaine and clove oil produced excess mucus. Exposure to all doses of AQUI-S™, benzocaine and clove oil elicited torsion. Exposure to 0.50 and 0.80 mL/L AQUI-S™, 0.20 mL/L benzocaine and 0.50 and 0.70 mL/L of clove oil and ethanol controls at 1 mL/L elicited violent torsion and an average of five abalone per treatment bucket were cut by the shell of the other abalones' during torsion. All

doses of $MgSO_4$ and benzocaine caused the foot to change from green to brown with marked darkening. Exposure to benzocaine at 0.10 and 0.20 mL/L or 1 mL/L ethanol caused the abalone foot to cell slough (lose epithelial tissue) and shrink.

Abalone responses to relaxant exposures are shown in Table 3.1. 2-PE, PPE, MS-222, $MgCl_2$ and benzocaine had relaxation times of <5 minutes. Mean REL for abalone exposed to AQUI-S™ at 0.20 mL/L was over 40 minutes. 2-PE, PPE, MS-222, $MgCl_2$ and benzocaine had mean REC of <20 minutes. $MgSO_4$ at 180.0 g/L had the longest mean REC of 25±3 minutes. Clove oil REC at all doses was longer than 20 minutes. AQUI-S™ at 0.80 mL/L had significantly shorter REC_{60} (P<0.002) than all other doses of AQUI-S™. Benzocaine at 0.20 mL/L had significantly shorter REC_{60} (P<0.002) than all other doses of benzocaine. $MgSO_4$ at 120 and 180 g/L had significantly shorter REC_{60} (P<0.001) than at 60 g/L.

Table 3.1 Responses of greenlip abalone (*Haliotis laevigata*) to relaxant exposure at 18 °C.

Relaxant	Units	Dose	Relaxation (seconds)	Recovery (seconds)	Recovery % (60 minutes)	Survival % (24 hours)	Survival % (90 days)
2-phenoxyethanol	mL/L	0.0	0±0	0±0	100[a]	97[a]	100[a]
		0.5	171±11[b]	455±76[a]	100[a]	97[a]	100[a]
		2.0	98±8[a]	401±125[a]	100[a]	100[a]	100[a]
		3.0	58±5[a]	530±97[b]	100[a]	97[a]	99[a]
AQUI-S™	mL/L	0.0	0±0	0±0	100[b]	97[a]	100[a]
		0.2	2416±191[c]	1368±283[b]	97[b]	100[a]	100[a]
		0.5	1154±107[b]	501±32[a]	97[b]	94[a]	98[a]
		0.8	441±42[a]	624±87[a]	85[a]	93[a]	98[a]
benzocaine	mL/L	0.0	0±0	0±0	100[b]	100[a]	100[a]
		0.05	438±37[b]	732±138[a]	97[b]	98[a]	100[a]
		0.1	264±33[a]	717±94[a]	91[b]	94[a]	99[a]
		0.2	261±16[a]	694±143[a]	79[a]	96[a]	99[a]
clove oil	mL/L	0.0	0±0	0±0	100[a]	97[a]	100[a]
		0.3	319±26[a]	904±122[a]	100[a]	97[a]	100[a]
		0.5	335±35[a]	1034±107[ab]	97[a]	96[a]	99[a]
		0.7	277±24[a]	1174±67[b]	97[a]	96[a]	99[a]
magnesium chloride ($MgCl_2$)	g/L	0.0	0±0	0±0	100[a]	97[a]	100[a]
		20.0	950±90[c]	837±61[b]	100[a]	100[a]	100[a]
		50.0	584±70[b]	815±78[b]	100[a]	100[a]	99[a]
		80.0	61±4[a]	502±50[a]	100[a]	97[a]	100[a]
magnesium sulphate ($MgSO_4$)	g/L	0.0	0±0	0±0	100[b]	97[b]	97[a]
		60.0	126±18[b]	978±143[a]	97[b]	100[b]	95[a]
		120.0	73±9[a]	1093±167[a]	85[a]	86[a]	82[b]
		180.0	45±6[a]	1519±159[b]	82[a]	93[b]	95[a]
MS-222	g/L	0.0	0±0	0±0	100[a]	100[a]	100[a]
		0.5	247±29[a]	378±63[b]	100[a]	100[a]	100[a]
		1.0	48±7[b]	211±19[a]	100[a]	97[a]	99[a]
		2.0	24±2[b]	244±26[a]	100[a]	94[a]	99[a]
propylene phenoxetol	mL/L	0.0	0±0	0±0	100[a]	100[a]	100[a]
		1.0	130±13[a]	689±85[a]	100[a]	97[a]	99[a]
		2.0	123±3[a]	789±83[a]	100[a]	100[a]	99[a]
		3.0	110±2[a]	1001±49[b]	100[a]	97[a]	99[a]

n=30 per dose. Small *H. laevigata* (18-28 mm shell length). Standard error of the means is presented. Different superscripts indicate significant differences in mean relaxation and recovery times ($P<0.05$). The relaxation time and recovery time were analysed separately.

3.4.3 *Post relaxation grow-out*

Growth is shown in Table 3.2. There were no significant differences in growth ($P>0.05$) following exposure to most relaxants. Abalone treated with benzocaine at 0.05 mL/L showed significantly higher SGRW, AGRW, AGRL and SGRL ($P<0.001$) than other

doses of benzocaine and untreated controls. Exposure to $MgSO_4$ at 120 g/L produced significantly lower survival at 90 days than other doses and untreated controls ($P<0.002$) and was the only dose for all relaxants and treatments that provided significantly lower survival at 90 days (Table 3.1).

Table 3.2 Growth of greenlip abalone (*Haliotis laevigata*) 90 days after exposure to relaxants.

Relaxant	Units	Dose	SGRW (% g/day)	AGRW (g/day)	AGRL (mm SL/day)	SGRL (% mm SL/day)
2-phenoxyethanol	mL/L	0.0	1.03±0.05	0.03±0.00	0.10±0.01	0.37±0.02
		0.5	1.00±0.04	0.03±0.00	0.09±0.01	0.34±0.01
		2.0	0.90±0.04	0.03±0.01	0.09±0.00	0.33±0.02
		3.0	0.98±0.06	0.03±0.00	0.10±0.01	0.37±0.02
AQUI-S™	mL/L	0.0	0.96±0.06	0.03±0.01	0.09±0.01	0.34±0.02
		0.2	0.91±0.05	0.02±0.00	0.08±0.01	0.32±0.02
		0.5	0.87±0.05	0.03±0.00	0.10±0.01	0.37±0.03
		0.8	0.94±0.06	0.03±0.00	0.10±0.01	0.35±0.02
benzocaine	mL/L	0.0	0.85±0.05[a]	0.02±0.00[a]	0.08±0.01[a]	0.32±0.02[a]
		0.05	1.19±0.05[b]	0.04±0.00[b]	0.12±0.01[b]	0.42±0.02[b]
		0.1	0.86±0.06[a]	0.02±0.00[a]	0.08±0.01[a]	0.31±0.02[a]
		0.2	0.82±0.04[a]	0.02±0.00[a]	0.08±0.00[a]	0.30±0.01[a]
clove oil	mL/L	0.0	0.99±0.05	0.03±0.00	0.10±0.01	0.40±0.02
		0.3	0.85±0.04	0.03±0.00	0.08±0.01	0.29±0.02
		0.5	0.95±0.04	0.03±0.00	0.09±0.01	0.32±0.01
		0.7	0.83±0.04	0.03±0.00	0.08±0.00	0.29±0.01
magnesium chloride ($MgCl_2$)	g/L	0.0	0.97±0.05	0.02±0.00	0.09±0.01	0.35±0.02
		20.0	0.98±0.04	0.03±0.00	0.09±0.00	0.34±0.01
		50.0	1.02±0.06	0.03±0.01	0.10±0.01	0.37±0.02
		80.0	0.94±0.05	0.03±0.01	0.10±0.01	0.35±0.02
magnesium sulphate ($MgSO_4$)	g/L	0.0	0.96±0.04	0.02±0.00	0.09±0.01	0.35±0.02
		60.0	0.79±0.03	0.02±0.00	0.07±0.00	0.30±0.02
		120.0	0.89±0.03	0.02±0.00	0.08±0.01	0.32±0.01
		180.0	0.79±0.04	0.02±0.00	0.07±0.01	0.28±0.02
MS-222	g/L	0.0	0.95±0.08	0.03±0.00	0.09±0.01	0.34±0.03
		0.5	0.96±0.05	0.02±0.00	0.09±0.00	0.35±0.01
		1.0	0.90±0.05	0.02±0.00	0.08±0.01	0.33±0.02
		2.0	1.04±0.04	0.03±0.00	0.10±0.01	0.38±0.01
propylene phenoxetol	mL/L	0.0	0.99±0.05	0.02±0.00	0.09±0.01	0.36±0.02
		1.0	1.00±0.05	0.03±0.00	0.10±0.01	0.37±0.02
		2.0	0.98±0.05	0.03±0.01	0.10±0.01	0.38±0.02
		3.0	0.94±0.05	0.02±0.00	0.09±0.01	0.36±0.02

n=30 per dose. Small *H. laevigata* (18-28 mm shell length). The specific growth rate for abalone weight (SGRW) (% body weight (g) per day), the average growth rate for abalone weight (AGRW) (g/day), the average growth rate for abalone shell length (SL) (AGRL) (mm/day) and the specific growth rate for SL (SGRL) (% shell growth (mm) per day). Standard error of the means is presented. Different superscripts indicate significant differences in SGRW, AGRW, AGRL and SGRL ($P<0.05$). The SGRW, AGRW, AGRL and SGRL were analysed separately within their relaxant categories.

3.4.4 *Water quality*

Water quality parameters are shown in Table 3.3. Seawater containing $MgSO_4$ at 60, 120 and 180 g/L had significantly higher salinity (P<0.05) than the control. The instrument could measure PSU only to 100 PSU so some $MgSO_4$ doses are expressed as >100 rather than a specific measurement of salinity. No significant differences (P>0.05) were observed in water quality parameters in 2-PE, $MgCl_2$, PPE, AQUI-S™, MS-222, benzocaine and clove oil. Exposing abalone to benzocaine at 0.10 and 0.20 mL/L resulted in the water in the test buckets turning blue.

Table 3.3 Effects of relaxants on water quality.

Relaxant	Units	Dose	DO (mg L-1)	DO (%)	Temperature (°C)	pH	SAL (PSU)
2-phenoxyethanol	mL/L	0.0	6.8±0.1	98±2	18.2±0.1	8.34±0.01	37±0
		0.5	6.8±0.2	93±4	18.5±0.0	8.33±0.01	38±1
		2.0	6.2±0.3	89±5	18.5±0.0	8.33±0.00	38±0
		3.0	6.2±0.2	88±2	18.4±0.1	8.33±0.00	38±1
AQUI-S™	mL/L	0.0	6.9±0.1	99±1	18.1±0.1	8.33±0.01	37±1
		0.2	6.9±0.1	98±1	18.1±0.1	8.34±0.00	37±1
		0.5	7.0±0.0	99±1	18.2±0.1	8.34±0.00	37±1
		0.8	7.0±0.1	99±1	18.3±0.1	8.33±0.01	38±1
benzocaine	mL/L	0.0	7.1±0.1	100±1	18.6±0.5	8.34±0.00	37±1
		0.05	7.1±0.1	101±1	18.6±0.4	8.35±0.01	37±1
		0.1	7.0±0.0	100±1	18.5±0.4	8.34±0.00	38±1
		0.2	7.0±0.1	99±1	18.6±0.3	8.34±0.00	38±1
clove oil	mL/L	0.0	7.0±0.1	100±1	18.2±0.1	8.34±0.01	37±1
		0.3	7.0±0.1	99±1	18.1±0.0	8.32±0.02	38±1
		0.5	6.9±0.1	98±2	18.2±0.0	8.31±0.02	38±1
		0.7	7.0±0.1	99±1	18.2±0.1	8.29±0.01	38±2
magnesium chloride ($MgCl_2$)	g/L	0.0	7.1±0.1	101±1	18.5±0.2	8.34±0.00	36±1
		20.0	7.0±0.0	99±1	18.6±0.2	8.08±0.07	38±1
		50.0	6.8±0.1	96±2	18.9±0.4	7.71±0.13	42±6
		80.0	6.6±0.2	93±4	18.3±0.3	7.42±0.23	48±5
magnesium sulphate ($MgSO_4$)	g/L	0.0	6.8±0.1	97±2	18.5±0.3	8.33±0.00	$37±1^a$
		60.0	6.8±0.2	98±1	19.6±0.8	8.02±0.16	$90±10^b$
		120.0	7.0±0.1	101±1	20.1±1.1	7.59±0.11	$>100^b$
		180.0	7.1±0.1	102±1	21.7±1.2	7.37±0.10	$>100^b$
MS-222	g/L	0.0	7.0±0.1	100±1	18.7±0.1	8.34±0.00	37±1
		0.5	6.6±0.2	92±3	18.5±0.2	8.33±0.01	39±1
		1.0	6.3±0.2	89±2	18.6±0.1	8.31±0.02	40±0
		2.0	5.9±0.3	87±3	18.2±0.3	8.30±0.01	40±1
propylene phenoxetol	mL/L	0.0	6.7±0.2	99±1	18.3±0.0	8.33±0.00	38±1
		1.0	6.8±0.1	98±1	18.4±0.1	8.35±0.01	37±0
		2.0	6.6±0.1	98±1	18.2±0.2	8.34±0.01	37±1
		3.0	6.7±0.1	97±2	18.4±0.1	8.34±0.01	38±1

Temperature (°C), dissolved oxygen (DO (%)), pH and salinity (SAL (PSU)). Standard error of the means is presented. Different superscripts indicate significant differences (P<0.05). The relaxants were analysed separately within their relaxant categories.

Table 3.4 Assessment of greenlip abalone (*Haliotis laevigata*) behaviour when exposed to relaxants.

	Abalone			Relaxant efficacy					90 day grow out	
Relaxant	Physiology and behaviour	Foot colour	Ease of release	Water quality	Easy admin	Relaxation time (<5 min)	Recovery time (<20 min)	% Recovery	% Survival	Growth
2-phenoxyethanol	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
AQUI-S™	x	x	x	✓	x	x	✓	x	✓	✓
benzocaine	x	x	x	✓	x	✓	✓	x	✓	✓
clove oil	x	✓	x	✓	x	✓	x	x	✓	✓
$MgCl_2$	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
$MgSO_4$	x	x	x	x	✓	✓	x	x	x	✓
MS-222	✓	✓	x	✓	x	✓	✓	✓	✓	✓
propylene phenoxetol	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Small *H. laevigata* (18-28 mm shell length) Animal behaviour and physiology; torsion, shrivelling and mucous production. Foot colour and ease of release from the substrate. Relaxant impacts on water quality; (temperature (°C), dissolved oxygen (DO (%)), pH and salinity (SAL (PSU)) and ease of administration. All assessments reported as acceptable (✓) or not acceptable (x) compared to industry relaxant criteria.90 day grow out assessment on % survival and growth after exposure including the specific growth rate (SGRW) (% body weight (g) per day), the average growth rate (AGRW) (g/day), the average growth rate for shell length (SL) (AGRL) (mm/day) and the specific growth rate for SL (SGRL) (% shell growth (mm) per day).

Table 3.5 Magnesium (Mg) ion concentration [Mg^{2+}] for doses of $MgCl_2$ and $MgSO_4$.

Relaxant	Dose (g/L)	Mg^{2+} (g/L)	Mg^{2+} (mol/L)
$MgSO_4$	60.0	12.11	0.50
	120.0	24.23	0.98
	180.0	36.34	1.50
$MgCl_2$	20.0	5.10	0.21
	50.0	12.80	0.53
	80.0	20.42	0.84

3.5 Discussion

$MgCl_2$, propylene phenoxetol and 2-phenoxythanol each met all response, behavioural, growth and water quality criteria for use as relaxants for abalone. Benzocaine, $MgSO_4$, MS-222, clove oil and AQUI-S™ all failed to meet one or more requirements for efficacy, behaviour, appearance, mortality or growth. The REL and REC for all relaxants was relaxant specific. Abalone stress responses to the relaxants was also relaxant specific, for example, some relaxants induced relaxed tentacles but with a contracted foot muscle and others exhibiting the relaxation effect in both.

Exposure to $MgCl_2$ met all the commercial requirements for an acceptable relaxant (Table 3.4). $MgCl_2$ is generally recognised as safe (FDA 2015) and has potential for rested harvest. $MgCl_2$ also induces REL adequately quickly, has safe REC of appropriate duration, causes no signs of stress and has low mortality in other molluscs including *Ostrea edulis* Linnaeus (Culloty & Mulcahy 1992), *Pecten fumatus* Reeve (Heasman et al. 1995), *Strombus gigas* Linnaeus, (Acosta-Salmon & Davis 2007), *Saccostrea glomerata* Gould (Butt et al. 2008) and *Crassostrea gigas* Thunberg (Suquet et al. 2009). As $MgCl_2$ dose increased, REL decreased and there was a decrease in REC. $MgCl_2$ did not induce relaxation in *Pinctada margaritifera* Linnaeus (Norton et al. 1996), however, with the oyster keeping its shell tightly closed to avoid contact with the relaxant, indicating that the effects of relaxants differ between mollusc taxa.

2-PE met all the commercial requirements for an acceptable relaxant (Table 3.4). 2-PE is also a successful muscle relaxant for *Haliotis midae* Linnaeus (White et al. 1996) with doses for juvenile *H. midae* comparable to those we identified as useful for *H. laevigata*. We observed REC increasing with dose as noted by White et al. (1996). Successful relaxation and recovery from 2-PE relaxation was also observed in *Pinctada albina* Lamarck (Norton et al. 1996), *H. iris* (Aquilina & Roberts 2000) and *P. maxima* Jameson (Mamangkey et al. 2009). Relaxation was not induced, however, in *S. gigas* exposed to 3 mL/L 2-PE (Acosta-Salmon & Davis 2007) with strong muscular contraction occurring during exposure, indicating that this relaxant induces different responses in different taxa.

Propylene phenoxetol met all the industry requirements for an acceptable relaxant (Table 3.4). Propylene phenoxetol exposure exhibited a longer REC at a higher dose and this was also observed in *P. albina* (Norton et al. 1996) and in *Pteria penguin* Röding (Kishore 2011). Successful relaxation and recovery was also recorded in *Pinctada imbricata* Gould, *P. albina* (O'Connor & Lawler 2002) and *P. maxima* (Mamangkey et al. 2009). Norton et al. (1996) and O'Connor & Lawler (2002) showed that relaxation time when exposed to propylene phenoxetol was independent of the size of *P. albina*, suggesting potential for a single dose to be useful for different abalone sizes. However, large (70-140 mm SL) *H. iris* exposed to propylene phenoxetol displayed muscle contractions and mortalities (Aquilina & Roberts 2000). Although propylene phenoxetol shows potential, species-specific differences need to be taken into account. Propylene phenoxetol shows potential for further investigation for use as an abalone relaxant.

No mortalities were recorded when $MgCl_2$, 2-PE or PPE were administered, even at the higher doses. The dose and duration at which mortality would occur and therefore the margin of safety of the optimal doses is unknown.

Abalone exposed to 0.05 mL/L benzocaine had significantly longer REL than other doses and SGRW was 19 % and significantly (P<0.002) higher than controls. Benzocaine possesses considerable antimicrobial activity (Morrow & Berry 1988) and antimicrobials are used in animal husbandry to promote growth (Butaye et al. 2003). The antimicrobial activity of benzocaine may have influenced growth. Benzocaine, however, failed to meet most of the efficacy criteria because it induced mortality following exposure (Table 3.1), induced behavioural stress response and caused the foot muscle to cell slough. This is probably caused by exposure to ethanol used to dissolve the product causing dehydration (Stickland 1975); control abalone exposed only to ethanol exhibited similar effects. Exposing abalone to benzocaine at 0.10 and 0.20 mL/L caused the seawater containing the abalone to become blue. This phenomenon was also observed by Garrey (1905) during osmotic pressure experiments on *Limulus* spp. following gill microrupture and hemocyanin release. Microrupture of abalone gill and foot epithelial tissue from ethanol exposure or dehydration probably caused the blue water observed during the trials. Norton et al. (1996) observed that increasing exposure to benzocaine caused increasing irritation and torsion in *H. iris*. Norton et al. (1996) Aquilina & Roberts (2000), Hagen (2003), Mamangkey et al. (2009) and Kishore (2011) noted that benzocaine induces relaxation in other invertebrates, but with substantial variation in optimal dose and exposure time between species. Our observations of abalone exposed to benzocaine suggests that the dose (0.2 -0.4 mL/L) recommended by PER 14638 (APVMA 2016c) induces adverse effects.

MS-222 did not meet most industry requirements for an acceptable relaxant at all doses trialled (Table 3.4). Muscle rigidity following MS-222 exposure was also observed in *P. albina* (Norton et al. 1996), *H. iris* (Aquilina & Roberts 2000) and *S. gigas* (Acosta-Salmon & Davis 2007). Abalone exposed to MS-222 showed adverse effects at all doses. Buffering the treatment solution prevented a decrease in seawater pH (Norton et al. 1996) but

excess mucus production was still observed. Adding MS-222 at 1.0 or 2.0 g/L to seawater caused product to precipitate on the surface of the treatment water and these treatments probably did not reach the target concentration. MS-222 is not suitable for further investigation for use as an abalone relaxant.

Abalone exposed to AQUI-S™ (mostly isoeugenol as its active component) and clove oil (a mixture of eugenol, isoeugenol and methyl eugenol) (Javahery et al. 2012) exhibited similar outcomes. Doses of AQUI-S™ of 0.80 mL/L and clove oil of 0.70 mL/L reached saturation and the product formed a film on the treatment water. AQUI-S™ at 0.20 mL/L had a mean REL which is too long for practical use. Burke et al. (2001) also found that AQUI-S™ was an unreliable relaxant for abalone, observing tentacle relaxation but an absence of foot relaxation and with subsequent difficulty in removing the abalone from the substrate. The adverse effects and difficulties associated with administration observed during the trials make AQUI-S™ unsuitable for industry use as an abalone relaxant. During all clove oil exposures adverse effects were observed and abalone exposed to clove oil displayed REC longer than the industry requirement of <20 minutes. Exposing *Haliotis tuberculata coccinea* Linnaeus to clove oil caused foot rigidity and Bilbao et al. (2010) and Burke et al. (2001) determined that it was not a suitable relaxant for facilitating handling. We did not observe significant mortality in this study after exposure to clove oil but mortality was observed following exposure to 1.5 mL/L for 10.6 ± 4.7 minutes in *P. maxima* (Mamangkey et al. 2009) and during unsuccessful attempts to relax *P. margaritifera* (Norton et al. 1996). Clove oil does not meet industry criteria when used to relax abalone and is unsuitable for further investigation for industry use as a relaxant.

$MgSO_4$ delivered in solution did not meet most relaxant efficacy criteria. All doses of $MgSO_4$ caused irritation, discoloration, foot rigidity, violent removal from substrate and mortality. There was, however, no adverse effect on growth in survivors. The significant

salinity increases associated with $MgSO_4$ exposure at the doses we investigated are likely to induce osmotic shock on introduction of the treatment solution, which, combined with epithelial irritation, probably induced the observed excess mucus production. $MgSO_4$ induces gradual, gentle muscle relaxation and facilitates easy removal of juvenile *H. midae* (White et al. 1996) and also successfully relaxed juvenile *H. gigantea* Gmelin (Sagara & Ninomiya 1970). It did not, however, induce relaxation in 3-year-old *O. edulis* (Culloty & Mulcahy 1992) further indicating that relaxant action is species specific. Large amounts of $MgSO_4$ are required for successful relaxation which is disadvantageous for industry use (Messenger et al. 1985, Culloty & Mulcahy 1992). Heasman et al. (1995) observed similar adverse effects in *Pecten fumatus* to those we observed and also high mortality.

$MgSO_4$ and $MgCl_2$ had dissimilar effects on abalone. Magnesium ions are effective relaxants for invertebrates because they block muscle action, competing with the calcium required for synaptic transmission (Acosta-Salmón & Davis 2007). Higher delivered doses of Mg^{2+} should induce relaxation more quickly. $MgCl_2$ contains more Mg^{2+} than $MgSO_4$ on an equivalent weight basis and the concentrations of Mg^{2+} ions provided by the $MgCl_2$ and $MgSO_4$ doses are shown in Table 3.5. The relative differences in ionic concentration of doses of each salt account for the differences in salinity observed in treatments. The greater proportional content of Mg^{2+} in $MgCl_2$ than $MgSO_4$ makes the product provide a greater effective dose while adding less product than $MgSO_4$. This greater dose/weight efficiency makes $MgCl_2$ more suitable for industry use and provides savings if the products are similarly priced. Similar Mg^{2+} concentrations in $MgCl_2$ at 50 g/L (0.53 mol/L Mg^{2+}) and $MgSO_4$ at 60 g/L (0.50 mol/L Mg^{2+}), provided disparate RELs of 10±1 and 2±0 minutes, respectively. The apparent irritation and rapid but violent release induced by $MgSO_4$ makes the REL of the two products difficult to compare. Exposure to $MgSO_4$ elicits a different effect on abalone than $MgCl_2$, but the cause is unknown. At higher doses of $MgSO_4$, the significantly increased

salinity is likely to have had a substantial effect on the osmotic balance of the abalone. Abalone are osmoconformers but upregulate potassium $[K]^+$ and downregulate sodium $[Na]^+$ relative to seawater (Prior & Pierce 1981) using Na^+/K^+ exchange pumps (Scemes & Cassola 1995). Potassium maintains osmotic pressure in cells which facilitates normal nervous system function (Willmer 1978) and supports function when the animal experiences osmotic stress (Prior & Pierce 1981). If potassium/sodium regulation is overwhelmed, nervous system function can be damaged and if sufficiently severe, can lead to mortality (Benson & Treherne 1978, Burke et al. 2001). The forceful behavioural responses and significant mortality observed in the abalone exposed to higher doses of $MgSO_4$ are likely to have been associated with osmotic shock and loss of normal potassium/sodium balance leading to serious changes in the nervous system.

3.6 Conclusion

Relaxants can facilitate improved management of abalone during handling and movement. We found that $MgCl_2$, propylene phenoxetol and 2-PE are effective, safe relaxants for abalone. These relaxants show potential for further investigation to obtain data to support regulatory authorizations for use, but dose optimisation studies are required. Effective doses of relaxants for abalone can be size specific (White et al. 1996), so dose optimisation studies need to include abalone of different relevant sizes to obtain information that can be extended and relevant to industry.

Chapter 4: Effect of dose, size and temperature on efficacy of relaxants for abalone.

Mercer G. J., J. O. Harris & M. R. Deveney. 2016. Effect of dose, size and temperature on efficacy of relaxants for abalone. J. Shellfish. Res. (in preparation)

4.1 Abstract

The efficacy of 2-phenoxyethanol, magnesium chloride and propylene phenoxetol as relaxants for two sizes (shell length 18-28 and 70-90 mm) of greenlip abalone (*Haliotis laevigata* Donovan) was evaluated. For each product 5 doses were assessed for small abalone and 4 doses for large abalone, each at three temperatures (14, 18 and 22 °C). Small abalone were exposed to the relaxants for 10 minutes and large abalone were exposed for 20 minutes. Relaxation and recovery times were used to evaluate the efficacy of the relaxants for industry use. The lowest effective dose was determined at each temperature for each relaxant. Magnesium chloride and 2-phenoxyethanol are good candidates for further investigation as relaxants for use in abalone aquaculture, with small and large abalone tolerating 10 or 20 minute exposures, respectively, without displaying behavioural signs of stress and with successful relaxation, recovery and no negative effects on long term growth or survival. Propylene phenoxetol did not meet the criteria for a successful relaxant; approximately 60 % of large abalone spawned on exposure and relaxation was not achieved after 20 minutes exposure.

4.2 Introduction

In abalone aquaculture, relaxation is required to facilitate handling without injury or undue stress. The large numbers of individual abalone in a commercial farm make mechanical removal of each abalone logistically impractical and costly. Mechanical dislodgment, moreover, often causes injury or death (Ross et al. 2007) principally as a consequence of slow healing rates, limited capacity to coagulate haemolymph, stress and opportunistic infections (Hooper et al. 2007, Hooper et al. 2011). Handling following relaxation is safer, less invasive and more economical than mechanical removal of individual abalone and the routine use of relaxants in abalone aquaculture is widely accepted (White et

al. 1996). In consultation with Australian abalone farmers, the following criteria for good/effective/useful relaxants were developed: recovery in <20 minutes, with exposures of 10 minutes for small abalone and 20 minutes for large abalone producing no mortality or negative effects on growth or behavioural indicators of stress. These characteristics facilitate effective, logistically manageable large scale use in industry including facilitating removal and handling during husbandry procedures without premature recovery and re-attachment to equipment or a substrate.

A broad range of mollusc relaxants from literature were screened for their potential use in abalone aquaculture (Chapter 3). Three candidate relaxants, which were magnesium chloride ($MgCl_2$), 2-phenoxyethanol (2-PE) and propylene phenoxetol (PPE), met industry criteria for relaxation, recovery and effects of exposure in screening trials (Chapter 3). Optimal doses need to be determined for the candidate relaxants, so industry can develop best practice guidelines for relaxation and minimise cost without influencing efficacy (White et al. 1996).

The optimal dose for a relaxant varies with water temperature (White et al. 1996). Relaxation in abalone is induced more quickly for a given dose at elevated temperature (White et al. 1996, Aquilina & Roberts 2000). Low temperatures require higher doses to achieve the same effects observed at lower doses at higher temperatures (White et al. 1996). Abalone are poikilotherms and temperature influences the metabolic rate, gas exchange and oxygen supply (Hooper et al. 2007) which influences the relaxation and recovery times. South Australian greenlip abalone (*Haliotis laevigata* Donovan) grow optimally at 22 °C (Stone et al. 2013) but there is substantial temperature variation on and between farms (Stone et al. 2013). Water temperatures in South Australian, Victorian and Tasmanian abalone farms range from 8 °C to 26 °C. Some abalone aquaculture systems have temperature control, but for most systems water volume and flow preclude altering water temperature from ambient.

Relaxant efficacy is also influenced by abalone size (White et al. 1996). Small abalone have a greater surface area for their volume than large abalone and absorb immersion medicines faster than larger individuals (Burke et al. 2001). White et al (1996) found that for three size classes of *H. midae* Linnaeus relaxed using 2-phenxyethanol, optimum dose increased with abalone size, regardless of temperature.

We aimed to optimise doses of relaxants and assess relationships between dose, temperature and abalone size, to establish patterns of use which meet industry criteria for relaxing Australian abalone. For a relaxant to be useful for abalone aquaculture, it must induce gradual loss of adherence to the substrate in <5 minutes, allow recovery in <20 minutes following cessation of treatment with no mortality or ongoing effects on behaviour, be easy to administer and be safe and effective at low concentrations. Data for small abalone from the screening trial (Chapter 3) and for large abalone from White et al. (1996), Aquilina & Roberts (2000), Burke et al. (2000) and Bilbao et al. (2010) were used to set doses aimed at achieving equivalent relaxation and recovery times across relaxant products, sizes and temperatures.

4.3 Materials and methods

4.3.1 *Abalone*

Small (18-28 mm shell length SL) one-year-old farmed *H. laevigata* were obtained from the Southseas Abalone Group, Kangaroo Island Abalone (KIAB), Kangaroo Island, Smith Bay, South Australia. Large (70-90 mm shell length SL) two and a half year old farmed *H. laevigata* were obtained from South Australian Mariculture (SAM) Pty Ltd, Port Lincoln, South Australia. Small abalone were maintained in three 5 000 L fibreglass flow through tanks and large abalone were maintained in nine 1 000 L fibre glass flow through tanks at the South Australian Research and Development Institute (SARDI), South Australian

Aquatic Sciences Centre (SAASC), West Beach, South Australia. Abalone were acclimated at 14, 18 or 22 °C for two months, over September and October, through the use of either immersion heaters (240 V, 3 kw, JQ20; Austin and Cridland, Carlton, NSW, Australia) or chillers (3 hp, 240 V, 50 Hz: Daeil Cooler Co., Ltd., Busan, Korea). During acclimation abalone were fed 3 mm ECO-feed chip (EP Aquafeeds, Lonsdale, South Australia, Australia) in excess of the industry standard of 3 % body weight per day (Stone et al. 2013). The abalone were fed and the tanks were maintained daily. Seawater was sand filtered to 50 µm. The experimental tanks' ambient conditions were 102±3 % O_2 saturation and 35±1 ppt salinity.

4.3.2 *Tagging*

To facilitate identification of individual abalone, 30 days into acclimation and 30 days prior to the trials commencing, each abalone was tagged with glue-on shellfish tags (Hallprint Fish Tags Pty Ltd, South Australia) using a protocol adjusted for abalone from Henry & Jarne (2007): abalone were mechanically removed from the holding tanks using a soft blunt scraper, the shell was blotted dry and tags were attached with cyanoacrylate gel glue, positioned on the apex of the shell as not to impede tremata, shell growth or movement during the duration of the experiment. Abalone were exposed to air for less than 2 minutes. Abalone were then returned to the holding tanks.

4.3.3 *Experimental description*

Abalone were mechanically removed from the holding tanks using a blunt scraper, blotted dry and the body weight of each abalone was measured to the nearest 0.01 g using a scientific electronic balance (A&D HT-120, compact precision scale A&D Company Ltd). SL was measured along the longest axis to the nearest 0.01 mm with Vernier callipers. Small

abalone were randomly assigned to one of 45 buckets with 10 abalone per bucket; temperature and dose treatments are described in Table 4.1. Large abalone were randomly assigned to one of 36 buckets with 10 abalone per bucket; temperature and dose treatments are described in Table 4.2. Each bucket contained 5 L of continuously aerated water from the holding tank with the temperature corresponding to the experimental treatment. Abalone were allowed to settle and re-adhere to the bucket sides for 10 minutes. The water was then removed from the buckets and the dose of relaxant was mixed in 5 L of seawater and added to the treatment buckets. Water temperature was measured before relaxants were added. For each size class an equivalent set of untreated control abalone were subjected to the same handling conditions (Table 4.1, Table 4.2). Control abalone were removed from the treatment buckets using a blunt scraper and returned to the holding tanks. After the relaxants were added, abalone were observed continuously. Following exposure, for each abalone, relaxation time (REL), defined as the time from initial exposure to when the abalone lost adhesion to the substrate, was recorded. Small abalone were exposed to the relaxants for a total of 10 minutes and large for 20 minutes. After the exposure, abalone were placed dorsal side downwards into the holding tank at the correct temperature. The time from cessation of treatment until each inverted abalone could right itself and re-adhere to the substrate was measured (Recovery time (REC)). REL and REC were recorded in seconds. Results for small abalone are shown in Table 4.1 and results for large abalone are shown in Table 4.2.

During exposure to the relaxants, abalone were observed for behavioural and physiological signs of stress such as irregular shell movement, torsion (raising the shell away from the foot while twisting rapidly) or behaviours indicating that the abalone was attempting to avoid contact with the relaxant such as trying to escape the treatment bucket. Mucus production was regarded as excessive if foaming was observed on the surface of the water in the bucket during exposure to the relaxant. Type of relaxation such as gradual loss of

adherence to the substrate with a gentle fall to the floor of the bucket, urgent movement with rapid self-removal or clamping without loss of adherence which required subsequent mechanical removal was noted. Tentacle relaxation was also noted as a sign of the relaxant having an effect on the abalone. The abalone foot was observed and any loss of epithelial tissue (cell sloughing) or size decrease that might indicate dehydration was noted. Abalone foot colour was observed during transfer from exposure to recovery and any colour changes, darkening or development of colour irregularity was recorded. Mortality was recorded at 24 hours.

4.3.4 *Post-exposure growth*

Following exposure, abalone were gradually acclimated over 2 weeks to ambient temperature (18 °C) and maintained for 90 days. Post exposure, abalone were monitored daily for mortality and at 90 days post exposure each abalone was weighed and measured. Absolute growth rate (AGR) was determined for both length (L) and weight (W) using the following formulae: AGRL (mm/day) = ((Lf – Li)/t) where Lf = the final mean abalone shell length (mm), Li = the initial mean abalone shell length (mm), ln = natural logarithm and t = grow out trial period (days). AGRW (g/day) = ((Wf – Wi)/t), where Wf = the final mean abalone weight (g), Wi= the initial mean abalone weight (g) and t = grow out trial period (days). Specific growth rate was determined for both length and weight and using the following formulae: SGRL (% shell growth (mm) per day) = (ln(Lf) - ln(Li))/t x 100 and SGRW (% body weight (g) per day) = (ln(Wf) - ln(Wi))/t x 100. AGRL, AGRW, SGRL and SGRW at 90 days were compared within each relaxant between dose.

4.3.5 *Water quality measures*

During the experiment and subsequent grow-out, temperature (°C) (glass thermometer, Aldrich®), dissolved oxygen (DO, % saturation) (Oxyguard meter, Handy Polaris H01P, OxyGuard®), pH (IC-MW-102 meter) and salinity (PSU) (RHS-10ATC refractometer, Magnum Media™) were assessed. Temperature and DO of each acclimation tank were assessed daily. All water quality parameters were measured in each bucket prior to exposure in each trial (Table 4.3). All equipment was calibrated as per manufacturer instructions.

4.3.6 *Analysis*

One-way ANOVA (Zar 1999) was used to assess differences in starting mass of the abalone. The proportion of abalone that re-adhered to the substrate by 60 minutes (REC_{60}) was calculated. Survival was expressed as the proportion of abalone alive at 24 hours and at 90 days post exposure. Survival at 90 days data was taken from surviving abalone after 24 hours. One-way ANOVA was used to determine differences between doses, water quality (salinity, dissolved oxygen and temperature) and growth within size class and relaxant treatment. Data was analysed separately for each relaxant and temperature. All data were tested for normality with the one-sample Kolmogorov-Smirnoff test and for homogeneity of variances with Levene's test (Zar 1999). Data that were non-normal were log transformed. A post-hoc Tukey's test (Zar 1999) was used to determine the homogenous subsets. For all tests statistical significance was judged at the alpha level of 0.05. All statistical analysis was performed using SPSS Software for Windows (SPSS Inc., Chicago, IL, USA). All values are reported as the mean ± standard error.

4.4 Results

4.4.1 *Effects of exposure*

Small (Table 4.1) and large (Table 4.2) abalone exposed to $MgCl_2$ or 2-PE and small abalone exposed to PPE showed no adverse reactions and exhibited gradual foot relaxation, mantle extension and gentle loss of adherence to the substrate. No mortality at 24 hours was observed in any dose with 10 or 20 minute exposures for small and large abalone respectively. Approximately 60 % of large abalone exposed to PPE spawned during exposure.

Table 4.1 Responses of small greenlip abalone (*Haliotis laevigata*) to relaxant exposure.

Relaxant	Temperature (°C)	Dose	Relaxation (seconds)	Recovery (seconds)	Recovery (60 minutes) %	Survival (24 hours) %	Survival (90 days) %
2-phenoxyethanol (mL/L)	14	0.0	0±0	0±0	100	97	100
		2.0	165±9^{a}	838±52^{a}	100	97	97
		3.0	128±6^{a}	711±9^{b}	100	100	100
		4.0	82±7^{b}	704±10^{b}	100	100	100
		5.0	81±9^{b}	646±12^{b}	100	97	100
		6.0	91±6^{b}	817±19^{a}	100	94	100
	18	0.0	0±0	0±0	100	100	100
		0.5	171±17^{a}	655±135^{a}	100	100	100
		2.0	138±8^{a}	601±22^{a}	100	100	100
		3.0	98±10^{b}	731±56^{ab}	100	97	97
		4.0	82±6^{b}	842±59^{b}	100	100	100
		5.0	58±6^{c}	690±85^{ab}	100	97	100
	22	0.0	0±0	0±0	100	100	100
		0.5	188±4^{a}	713±10^{a}	100	97	97
		1.0	147±11^{ab}	703±24^{a}	100	100	100
		2.0	124±4^{ab}	614±13^{b}	100	100	100
		3.0	80±5^{b}	506±14^{c}	100	100	100
		4.0	84±6^{b}	608±56^{b}	100	100	100
magnesium chloride ($MgCl_2$) (g/L)	14	0.0	0±0	0±0	100	97	100
		50.0	733±6^{a}	1064±31^{a}	100	97	100
		80.0	154±16^{b}	824±34^{b}	100	100	100
		100.0	89±13^{b}	811±19^{b}	100	100	100
		150.0	95±7^{b}	468±40^{c}	100	100	100
		200.0	124±10^{b}	464±44^{c}	100	97	100
	18	0.0	0±0	0±0	100	100	100
		20.0	950±47^{a}	837±125^{a}	100	97	97
		50.0	584±151^{b}	815±72^{a}	100	100	100
		80.0	61±4^{c}	702±50^{b}	100	100	100
		100.0	48±5^{c}	834±101^{ab}	100	100	100
		150.0	76±4^{c}	783±47^{a}	100	100	100
	22	0.0	0±0	0±0	100	100	100
		20.0	962±78^{a}	817±30^{a}	100	100	100
		50.0	286±34^{b}	897±56^{a}	100	100	100
		60.0	71±5^{c}	613±16^{b}	100	100	97
		80.0	50±5^{c}	610±14^{b}	100	100	100
		100.0	40±2^{c}	659±19^{b}	100	100	97
propylene phenoxytol (mL/L)	14	0.0	0±0	0±0	100	97	100
		1.0	185±10^{a}	953±8^{b}	100	97	100
		2.0	165±13^{a}	1111±11^{ab}	100	100	97
		3.0	133±10^{ab}	1310±36^{a}	100	100	97
		4.0	97±4^{b}	1001±7^{b}	100	100	100
		5.0	82±2^{b}	807±24^{b}	100	97	97
	18	0.0	0±0	0±0	100	100	100
		1.0	130±13^{a}	889±178^{ab}	100	100	100
		2.0	123±3^{a}	789±96^{ab}	100	100	100
		3.0	110±2^{a}	1001±63^{a}	100	97	97
		4.0	73±19^{ab}	830±140^{ab}	100	100	100
		5.0	41±8^{b}	636±57^{b}	100	100	97
	22	0.0	0±0	0±0	100	100	97
		0.5	215±16^{a}	655±7^{b}	100	100	100
		1.0	112±10^{ab}	707±10^{b}	100	100	100
		2.0	92±3^{b}	800±16^{a}	100	100	97
		3.0	71±3^{b}	749±8^{a}	100	100	100
		4.0	47±3^{b}	614±43^{b}	100	100	97

n=30 per dose. Small *H. laevigata* (18-28 mm shell length). Dose 0.0 is untreated control. Standard error of the means is presented. Superscripts indicate significant differences in mean relaxation and recovery rates (P<0.05). The relaxation rate and the recovery rate were analysed separately within their temperature categories.

Table 4.2 Responses of large greenlip abalone (*Haliotis laevigata*) to relaxant exposure.

Relaxant	Temperature (°C)	Dose	Relaxation (seconds)	Recovery (seconds)	Recovery (60 minutes) %	Survival (24 hours) %	Survival (90 days) %
2-phenoxyethanol (mL/L)	14	0.0	0±0	0±0	100	100	100
		6.0	528±18^{c}	1691±66^{c}	100	97	100
		10.0	357±7^{b}	1257±83^{b}	100	100	100
		15.0	326±9^{b}	1023±82^{b}	100	100	97
		20.0	157±8^{a}	630±48^{a}	100	97	100
	18	0.0	0±0	0±0	100	100	100
		3.0	595±18^{c}	1190±97^{c}	100	97	100
		6.0	387±14^{b}	1204±99^{c}	100	100	100
		10.0	329±3^{b}	810±114^{b}	100	100	100
		20.0	75±11^{a}	260±9^{a}	100	100	100
	22	0.0	0±0	0±0	100	100	100
		3.0	583±14^{c}	736±51^{b}	100	100	100
		5.0	387±16^{b}	808±79^{b}	100	100	100
		6.0	315±16^{b}	738±49^{b}	100	100	97
		10.0	238±13^{a}	465±18^{a}	100	100	100
magnesium chloride ($MgCl_2$) (g/L)	14	0.0	0±0	0±0	100	100	100
		160.0	567±29^{a}	1258±117^{a}	100	100	100
		180.0	347±10^{b}	996±58^{b}	100	100	100
		200.0	342±10^{b}	1012±34^{b}	100	100	100
		220.0	293±5^{b}	1087±90^{b}	100	97	100
	18	0.0	0±0	0±0	100	100	100
		100.0	1383±30^{a}	1171±74^{a}	100	100	100
		120.0	884±95^{b}	1049±63^{ab}	100	100	100
		160.0	374±49^{c}	935±42^{b}	100	100	100
		200.0	281±37^{c}	967±50^{b}	100	100	100
	22	0.0	0±0	0±0	100	100	100
		100.0	932±35^{a}	713±18^{a}	100	100	100
		120.0	517±27^{ab}	635±32^{b}	100	100	97
		160.0	330±19^{b}	618±15^{b}	100	100	100
		200.0	224±28^{b}	613±20^{b}	100	100	100

n=30 per dose. Large *H. laevigata* (70-90 mm shell length). Dose 0.0 is untreated control. Standard error of the means is presented. Superscripts indicate significant differences in mean relaxation and recovery rates (P<0.05). The relaxation rate and the recovery rate were analysed separately within their temperature categories.

Table 4.3 Optimal doses of 2-phenoxyethanol, magnesium chloride and propylene phenoxetol for greenlip abalone (*Haliotis laevigata*).

Relaxant	Abalone	Temperature (°C)	Duration	Optimal dose
2-phenoxyethanol (mL/L)	Small	14	10	3.0
		18	10	2.0
		22	10	2.0
	Large	14	20	10.0
		18	20	6.0
		22	20	5.0
magnesium chloride ($MgCl_2$) (g/L)	Small	14	10	100.0
		18	10	80.0
		22	10	60.0
	Large	14	20	180.0
		18	20	160.0
		22	20	160.0
propylene phenoxetol (mL/L)	Small	14	10	3.0
		18	10	1.0
		22	10	1.0

Small *H. laevigata* (18-28 mm shell length), large *H. laevigata* (70-90 mm shell length).

4.4.2 *Temperature effect*

At 14 ºC REL was longer and higher doses were required to meet industry REL criteria than at other temperatures (Table 4.3). This was particularly evident for small abalone where $MgCl_2$ optimal doses for 14, 18 and 22 °C were 100, 80 and 60 g/L, for 2-PE where optimal doses were 3.0, 2.0 and 2.0 mL/L and for 2-PE for large abalone where optimal doses were 10.0, 6.0 and 5.0 mL/L at 14, 18 and 22 °C respectively. REC was shorter at higher temperatures and longer at lower temperatures. At 14 °C REC was longer for all relaxants. The longest REC for small abalone was 21-23 minutes following exposure to 3 mL/L PPE at 14 °C. This REC was longer than industry REC criteria. REC was shorter at 18 and 22 °C than at 14 °C. Large abalone exposed to 2-PE at 20 mL/L at 18°C or 10 mL/L at 22°C had mean REC of 4 and 8 minutes respectively. These were shorter than industry REC criteria.

4.4.3 *Size effect*

Abalone size was positively associated with safe REL; higher doses were required for larger abalone to achieve industry criteria for safe REL and REC. REL could not be determined for PPE for the large abalone at any temperature because spawning prevented observation of the abalone, but on cessation of treatment all large abalone exposed to PPE still adhered to the buckets. There was no clear relationship between REC and size.

4.4.4 *Post relaxation grow-out*

There were no significant differences (P>0.05) in abalone AGRW, AGRL, SGRW or SGRL between temperatures or doses for each relaxant. There were no significant differences (P>0.05) in growth between treated and control abalone. All small abalone across all treatments and controls grew 0.10±0.02 - 0.11±0.06 mm/day and 0.10±0.01 - 0.12±0.06 g/day. All large abalone across all treatments and controls grew 0.09±0.00 - 0.10±0.04 mm/day and 0.05±0.02 - 0.06±0.01 g/day. There were no significant differences (P>0.05) in 24 hour survival between relaxants. There were no significant differences (P>0.05) in mortality between relaxants over the 90 days post exposure.

4.4.5 *Water quality*

No significant differences (P>0.05) were observed for any water quality parameter, including control test buckets, for any relaxant or dose.

4.5 Discussion

$MgCl_2$ and 2-PE met all industry criteria for a safe, effective relaxant for all sizes of abalone at all temperatures. Optimal doses met safe REC industry criteria of <20 minutes with exposure times of 10 minutes for small abalone and 20 minutes large abalone. No behavioural signs of stress or mortalities were observed and there were no adverse effects on growth. Optimal doses are outlined in Table 4.3. Each is the lowest dose that met industry time and behaviour criteria and had REC which was not statistically significantly different to REC for a higher dose.

$MgCl_2$ successfully relaxes other molluscs with no adverse effects including: *Ostrea edulis* Linnaeus (Culloty & Mulcahy 1992), *Pecten fumatus* Reeve (Heasman et al. 1995), *Strombus gigas* Linnaeus (Acosta-Salmon & Davis 2007), *Saccostrea glomerata* Gould (Butt et al. 2008) and *Crassostrea gigas* Thunberg (Suquet et al. 2009). 2-PE induces relaxation successfully with good recovery in a variety of molluscs including *Pinctada albina* Lamarck (Norton et al. 1996), *H. rubra* Gmelin (Burke et al. 2001) and *P. maxima* Jameson (Mamangkey et al. 2009). Large (60-90 mm SL) *H. midae* exposed to 3 mL/L 2-PE, a lower dose to the proposed optimal from our study, were not relaxed and exhibited rigid contraction of the adductor muscle during exposure with subsequent 100% mortality; demonstrating that responses to 2-PE appear to vary between abalone species (White et al. 1996).

The dose of $MgCl_2$ or 2-PE required to achieve acceptable REL at 18 or 22 ºC was less than that required at 14 ºC, probably due to increased absorption of the relaxant with rising temperature. Increased metabolic rate at higher temperatures (Fujino et al. 1984, Norton et al. 1996) facilitates more rapid uptake of relaxants. Faster relaxation with increasing temperature was also observed in scallops, *Pecten fumatus* Müller relaxed with chloral hydrate (Heasman et al. 1995). Effects of temperature on metabolism may also

facilitate more rapid elimination of relaxants from abalone. Faster REC was observed at 18 and 22 ºC compared to REC at 14 ºC for all tested relaxants.

No doses produced mortality, even though some were 4x the optimal dose (for $MgCl_2$) and 3.2x for 2-PE. This suggests that the optimal doses and the lowest dose with toxic effects are substantially different, suggesting a broad margin of safety (MOS). Further investigation is needed to assess host safety of the recommended doses.

PPE successfully relaxed small abalone with acceptable REL and REC. PPE did not, however, induce relaxation reliably in large abalone. All doses of PPE for large abalone derived from the screening trial and Aquilina & Roberts (2000) caused approximately 60 % of exposed abalone to spawn at all temperatures. Induction of spawning may have affected the ability of the exposures to achieve reliable REL or REC because of interactions between the gametes in the water and the available product in the treatment solution. Propylene phenoxetol is not a suitable relaxant for large abalone and will not be investigated further.

4.6 Conclusion

These data provide the abalone industry with optimised doses and information to understand the relationships between effective dose of $MgCl_2$ or 2-PE, temperature and abalone size. The optimal doses are useful for industry to manage abalone relaxation with greater control and improved understanding of the environmental influences on relaxation for each product. $MgCl_2$ and 2-PE are successful candidates for abalone relaxation and require further investigation. Target animal safety (relaxant lethal concentration, LC and LT) and residue studies need to be completed, particularly to identify products suitable for rested harvest. Field validation of products and delivery methods is also required.

Chapter 5: Dose and duration safety studies for immersion treatment of abalone using 2-phenoxyethanol and magnesium chloride.

Mercer G. J., J. O. Harris & M. R. Deveney. 2016 Dose and duration safety studies for immersion treatment of abalone using 2-phenoxyethanol and magnesium chloride. J. Shellfish. Res. (in preparation)

5.1 Abstract

Regulatory approval is being sought to use 2-phenoxyethanol (2-PE) and magnesium chloride ($MgCl_2$) as relaxants for use in abalone aquaculture in Australia, but host safety data for abalone were absent. For greenlip abalone (*Haliotis laevigata* Donovan), dose optimisation studies indicated that optimal doses were 2 mL/L 2-PE or 80 g/L $MgCl_2$ for 10 minutes for small (18-28 mm shell length) abalone and 6 mL/L 2-PE or 160 g/L $MgCl_2$ for 20 minutes for large (70-90 mm shell length) abalone. We investigated the effect of increasing dose (1, 2, 3, 4, 5x) or prolonging exposure (1, 2, 4, 6, 12x for small abalone and 1, 2, 3, 6x for large abalone) on behaviour, recovery time and mortality. Significant increases in mortality (P<0.05) were observed at 4x the optimal dose for small abalone exposed to 2-PE and small and large abalone exposed to $MgCl_2$. Large abalone exposed to 2-PE displayed a significant increase in mortality (P<0.05) at 3x the optimal dose and at 5x the optimal dose 100 % mortality was observed at 24 hours. LC_{50} values for overdose at optimal exposure time for abalone exposed to 2-PE were 10-min LC_{50} 9.73 mL/L (small *H. laevigata*) and 20-min LC_{50} 20.81 mL/L (large *H. laevigata*). LC_{50} values for overdose at optimal exposure time for abalone exposed to $MgCl_2$ were 10-min LC_{50} 400 g/L (small *H. laevigata*) and 20-min LC_{50} 778 g/L (large *H. laevigata*). Significant increases in mortality (P<0.05) were observed at 6x the optimal exposure time for small abalone exposed to 2-PE and small and large abalone exposed to $MgCl_2$. A significant increase in mortality (P<0.05) was observed at 3x the optimal exposure time for large abalone exposed to 2-PE. LT_{50} values for overexposure at optimal dose for abalone exposed to 2-PE were 2 mL/L LT_{50} 103.2-min (small *H. laevigata*) and 6 mL/L LT_{50} 93.1-min (large *H. laevigata*). LT_{50} values for overexposure at optimal dose for abalone exposed to $MgCl_2$ were 80 g/L LT_{50} 108.4-min (small *H. laevigata*) and 160 g/L LT_{50} 115.3-min (large *H. laevigata*). There were no effects of any treatment on growth of abalone that survived 90 days subsequent to exposure. These

safety margins are adequate for growers to administer 2-PE or $MgCl_2$ without risk of mortality with normal farm infrastructure, controls and good management. Small and large abalone can be effectively and safely relaxed using either 2-PE or $MgCl_2$ without long term mortality or effects on growth.

5.2 Introduction

Farming practices such as grading, adjusting stock density, transfer between tanks, system maintenance and harvesting require periodic removal of abalone from their holding tanks (Ross et al. 2007). Abalone possess a large, muscular foot, which functions as an adhesive organ, allowing it to adhere to or pull the shell down tightly onto substrates (Aquilina & Roberts 2000, Bilbao et al. 2010). Dislodgement by mechanical means during farm practice often results in injury and subsequent mortality principally as a consequence slow healing rates and the limited capacity of abalone to coagulate haemolymph (Hooper et al. 2007, Hooper et al. 2011). Relaxants can reduce abalone mortality, decrease stress and enhance product quality by preventing shell and muscle damage during removal from substrates. Relaxant use is a widely accepted practice in abalone aquaculture (White et al. 1996, Ross & Ross 2008). For a relaxant to be useful for abalone aquaculture, it must have a safe 10 minute exposure for small (18-28 mm Shell length SL) abalone and 20 minute exposure for large (70-90 mm SL) abalone with no mortality or negative effects on growth. These characteristics would facilitate effective, logistically manageable large scale use in industry.

Regulatory approval for veterinary medicines for use on animals for human consumption requires safety studies to provide insight into the relationship between the doses provided by efficacy and optimisation studies and the lowest toxic dose, facilitating determination of a margin of safety (MOS) for the target animal. MOS is the ratio of the

range between the lowest therapeutic dose and the lowest toxic dose of a drug. This information can then be used to assess if the planned pattern of use is safe and to define the likely adverse effects of overdose and/or increased duration of exposure. The MOS is an effective tool to compare safety of different relaxants.

2-Phenoxyethanol (2-PE) and magnesium chloride ($MgCl_2$) are two candidate relaxants identified for further research for use on abalone (see Chapters 3 and 4). These relaxants induce a gradual loss of adherence to the substrate with no mortality or ongoing effects on abalone growth (see Chapters 3 and 4) and appear suitable for all husbandry procedures during abalone grow-out in aquaculture. Dose determination and dose optimisation studies indicated that 2 mL/L 2-PE or 80 g/L $MgCl_2$ for 10 minutes exposure for small (18-28 mm shell length (SL)) abalone and 6 mL/L 2-PE or 160 g/L $MgCl_2$ for 20 minutes for large (70-90 mm SL) abalone were the lowest doses that provided effective relaxation (see Chapters 3 and 4). The MOS for these products and doses for use on abalone is, however, unknown and needs to be determined.

In chapters 3 and 4, relaxation time and optimal dose have been shown to vary with abalone size, but relaxation time is also subject to variability caused by stress, environmental factors and abalone health (Norton et al. 1996, White et al. 1996, Burke et al. 2001). While assessment of recovery time (REC) is a good indicator for the utility of optimised doses, no mortality was observed when using optimised doses so those studies do not inform understanding the potential adverse impacts of relaxant overdose or overexposure on abalone health.

Understanding the MOS of proposed relaxants is critical for preventing mortality and potential negative impacts of relaxant misuse in industry application. Following Australian Pesticides and Veterinary Medicines Authority data guidelines (APVMA 2016a), we

undertook dose and duration safety studies for immersion exposure of small and large abalone using 2-PE and $MgCl_2$ and determined the MOS for these products.

5.3 Materials and methods

5.3.1 *Abalone*

Small (18-28 mm shell length SL) one-year-old farmed greenlip abalone (*H. laevigata*) were obtained from the Southseas Abalone Group, Kangaroo Island Abalone (KIAB), Smith Bay, Kangaroo Island, South Australia. Large (70-90 mm shell length SL) two and a half year old farmed greenlip abalone (*H. laevigata*) were obtained from SAM Abalone Pty Ltd, Port Lincoln, South Australia. Small abalone were maintained in a 5 000 L fibreglass flow through holding tank and large abalone were maintained in eight 1 000 L fibre glass flow through holding tanks at the South Australian Aquatic Sciences Centre (SAASC), South Australian Research and Development Institute (SARDI), West Beach, South Australia. Seawater was sand filtered to 50 µm. Holding conditions were 18±1 °C, 102±3 % O_2 saturation and 35±1 ppt salinity. Abalone were acclimated to trial conditions for two months. During holding abalone were fed 3 mm ECO-feed chip (EP Aquafeeds, Lonsdale, South Australia, Australia) in excess of the industry standard of 3 % body weight per day (Stone et al. 2013). Abalone were fed and the holding tanks were maintained daily.

5.3.2 *Tagging*

To facilitate identification of individual abalone, 30 days into acclimation and 30 days prior to the trials commencing, each abalone was tagged with glue-on shellfish tags (Hallprint Fish Tags Pty Ltd, South Australia) using a protocol adjusted for abalone from Henry & Jarne (2007): abalone were mechanically removed from the holding tanks using a soft blunt scraper, the shell was blotted dry and tags were attached with cyanoacrylate gel glue,

positioned on the apex of the shell as not to impede shell growth or movement during the duration of the experiment. Abalone were exposed to air for less than 2 minutes. Abalone were then returned to the holding tanks.

5.3.3 *Allocation of abalone to the experimental tanks*

Abalone were mechanically removed from the holding tanks using a soft, blunt scraper and blotted dry to remove excess water. Body weight was measured to the nearest 0.01 g using a calibrated scientific electronic balance (A&D HT-120, compact precision scale A&D Company Ltd). SL was measured along the longest axis of the abalone shell to the nearest 0.01 mm using Vernier calipers. Water in all test buckets was 18±1 °C, 102±2 % O_2 saturation and 35±2 ppt salinity, with water taken from holding tanks.

5.3.4 *Duration and dose studies*

For each of the relaxants, 300 small and 300 large abalone from the source populations were manually removed using a blunt scraper and placed in treatment buckets, allowed to settle and re-adhere to the bucket sides. Three replicate buckets were used for each treatment, each containing 10 abalone. All buckets were aerated continuously. The water was then removed from the buckets and a pre-mixed dose of relaxant in 5 L of holding tank water was added (10 L bucket containing 5 L of solution). For the dose study, small abalone were exposed to 2, 4, 6, 8 or 10 mL/L 2-PE and 80, 160, 240, 320 or 400 g/L $MgCl_2$ and large abalone were exposed to 6, 12, 18, 24 or 30 mL/L 2-PE or 160, 320, 480, 640 or 800 g/L $MgCl_2$. Exposure time was 10 minutes for small abalone and 20 minutes for large abalone.

For the duration study, small abalone were exposed to 2 mL/L 2-PE or 80 g/L $MgCl_2$ and large abalone were exposed to 6 mL/L 2-PE or 160 g/L $MgCl_2$ for 10, 20, 30, 60 and 120 minutes. For both duration and dose studies a set of untreated controls for both small and

large abalone, comprised 3 replicate buckets containing 10 abalone per bucket. Control abalone were subjected to handling as per other treatments, but were returned to holding tanks after handling and maintained for subsequent mortality and growth analysis. After treatment, abalone were placed dorsal side down into the holding tank. The time from cessation of treatment until each inverted abalone could right itself and re-adhere to the substrate was measured (Recovery time (REC)). REC was recorded in seconds.

Non-recovery was determined to have occurred when abalone oriented dorsal side down were not able to right themselves and re-adhere to the substrate by 120 minutes post-treatment and mortality was determined to have occurred when abalone remained dorsal side down 24 hours post-exposure (Table 5.1 and 5.2).

5.3.5 *Reference toxicant*

Zinc (Zn) as $ZnCl_2$ was used as a health reference toxicant following Liao et al. (2002) who demonstrated a 24-h LC_{50} of 1.8 mg L-1 . Small abalone were exposed to 0.5, 1.0, 2.0 and 4.0 mg L-1 $ZnCl_2$ with 3 replica buckets for each dose and 10 abalone per bucket were used. Abalone were exposed to zinc for 24 hours. After treatment, abalone were placed dorsal side down into the holding tanks. No reference toxicant data were available for large abalone.

5.3.6 *Effects of exposure*

During exposure to the relaxants, abalone were observed for behavioural and physiological signs of stress such as irregular shell movement, torsion (raising the shell away from the foot while twisting rapidly) or behaviours indicating that the abalone was attempting to avoid contact with the relaxant such as trying to escape the treatment bucket. Mucus production was regarded as excessive if mucus was observed on the surface of the water in

the bucket during exposure to the relaxant. Type of relaxation such as gradual loss of adherence to the substrate with a gentle fall to the floor of the bucket, urgent movement with rapid self-removal or clamping without loss of adherence which required subsequent mechanical removal was noted. Tentacle relaxation was also noted as a sign of the relaxant having an effect on the abalone. The abalone foot was observed and any loss of epithelial tissue (cell sloughing) or size decrease that might indicate dehydration was noted. Abalone foot colour was observed during transfer from exposure to recovery and any colour changes, darkening or development of colour irregularity was recorded.

5.3.7 *Post-exposure growth*

Following exposure, abalone were returned to the holding tanks and maintained for 90 days. After 90 days, the abalone were removed from the holding tanks using a soft, blunt scraper and blotted dry to remove excess water and body weight and SL were recorded. Growth after 90 days was compared within each relaxant between each dose and time of exposure. Absolute growth rate (AGR) was determined for both length and weight using the following formulae: AGRL (mm/day) = ((Lf – Li)/t), where Lf = the final mean abalone shell length (mm), Li = the initial mean abalone shell length (mm), ln = natural logarithm and t = grow out trial period (days) and AGRW (g/day) = ((Wf – Wi)/t), where Wf = the final mean abalone weight (g), Wi= the initial mean abalone weight (g) and t = grow out trial period (days). Specific growth rate SGRL (% shell growth (mm) per day) and SGRW (% body weight (g) per day) were determined for both length and weight using the following formulae: (ln(Lf) - ln (Li))/t x 100 and (ln(Wf) - ln(Wi))/t x 100 respectively.

5.3.8 *Analysis*

Abalone body weight was subjected to a one-way ANOVA test (Zar 1999) for all treatments prior to the trial confirmed no significant differences in the length or mass of abalone between different treatments of dose or duration for each relaxant (P>0.05). For each relaxant, REC, % non-recovery and % mortality of all treatments were compared against relaxant and study type (Table 5.1 and 5.2). One-way ANOVA (Zar 1999) was used to assess differences in growth between exposure treatments. All data were tested for normality using the one-sample Kolmogorov-Smirnoff test and for homogeneity of variances with Levene's test (Zar 1999) and log transformed when required to meet normality. A post-hoc Tukey test (Zar 1999) was used to determine the homogenous subsets. For all tests statistical significance was judged at the alpha level of 0.05. All statistical analyses were performed using SPSS Software for Windows (SPSS Inc., Chicago, IL, USA). All values are reported as the mean ± standard error. The MOS was calculated using MOS = lowest effective dose/lowest dose that caused significant mortality. LC_{50} and LT_{50} values were determined using a Trimmed Spearman-Karber estimate following Hamilton et al. (1977) (Table 5.3).

5.4 Results

5.4.1 *Dose study*

No significant differences in the length or mass of abalone was found between different treatments of dose or duration for each relaxant (P>0.05). Small abalone exposed to 4x the optimal dose of 2-PE (8 mL/L 2-PE) showed a significant (P<0.05) increase in mortality compared to controls. Small and large abalone exposed to 4x the optimal dose of $MgCl_2$ (320 and 640 g/L $MgCl_2$) showed significant (P<0.05) increases in mortality compared to controls. Large abalone exposed to 3x the optimised dose of 2-PE (18 mL/L 2-PE) showed a significant (P<0.05) increase in mortality compared to controls. Small and

large abalone exposed to 3x the optimal dose of $MgCl_2$ (240 and 480 g/L $MgCl_2$) showed no significant increase (P>0.05) in mortality compared to controls. Small and large abalone exposed to 3x and 2x the optimal dose of 2-PE (6 and 12 mL/L) showed no significant increases (P>0.05) in mortality compared to controls. Large abalone exposed to 5x the optimal dose of 2-PE (30 mL/L 2-PE) resulted in 100 % mortality at 24 hours. Table 5.1 includes study data for 2-PE and Table 5.2 includes data for $MgCl_2$.

LC_{50} values after $MgCl_2$ overdose at optimal times are 10-min LC_{50} 400.2 g/L (small *H. laevigata*) and 20-min LC_{50} 778.1 g/L (large *H. laevigata*) (see Table 5.3). LC_{50} values after 2-PE overdose at optimal times are 10-min LC_{50} 9.73 mL/L (small *H. laevigata*) and 20-min LC_{50} 20.81 mL/L (large *H. laevigata*) (Table 5.3).

5.4.2 *Duration study*

Small abalone exposed to 2-PE (2 mL/L 2-PE) for 6x the optimal time of 10 minutes showed significant (P<0.05) increase in mortality compared to controls. Large abalone exposed to 2-PE (6 mL/L 2-PE) for 3x optimal time of 20 minutes showed significant (P<0.05) increase in mortality compared to controls. Small and large abalone exposed to $MgCl_2$ (80 and 160 g/L $MgCl_2$) for 6x the optimal times of 10 and 20 minutes respectively, showed significant (P<0.05) increase in mortality compared to controls. Small and large abalone were exposed to of 2-PE (2 and 6 mL/L 2-PE) 4 and 2x the optimal times of 10 and 20 minutes respectively, with no significant (P>0.05) increase in mortality compared to controls. Small and large abalone were exposed to $MgCl_2$ (80 and 160 g/L $MgCl_2$) for 4 and 3x optimal times of 10 and 20 minutes respectively, with no significant (P>0.05) increase in mortality compared to controls.

LT_{50} values from exposure to $MgCl_2$ at optimised doses are 108.1-min LT_{50} 80 g/L (small *H. laevigata*) and 115.3-min LT_{50} 160 g/L (large *H. laevigata*) (Table 5.3). LT_{50}

values from exposure to 2-PE at optimised times are 103.2-min LT_{50} 2 mL/L (small *H. laevigata*) and 93.1-min LT_{50} 6 mL/L (large *H. laevigata*) (Table 5.3).

Table 5.1 Responses of greenlip abalone (*Haliotis laevigata*) to 2-phenoxyethanol overdose and overexposure.

Abalone size (mm shell length)	Assessment	Dose (mL/L)	Exposure time (minutes)	Time to recovery (minutes)	% Non-recovery (120 minutes)	% Mortality (24 hours)
Small (18-28)	Duration	2	10	21±6^{a}	0.0^{a}	0.0^{a}
		2	20	25±12^{a}	0.0^{a}	0.0^{a}
		2	40	86±42^{b}	10.0^{a}	3.3^{a}
		2	60	121±49^{bc}	40.0^{b}	20.0^{b}
		2	120	179±34^{c}	100.0^{c}	73.3^{c}
	Dose	2	10	18±4^{a}	0.0^{a}	0.0^{a}
		4	10	22±6^{a}	0.0^{a}	0.0^{a}
		6	10	78±48^{b}	13.3^{a}	3.3^{a}
		8	10	113±62^{b}	43.3^{b}	16.7^{b}
		10	10	135±14^{b}	100.0^{c}	56.7^{c}
Large (70-90)	Duration	6	10	20±2^{a}	0.0^{a}	0.0^{a}
		6	20	23±5^{a}	0.0^{a}	0.0^{a}
		6	40	92±31^{b}	20.0^{b}	3.3^{a}
		6	60	146±35^{bc}	93.3^{c}	16.7^{b}
		6	120	213±78^{c}	100.0^{c}	70.0^{c}
	Dose	6	20	22±3^{a}	0.0^{a}	0.0^{a}
		12	20	103±36^{b}	26.7^{b}	6.7^{a}
		18	20	126±38^{b}	53.3^{b}	30.0^{b}
		24	20	183±56^{c}	100.0^{c}	83.3^{c}
		30	20	NR	100.0^{c}	100.0^{d}

NR = no result. n=30 per dose. Different superscripts indicate significant differences (P<0.05). Mean and standard error is presented for time to recovery. Time to recovery (minutes), % non-recovery (120 minutes) and % mortality (24 hours) (minutes) were analysed separately.

Table 5.2 Responses of greenlip abalone (*Haliotis laevigata*) to magnesium chloride overdose and overexposure.

Abalone size (mm shell length)	Assessment	Dose (g/L)	Exposure time (minutes)	Time to recovery (minutes)	% Non-recovery (120 minutes)	% Mortality (24 hours)
Small (18-28)	Duration	80	10	15±3^{a}	0.0^{a}	0.0^{a}
		80	20	23±11^{a}	0.0^{a}	0.0^{a}
		80	40	79±43^{ab}	6.7^{a}	3.3^{a}
		80	60	111±26^{b}	33.3^{b}	16.7^{b}
		80	120	164±22^{c}	100.0^{c}	66.7^{c}
	Dose	80	10	14±5^{a}	0.0^{a}	0.0^{a}
		160	10	39±13^{a}	0.0^{a}	0.0^{a}
		240	10	103±28^{a}	16.7^{b}	0.0^{a}
		320	10	118±18^{b}	40.0^{b}	20.0^{b}
		400	10	128±6^{b}	100.0^{c}	50.0^{c}
Large (70-90)	Duration	160	10	14±2^{a}	0.0^{a}	0.0^{a}
		160	20	18±3^{a}	0.0^{a}	0.0^{a}
		160	40	78±46^{b}	13.3^{a}	0.0^{a}
		160	60	124±29^{bc}	76.7^{b}	3.3^{a}
		160	120	152±27^{c}	100.0^{c}	56.7^{b}
	Dose	160	20	18±9^{a}	0.0^{a}	0.0^{a}
		320	20	94±33^{b}	13.3^{a}	0.0^{a}
		480	20	115±27^{b}	20.0^{a}	3.3^{a}
		640	20	132±10^{b}	68.0^{b}	20.0^{b}
		800	20	136±12^{b}	100.0^{c}	63.3^{c}

n=30 per dose. Different superscripts indicate significant differences (P<0.05). Mean and standard error is presented for time to recovery. Time to recovery (minutes), % non-recovery (120 minutes) and % mortality (24 hours) (minutes) were analysed separately.

Table 5.3 Toxicity and margin of safety of 2-phenoxyethanol and magnesium chloride for greenlip abalone (*Haliotis laevigata*).

Relaxant	Units	Abalone size	Effective dose	Time exposure (minutes)	Margin of safety (overexposure)	Margin of safety (overdose)	LT_{50} (minutes) (optimal dose, extended duration)	LC_{50} dose (optimal time, overdose)
2-phenoxyethanol	mL/L	Small	2	10	0.4	0.3	103.2	9.73
		Large	6	20	0.2	0.2	93.1	20.81
magnesium chloride	g/L	Small	80	10	0.4	0.3	108.4	400.2
		Large	140	20	0.3	0.3	115.3	778.1

Margin of safety: (dose/time with no adverse effect / dose/time optimal). Small *H. laevigata* (18-28 mm shell length), large *H. laevigata* (70-90 mm shell length).

5.4.3 *Effects of exposure*

All small and large abalone exposed to $MgCl_2$ or 2-PE displayed gradual muscle relaxation with foot tone that became increasingly flaccid with relaxation. Abalone treated with either $MgCl_2$ or 2-PE showed no signs of irritation, produced no excess mucus and no change in foot colour was observed. There were no unusual or adverse behaviours observed in abalone on exposure to any treatment or during the handling of untreated controls at sub lethal doses.

5.4.4 *Reference control toxicant*

The zinc LC_{50} was 24-h LC_{50} 2.0 mg L-1 $ZnCl_2$ (small *H. laevigata*). There were no unusual or adverse behaviours observed in abalone on or during exposure to $ZnCl_2$.

5.4.5 *Post relaxation grow-out*

No significant difference (P>0.05) was observed in survival between all treatments and untreated control at 90 days. No significant difference (P>0.05) was observed at 90 days post exposure to either relaxant at any dose for SGRL, SGRW, AGRL or AGRW (data not shown). AGRL for all small abalone was 0.10 (0.09-0.11) - 0.11 (0.07-0.15) mm/day and AGRW was 0.09 (0.09-0.09) - 0.13 (0.12-0.14) g/day. AGRL for large abalone was 0.09 (0.08-0.10) - 0.10 (0.10-0.10) mm/day and AGRW was 0.04 (0.04-0.04) - 0.06 (0.04-0.08) g/day.

5.5 Discussion

Small and large abalone were safely exposed to $MgCl_2$ and 2-PE at doses in excess of those provided by optimisation studies. An increase in REC was observed as dose increased, consistent with observations of White et al. (1996) of relaxants on *H. midae*. The RECs observed at optimal doses and exposures were comparable with results from the screening trial (see Chapter 4), demonstrating consistency of response to exposure.

These are the first data that describe acute toxicity or the MOS for $MgCl_2$ or 2-PE in any abalone species. The effects of overexposing *H. midae* to $MgSO_4$ were assessed by White et al. (1996), who found that 18x (6 hours) the recommended exposure (20 minutes) to 140 g/L $MgSO_4$ was required before significant mortality was observed. $MgSO_4$ did not, however, induce successful relaxation in *H. laevigata* (see Chapter 3). In a relaxant exposure study on the gastropod *Strombus gigas* Linnaeus no mortality was recorded after exposure to 30 g/L $MgCl_2$ or 3 mL/L 2-PE for 30 minutes (Acosta-Salmon & Davis 2007). No mortality was observed in *Saccostrea glomerata* Gould, after 3 hours exposure to 50 g/L $MgCl_2$ (Butt et al. 2008). These demonstrate that mortality following exposure to relaxants, like efficacy and dose, is species specific and that broad assumptions about safety cannot be made for distantly related taxa.

Few guidelines exist for MOS: the highest normally acceptable MOS for oral administration of a medicine in humans is 0.5 (Number of times over optimal dose) (Larsen 2006). The relaxants examined here have an MOS of 0.2 - 0.4. In practice, overdose or overexposure are unlikely to occur. In Australian abalone aquaculture, tank systems are shallow, volume can be calculated precisely and abalone can be constantly visually assessed (Burke et al. 2001). Flow can be closely regulated and immersion treatments can be rapidly removed from the system (AAGA unpublished data). Dose can be carefully determined and

administered and overdose and/or overexposure are extremely unlikely to occur. Close supervision of delivery is, however, required to ensure good control over the administration and animals need to be observed during all procedures.

Differences in MOS were observed between small and large abalone (Table 5.3) for both relaxants. Small abalone are more tolerant of overdose than large abalone, possibly as a result of the lower optimal dose and duration. This size specific information is important for practical application to ensure safe use. The only relaxant with a regulatory authority for use on abalone in Australia, benzocaine (APVMA 2016c), has no available safety studies, and the assumed safety margin is based on data for finfish sedation. The lack of availability of these data greatly increases the risk of adverse effects during use. On-farm use of benzocaine for relaxation of abalone has often caused mortality (Burke et al. 2001) and the MOS is probably narrow, which is compounded by the label doses being higher than required for abalone (AAGA unpublished data).

The zinc reference toxicant was used to assess the resilience of the abalone and the small abalone were of normal health. The LC_{50} value from exposure to zinc was within the range of the values from Liao et al. (2004). The abalone used in the study had no apparent health problems; no experimental animals died and normal growth rates were observed. Abalone exposed to $MgCl_2$ and 2-PE displayed growth rates over the 90 days post exposure of 100 µm/day SL, comparable to commercial and experimental (Stone et al. 2013) growth rates at optimal temperatures. Exposure to these relaxants did not negatively affect the long term growth of abalone during the experiment and appear to be suitable for use in the abalone aquaculture industry.

5.6 Conclusion

It is important for abalone farmers to understand the relationships between relaxant dose and exposure and mortality to prevent loss of farm stock and to ensure that adequate care is taken when administering relaxants. Small or large abalone can be effectively relaxed using 2-PE or $MgCl_2$ with optimised doses and exposure time without long term sub lethal effects on growth or mortality. Small and large abalone can be safely exposed to 2-PE or $MgCl_2$ for periods in excess of those required for routine farming procedures and the observed margin of safety is adequate for relaxation to be successfully achieved without mortality in normal farming conditions. 2-PE and $MgCl_2$ are suitable candidates for use as relaxants in commercial abalone aquaculture.

Chapter 6: Magnesium residue in abalone muscle after exposure to magnesium chloride.

Mercer G. J., J. O. Harris & M. R. Deveney. 2016. Magnesium residue in abalone muscle after exposure to $MgCl_2$. J. Shellfish. Res. (in preparation)

6.1 Abstract

Relaxants are used in abalone aquaculture to facilitate handling, transport and grading stock and to minimise injury and mortality associated with handling. Magnesium chloride ($MgCl_2$) is a candidate relaxant that successfully induces safe relaxation in abalone. Most relaxants used in aquaculture are applied by immersion and absorbed through the skin and gills of the animal. Abalone tissue after exposure to $MgCl_2$ will contain residues until depurated. Residue data generated from exposure to $MgCl_2$ must therefore be assessed for residues and food safety of treated animals and this can be done through inductively coupled plasma mass spectrometry (ICP-MS) analysis. Large *Haliotis laevigata* were relaxed using 160 g/L $MgCl_2$ for 20 minutes and edible tissues were tested for magnesium residues. Immediately following exposure muscle tissue contained 1903±98 mg Mg/kg, significantly higher than untreated controls. Three hours after being returned to seawater abalone muscle contained 1072±170 mg Mg/kg, which was not significantly different to untreated control animals which contained 1306±51 mg Mg/kg. Abalone are osmoconformers and display rapid equilibration of Mg^{2+} following cessation of exposure to elevated Mg^{2+}. The total amount of Mg immediately post-treatment in a standard 75 g serve of abalone muscle is safe for human consumption and provides 44 % and 34 % of the recommended daily intake of Mg for adult women and men respectively. No withholding period is required for $MgCl_2$ for use as a relaxant for abalone and $MgCl_2$ relaxation is therefore safe for rested harvest, but organoleptic assessment is required to ensure no decrease in product quality.

6.2 Introduction

Relaxants are used in the abalone aquaculture industry to facilitate rapid, safe removal of abalone from substrates prior to husbandry procedures such as grading, movement between tanks and harvest. Many veterinary medicines used in aquaculture are applied through immersion and absorbed through the skin and gills of the animal, so the tissues are likely to contain residues until depurated (Redshaw 1995, Neiffer & Stamper 2009). The Australian Pesticides and Veterinary Medicines Authority (APVMA) data guidelines for applications to register veterinary medicines (APVMA 2016a) include requirements for data on chemistry and manufacture, toxicology, metabolism and kinetics, residues, overseas trade, occupational health and safety, environment and target animal safety (APVMA 2016a). The APVMA defines a residue as 'the chemical, its metabolites and related compounds to which the maximum residue limit (MRL) applies' (APVMA 2016b). The inclusion of specific metabolites or degradation products in the expression of a residue depends on their toxicological profile and the extent to which they occur (APVMA 2016b). There is, however, no residue definition for magnesium. The residue concentration in a product depends on the dose, application method and subsequent time to harvest of food for human consumption. Irrespective of existing residue definitions, APVMA requires data to show that the residues in the edible portion of animals destined for human consumption are safe by the time it reaches the consumer (APVMA 2016b).

Magnesium chloride ($MgCl_2$) is a salt that is commercially extracted from brine or salt water (Eliezer et al. 1998). Magnesium ions (Mg^{2+}) have desirable muscle relaxation properties (Culloty & Mulcahy 1992, Heasman et al. 1995, Acosta-Salmon & Davis 2007, Butt et al. 2008, Suquet et al. 2009) and $MgCl_2$ is a good candidate relaxant for abalone which has met industry criteria for aquaculture use (Chapters 3,4 and 5) and Magnesium is not listed on the US Agency for Toxic Substances and Disease Registry.

Abalone muscle is exposed to relaxants during immersion treatment, with only epithelia as a barrier between the abalone tissues and the surrounding environment. Abalone can upregulate [K^+] and downregulate [Na^+] relative to seawater (Prior & Pierce 1981) but osmoconform to other ions; when exposed to a concentration imbalance, passive solution transfer and diffusion across the epithelial membrane occurs until equilibrium is reached. If the abalone are exposed to high concentrations of soluble, ionic products that change the osmotic balance, passive movement of water and ions in solution across the membranes increases the concentration of product in the abalone. White et al. (1996) examined [Mg^{2+}] in *Haliotis midae* Linnaeus tissue following single and multiple immersion exposures to magnesium sulphate ($MgSO_4$). $MgSO_4$ treatment did not significantly increase the [Mg^{2+}] in muscle following exposure to 300 g/L for 20 minutes and they concluded that no withholding period (WHP) was required and the product was safe for rested harvest (White et al. 1996). Residue depletion and safety of $MgCl_2$ has not been assessed, however and it is unknown if data for *H. midae* is relevant for Australian abalone.

Magnesium (Mg) is an essential element in biological systems (Wahlqvist & Darmadi-Blackberry 2002) and human recommended daily intake (RDI) of Mg is 320 and 420 mg/day for adult women and men respectively (Kelsay et al. 1979, Greger & Baier 1983, Kelsay & Prather 1983, Mahalko et al. 1983, Lakshmanan et al. 1984, Schwartz et al. 1986, Wisker et al. 1991, Spencer et al. 1994). Mg is a biologically essential nutrient and is found widely in foods. Adequate Mg intake benefits enzyme systems that regulate protein synthesis, muscle and nerve function, blood glucose control and blood pressure (Institute of Medicine 1997, Rude et al. 2009, Rosanoff et al. 2012). Mg also regulates active transport of calcium and potassium ions for nerve impulse conduction, muscle contraction and normal heart rhythm (Rosanoff et al. 2012). Green vegetables, legumes, peas, beans and nuts are rich in Mg, as are some shellfish and spices. In adult humans on conventional diets, the efficiency of

absorption, however, varies with Mg content of food (Spencer et al. 1980, Seelig 1982). The homeostatic capacity of the body to adapt to a wide range of intakes is high (Abrams et al. 1997, Sojka et al. 1997). Excess Mg in food does not pose a risk in healthy individuals because the kidneys eliminate excess in urine (Musso 2009).

A general upper limit (UL) intake of 350 mg/day of Mg^{2+} including non-food sources is recommended for adults including pregnant and lactating women and children over 8 years (Marken et al. 1989, Fine et al. 1991, Ricci et al. 1991). Doses of Mg supplements in excess of 5,000 mg/day can cause diarrhoea, nausea and abdominal cramping (Institute of Medicine 1997, Kutsal et al. 2007). The RDIs, UL and toxicity data can be used to determine if the Mg residues in abalone muscle after exposure to $MgCl_2$ using the proposed pattern of use are safe for human consumption. The total amount of Mg in a standard size portion of abalone for human consumption can be determined and compared to RDIs, UL and toxicity data to assess if the residual Mg in abalone muscle following $MgCl_2$ relaxation is safe for consumption. Residues of any product remain in the tissues until they are excreted or metabolized (White et al. 1996). Mg is not metabolised and its rate of loss in abalone by diffusion is unknown. Improved analytical methods and technology have driven heightened awareness of residues and in some cases has substantially decreased maximum residue limits (MRLs) (Alderman 1988, Treves-Brown 2000); current effective quantitative methods, such as inductively coupled plasma mass spectrometry (ICP-MS), need to be used to assess residues.

WHPs are the post treatment intervals required for veterinary medicine residues to decrease to the MRL in edible parts of animals. The WHP is the minimum period permissible between treatment and harvest. MRLs are based on the tolerable daily intake (TDI), which is determined from human or mammalian toxicological data and average portion size (Neiffer & Stamper 2009). WHPs are usually described for poikilotherms in degree-days. Each withdrawal day is 24 hours, starting from cessation of treatment, multiplied by the average

temperature (Costello et al. 2001). Ten days at 25 °C, therefore, is 250 degree-days. The ideal aquaculture relaxant, including for abalone aquaculture, would have negligible residues or be safe for human consumption immediately following use, so that it requires no WHP and can be used to harvest animals while relaxed (rested harvest). Rested harvest of can benefit the abalone industry by decreasing shell and muscle damage during removal from substrates, reducing mortality during transport and enhancing product quality (Wilkinson et al. 2008). Benzocaine has a WHP of 500 degree-days (PER 14638) (APVMA 2016c) which precludes its use for rested harvest.

We measured Mg residues in abalone muscle relaxed with $MgCl_2$ to determine if the residual Mg after treatment required a WHP or if it was safe for rested harvest. We assessed Mg residues in muscle of harvest size *H. laevigata* following exposure to the pattern of use (160 g/L $MgCl_2$ for 20 minutes) developed in dose optimisation studies (Chapter 4), using inductively coupled plasma mass spectrometry (ICP-MS). These data compared to the acceptable UL and RDI for Mg consumption in humans will help determine if the relaxant pattern of use is safe for rested harvest and human consumption.

6.3 Materials and methods

6.3.1 *Abalone*

Two hundred and forty large (70-90 mm shell length SL) two and a half year old farmed greenlip abalone (*H. laevigata*) were obtained from SAM Abalone Pty Ltd, Port Lincoln, South Australia and maintained in eight 1 000 L fibre glass flow through holding tanks at the South Australian Research and Development Institute (SARDI), South Australian Aquatic Sciences Centre (SAASC), West Beach, South Australia in seawater sand filtered to 50 µm. Abalone were fed 3 mm ECO-feed chip (EP Aquafeeds, Lonsdale, South Australia, Australia), in excess of the industry standard 3 % body weight per day (Stone et al. 2013) and

acclimated for two months. Abalone were fed and holding tanks were cleaned and maintained daily. Water quality in the experimental system was 18±1 °C, 101±3 % O_2 saturation and 35±1 ppt salinity.

6.3.2 *Tagging*

To facilitate identification of individual abalone, 30 days into acclimation and 30 days prior to the trials commencing, each abalone was tagged with glue-on shellfish tags (Hallprint Fish Tags Pty Ltd, South Australia) using a protocol adjusted for abalone from Henry & Jarne (2007): abalone were mechanically removed from the holding tanks using a soft blunt scraper, the shell was blotted dry and tags were attached with cyanoacrylate gel glue, positioned on the apex of the shell as not to impede tremata shell growth or movement during the duration of the experiment. Abalone were exposed to air for less than 2 minutes. Abalone were then returned to the holding tanks.

6.3.3 *Experimental description*

Two hundred and forty abalone were mechanically removed from the holding tanks using a blunt scraper and blotted to remove excess water. Body weight was measured to the nearest 0.01 g using a scientific electronic balance (A&D HT-120, compact precision scale A&D Company Ltd). SL was measured along the longest axis of the abalone shell to the nearest 0.01 mm with Vernier calipers. No significant differences in the length or mass of abalone was found between different treatments of dose or duration for each relaxant (P>0.05). Abalone were randomly assigned to 24 individual 10 L buckets each containing 5 L of aerated seawater from the holding tanks and 10 abalone which were allowed to firmly adhere to the internal surface of the bucket. Each of the eight treatments, including controls (all treatments shown in Table 6.1), had three replicate buckets, containing 10 abalone each.

Three replicate buckets containing 10 abalone each were destructively sampled at each time point. Water was sampled from all trial buckets to assess mean [Mg^{2+}] of the seawater prior to treatment and introduction of the abalone. Thirty abalone from the untreated control group (T_{-1}) were mechanically removed from the bucket surface, placed onto ice and maintained at -4 °C. Seawater was removed from the 21 treatment buckets, replaced with 5 L of pre-mixed 160 g/L $MgCl_2$ in seawater and exposed for 20 minutes. Thirty abalone were sampled for analysis at the end of the exposure (T_0). 180 abalone were then returned to the holding tanks. Thirty abalone were then sampled at each time point; 3 (T_3), 6 (T_6), 12 (T_{12}), 24 (T_{24}), 48 (T_{48}) and 72 (T_{72}) hours post exposure (Table 6.1), rinsed in distilled water, placed onto ice and maintained at -4 °C prior to processing for Mg analysis. Foot muscle tissue was sampled.

ICP-MS analysis was conducted with an Agilent 7500 auto sampler (Agilent Technologies™, USA) (RF Power 1370 W, Wavelength 213nm). The calibration solution for Mg analysis was prepared in de-ionized water for a calibration curve analysis for quantification. Three 1.5 g of foot muscle tissue sections were sampled from each abalone. Moisture content was analysed to determine water : flesh ratio to ensure test accuracy. One sample from each abalone was weighed, heated to 90 °C for 48 hours and water content was determined by gravimetric loss. Two samples from each abalone were weighed and added to 5 mL of concentrated HNO_3 in a pyrex test tube and then heated to 90 °C for 1.5 hours. 1 mL of 1% hydrogen peroxide was then added and the sample was heated to 90 °C for a further 1 hour. After cooling, the sample was made up to 50 mL with Milli Q water, then diluted 1:10,000 for ICP-MS analysis. Tank seawater samples were diluted at 1 in 2000 for ICP-MS analysis.

6.3.4 *Analysis*

Abalone body weight was subjected to a one-way ANOVA (Zar 1999) prior to the trial to ensure random weight distribution of animals. No significant difference (P>0.05) was found between start weights for all groups.

Moisture content and Mg residues were analysed using one-way Analysis of Variance with sample time as the main effect. For all tests, statistical significance was judged at the alpha level of 0.05. All statistical analyses were performed using SPSS Software for Windows (SPSS Inc., Chicago, IL, USA). All data were tested for normal distribution with the one-sample Kolmogorov-Smirnoff test and for homogeneity of variances with Levene's test (Zar 1999). Means were compared using Tukey's Multiple Range Test at 5% error probability (SPSS Software for Windows, SPSS Inc., Chicago, IL, USA). All values are reported as the mean ± standard error.

6.4 Results

6.4.1 *Moisture*

One-way ANOVA analysis of moisture content revealed no significant differences (P>0.612) in moisture content between samples (content value 73.4±.5 moisture %).

6.4.2 *Mg residues*

There were no mortalities during the experiment. All abalone exposed to the relaxant recovered after being returned to untreated seawater. Mg residues are shown in Table 6.1. Mg residues ranged from 1903±98 mg/kg at T_0 to 1028±53 at T_{72}. One-way ANOVA showed a significant difference in Mg residues between T_0 and all other sample times and the seawater sample (P<0.002) (Table 6.1). One-way ANOVA and post-hoc showed that there were no

significant differences (P>0.640) between Mg residues in the (T_{-1}) abalone muscle samples and seawater from the untreated buckets.

Table 6.1 Magnesium residues (mg/kg) in large greenlip abalone (*Haliotis laevigata*) muscle after 20 minute exposure to 160 g/L magnesium chloride.

Sample time (hour)	$[Mg^{2+}]$ (mg/kg)
-1	1306±51[a]
0	1903±98[b]
3	1072±170[a]
6	1349±59[a]
12	1413±73[a]
24	1190±85[a]
48	1141±64[a]
72	1028±53[a]
Seawater	1402±46 [a]

Large *H. laevigata* (80-90 mm shell length). -1 sample is the untreated control, 0 sample is immediately after exposure (no depuration period) and following samples taken at graded time levels of depuration period after singular exposure. $[Mg^{2+}]$ level in the seawater sample taken from the test tank is shown. Standard error of the means is presented. Superscripts indicate significant differences in mean $[Mg^{2+}]$ between sample times (P<0.05).

6.5 Discussion

Large abalone exposed to 160 g/L $MgCl_2$ for 20 minutes showed a significant 43±2 % increase in $[Mg^{2+}]$ in the muscle compared to untreated control abalone and seawater. Three hours post return to seawater, Mg residues in the muscle were not significantly different from those observed in the untreated controls (T_{-1}) or seawater. Abalone osmoconform to ambient $[Mg^{2+}]$ and the Mg residues data shows passive movement of Mg^{2+} from the higher concentration in the external environment during treatment into the abalone and subsequent loss of Mg^{2+} to the seawater when returned to seawater. Mg residues in muscle did not reach $[Mg^{2+}]$ in the treatment. This indicates that overexposure to $MgCl_2$ is also likely to cause overdose because the $[Mg^{2+}]$ has not reached equilibrium with the treatment after 20 minutes exposure. White et al. (1996), however, found no significant difference in Mg residues in *H. midae* muscle between abalone exposed to 300 g/L $MgSO_4$ for 20 minutes

(209±53 mg Mg/kg) and untreated controls (199±41 mg Mg/kg). These residues differ substantially from our data of 1903±98 mg Mg/kg after exposure to 160 g/L $MgCl_2$ for 20 minutes and 1306±51 mg Mg/kg for untreated controls. This may be due to ICP-MS having greater sensitivity than the atomic absorption spectroscopy used by White et al. (1996). White et al. (1996) recommended that no WHP was required for $MgSO_4$ use on *H. midae*, but it displays other undesirable properties including causing mortality at therapeutic doses, irritation, loss of epithelial tissue (cell sloughing) and discoloration during *H. laevigata* exposure (Chapter 3).

There is no standard portion size for shellfish for food safety assessment. A conservative estimate is 75 g/day based on daily consumption for people who eat crustaceans and molluscs (ABS 2012). 75 g/day of abalone can therefore be used to assess the potential toxicity of the total Mg found in this portion size in abalone treated following the proposed pattern of use. Magnesium in food was used to calculate the human RDI of 320 and 420 for women and men respectively (Wisker et al. 1991) and the 350 mg/day UL for Mg consumption for adults and children over 8 was based on intake from dietary supplements. Residue data, the assumed standard portion size for abalone and the human Mg UL can be used to assess the safety of Mg residues in treated abalone. At T_0 abalone muscle contained 1903±98 mg Mg/kg which equates to 142±7 mg Mg in a 75 g serve, a 43 % increase in Mg compared to 99±6 mg Mg for a 75 g serve of untreated abalone. Total Mg intake from these portions are below the UL of 350 mg/day of Mg (Marken et al. 1989, Fine et al. 1991, Ricci et al. 1991). The 142±7 mg of Mg from a 75 g serve of treated abalone is 41 % of the UL, 44 % and 34 % of the RDI of Mg for adult women and men respectively and is <3 % of the 5,000 mg/day Mg associated with toxic effects in humans (Kutsal et al. 2007). Almonds are a rich natural source of Mg and a 30 g serve contains 80 mg of Mg (USDA 2012). Only 30 - 40 % of dietary Mg consumed, moreover, is absorbed (Abrams et al. 1997, Sojka et al. 1997)

with highly selective absorption with luminal and tissue regulation of metal ion uptake from the gut of single-stomached mammals (Powell et al. 1999). These data indicate that the additional Mg in treated abalone will have negligible health impacts.

The proposed pattern of use as a relaxant in the Australian abalone aquaculture industry of 160 g/L $MgCl_2$ for 20 minutes with no repeat treatments occurring before tissue Mg normalises, does not create toxic or unsafe Mg residues in the edible portion of abalone. No WHP is therefore required for use on abalone destined for human consumption. $MgCl_2$ is suitable for rested harvest of abalone for live transport, including for animals transported live chilled in damp packages. For transport of fresh dead product following rested harvest, abalone are placed into a fresh seawater ice slurry, which may facilitate depuration of some Mg^{2+}. Use of $MgCl_2$ relaxation for live harvest may also reduce mortality by preventing shell and muscle damage during the removal from substrates. That $MgCl_2$ is suitable for rested harvest is of substantial benefit to the industry because benzocaine has a 500 degree-day WHP and is not suitable for rested harvest.

Residue analysis was not performed for chloride. Chloride cannot be determined by ICP-MS because it causes serious signal drift by depositing material on the interface cones and biasing all sample readings throughout the ICP-MS determination (Neubauer & Völlkopf 1999). Chloride in ICP-MS samples also generates ions in the plasma that combine with argon, solvent and acid based species to produce several polyatomic spectral interferences on many of the analytes. Human RDI is 23,000 mg Cl^-/day; chloride is broadly tolerated in biological systems (Whiting & Barabash 2006) and the additional chloride from treatment is of negligible food safety concern.

6.6 Conclusion

Abalone are osmoconformers and *H. laevigata* relaxed using 160 g/L $MgCl_2$ for 20 minutes display a 43 % increase in Mg in the muscle. Within three hours of being returned to seawater the Mg in the abalone muscle returned to that observed in untreated controls and seawater. Mg is a necessary component of a healthy diet, but doses in excess of 5,000 mg of Mg a day can cause negative effects and a daily RDI of 320 and 420 mg of Mg for adult women and men respectively, is recommended. Up to 142±7 mg of Mg could be contained in a standard 75 g serve of abalone from a treated animal, but this is less than 3 % of the dose that causes negative effects and 41 % of the daily UL of 350 mg of Mg. The proposed pattern of use of $MgCl_2$ as a relaxant in the Australian abalone aquaculture industry (160 g/L $MgCl_2$ for 20 minutes) does not create Mg residues in the edible portion of abalone that are toxic or unsafe for human consumption and this treatment is safe for rested harvest.

Chapter 7: General discussion

7.1 Introduction

Data collation was undertaken for the Australian Abalone Growers' Association (AAGA) required by the Australian Pesticides and Veterinary Medicines Authority (APVMA) to obtain regulatory approvals for relaxants for use in the Australian abalone aquaculture industry. The main research objectives were to: (i) find suitable relaxant(s) as alternatives to benzocaine for mass immersion relaxation; and (ii) to obtain sufficient information on the identified candidate relaxants to generate applications to the APVMA to obtain Minor Use Permits (MUPs). Specific objectives were to: (i) identify candidate relaxants for use on Australian abalone from literature and identify target species data gaps for permitting by APVMA; (ii) compare the efficacy of current and candidate relaxants for use in Australian abalone; (iii) optimise relaxant dose for candidate relaxants for use on Australian abalone; (iv) assess host impact for the candidate relaxants; (v) analyse the residues of administered relaxants; and (vi) determine the potential of candidate products for rested harvest.

The use of the only legal chemical relaxant benzocaine on Australian abalone farms is deeply concerning. Consultation with members of the industry proved to be essential throughout this project. The Australian group AAGA is a unique collaboration of farms and farmers willing to share observations and ideas. Consultation with international abalone farmers proved challenging at times, due to the ad hoc use of chemical relaxants and readiness to share information. We were able to observe and document the physiological issues raised by AAGA during our relaxant experiments and were able to covert this into observational analysis for our trials which could be readily replicated on farms.

7.2 Summary of major findings

Magnesium chloride ($MgCl_2$), 2-Phenoxyethanol and propylene phenoxetol met industry relaxation, recovery and behaviour criteria for small (18-28 mm shell length SL) *Haliotis laevigata* (abalone). These products induced gradual loss of adherence to the substrate in <5 minutes, recovery occurred in <20 minutes after cessation of exposure without mortality or negative effects on behaviour or growth. Negative effects were observed in abalone exposed to AQUI-S™, clove oil, magnesium sulphate or MS-222. Exposing abalone to benzocaine elicited negative behavioural and physiological effects, supporting anecdotal reports from farmers.

Small and large abalone can be safely exposed to $MgCl_2$ or 2-phenoxyethanol for periods in excess of those required for routine farming procedures without mortality or long term effects on growth. Small abalone can be exposed to $MgCl_2$ or 2-phenoxyethanol in excess of 3x the optimal dose. Large abalone can safely be exposed to 3x the optimal dose of $MgCl_2$ or 2x the optimal dose of 2-phenoxyethanol. Small abalone can be safely exposed to $MgCl_2$ or 2-phenoxyethanol for 4x the optimal duration. Large abalone can safely be exposed to $MgCl_2$ or 2-phenoxyethanol for 3 and 2x the optimal duration, respectively. The proposed pattern of use for the candidate relaxants is safe for use on farms because normal practices prevent overdose or overexposure.

The margin of safety of 2-phenoxyethanol for large abalone differed between experiments in chapters 4 and 5. The more conservative 2x optimal dose will be used as the margin of safety included in label cautions.

Large abalone exposed to 160 g/L of $MgCl_2$ for 20 minutes demonstrated a 43 % increase in $[Mg^{2+}]$ in the muscle. The highest total Mg that could be contained in a standard 75 g serve of abalone, 142±7 mg Mg, is less than 3 % of the dose that causes negative effects in humans and 41 % of the 350 mg recommended daily upper limit (UL) for Mg. The

proposed pattern of use of $MgCl_2$ as a relaxant for harvest size animals in the Australian abalone aquaculture industry (160 g/L $MgCl_2$ for 20 minutes) does not create toxic or unsafe $[Mg]^{2+}$ concentrations in the edible portion of abalone and $MgCl_2$ is therefore safe for rested harvest and treated abalone can be safely consumed immediately following relaxation.

7.3 Recommendations for management

1. When relaxing abalone in aquaculture systems the flow needs to be stopped or minimised to ensure that the abalone are exposed to the correct dose for the optimal duration. The outlet of the section of the culture unit being treated needs to be isolated from the rest of the system so only target animals are treated. Aeration must also be maintained to prevent hazardous decreases in DO. The correct dose needs to be calculated and products need to be mixed with seawater from the system before application. Products must be mixed with an adequate volume of water to ensure even distribution of the product to the entire culture unit to prevent overdose or having stock that do not receive any treatment. System designs differ from farm to farm and system-specific procedures should be developed based on experience.

2. Doses for abalone can be determined from the table of optimised doses (Chapter 4) including consideration of size and water temperature to ensure that optimum efficacy is achieved for each relaxant administration.

3. For large abalone, $MgCl_2$ displayed a wider margin of safety than 2-phenoxyethanol and residue data showed abalone treated with $MgCl_2$ are safe for consumers. $MgCl_2$ is a suitable relaxant for both small and large abalone but 2-phenoxyethanol has a lower

effective dose and is more suitable for small abalone although it can be safely used on large abalone.

4. The margin of safety data informs an understanding of the likelihood of risk of overdose or increased duration of exposure and can facilitate avoidance of mortalities or other adverse effects. The time for which abalone are exposed to relaxants needs to be effectively and carefully managed, due to the relatively narrow safety margins. Quality farm practice facilitates confidence in application and users need to be aware that poorly monitored use can be hazardous.

5. Product type needs to be taken into consideration. The $MgCl_2$ used in this project was food grade anhydrous powder for easy dissolution in seawater. Industrial grades of $MgCl_2$ contain more impurities including metals such as iron, arsenic and lead, which are toxic and can bioaccumulate. 'Cosmetic grade' 2-phenoxyethanol (CAS 122-99-6) is synthesised by hydroxylation of phenol (Williamson synthesis) at high >99 % purity (Chenxin 1999) and is also readily available in bulk from Australian chemical suppliers.

7.4 Recommendations future research

1. Farm trials of the proposed relaxants and doses are underway. These large scale trials will help build on the knowledge we have obtained through our facility trials. While our facility trials were designed to help negate compounding factors limiting results, the quantity and size of the on-farm trials will enable a more specific method development aimed towards site-specific farms. The data collected from these trials will give a significant insight into on-site specific relaxant use and associated

interaction with production factors; stress, water quality etc. Future relaxant site specific research and trials can then be focused on these findings.

2. 2-Phenoxyethanol residue analysis needs to be completed, but there was no analytical test for 2-phenoxyethanol in abalone tissue and the development of a test was beyond the scope and resourcing of this project. Some work has been done previously on the detection of phenoxyethanol (Klimankova et al. 2008, Shabir 2010), however, the laboratory techniques used in these experiments were aimed towards finfish and are potentially expensive in Australia. An assay for 2-phenoxyethanol in abalone tissue is in development at the Australian Government National Measurement Institute (Port Melbourne, Victoria); AAGA will continue to pursue this work. The assay will be designed towards being affordable and available for routine checking of stock. This assay could also facilitate studies on uptake and metabolism of 2-phenoxyethanol in abalone.

3. Environmental toxicity data need to be obtained to ensure that any product released from farms into the environment does not have adverse environmental effects. Concentrations of relaxants in discharge water need to be estimated and if necessary, tested to assess environmental safety. The feasibility of retaining treatment water until products have decomposed needs to be assessed for each farm. While $MgCl_2$ is likely to have limited environmental effects and to disperse rapidly (White et al. 1996), the environmental risks associated with releasing seawater containing 2-phenoxyethanol need to be assessed.

4. Data for veterinary medicine permitting are being collated for $MgCl_2$ and 2-phenoxyethanol. Other data required for permitting include information on the chemistry and manufacture of the product; this requires AAGA to identify a supplier and obtain information for that product. Standard operating procedures need to be developed to ensure workplace health and safety of farm workers during administration of these products.

5. There are no data available about the neurological effects of relaxants and in particular if they have analgesic or anaesthetic effects on abalone. Mollusc neurobiology is unlikely, however, to advance to provide a basis for assessing this in the near future. Stress and immune suppression can be investigated using biochemical and immune parameters and have been to some degree for benzocaine (Hooper et al. 2011 and Hooper et al. 2014). Stress and immune response observations can be undertaken on individual farms. This will also give insight in responses by all abalone species and not just Australian species. All abalone species would require experiments with observational based measuring parameters such as the ones developed in our study for relaxant screening assessment.

6. Improved understanding of the effects of relaxants has implications for animal welfare. While welfare studies of mollusc species is an emerging area, preliminary experiments on gastropod and cephalopod species have been undertaken and may provide an insight into future studies involving the pain and sense assessment of abalone (Winlow et al. 2018).

7. $MgCl_2$ and 2-phenoxyethanol have potential uses for development of an abalone pearl industry which has been limited by stress induced mortality and pearl rejection. Investigating the suitability of these candidate relaxants for pearl seeding in abalone utilizing a similar screening trial undertaken in our project could facilitate safe insertion of pearl seed material and expansion of this industry (Granados et al. 2017).

8. Propylene phenoxetol induced spawning of large abalone. Propylene phenoxetol has potential for inducing spawning in abalone broodstock and improving control of abalone breeding. Current methods include expensive hormone addition and temperature, water changes and can be temperamental (Babcock & Keesing. 1999, Uki & Kikuchi 1984). The dose and abalone exposure to propylene phenoxetol could be optimised to assess effects of varying exposure on reproduction and its potential for use as an agent to induce spawning.

9. Compound treatments for relaxation; eg. cold temperature with relaxant or elevated CO2 or low DO combination with relaxant, were not considered during this study. Consultation with AAGA resulted with complicated procedures not being endorsed by the industry. Complicated procedures may be at risk of misinterpretation during on farm use (White et al. 1996). Temperature shock is avoided due to the stress associated mortality events. Cold water can cause the abalone to bleed out as seen during some of the 14 degree trials. Getting a CO2 permit would be a costly and length process with work, health and safety implications and low DO is never an option on commercial farms and can cause long term reduced immunity.

10. Further insight into how stress can affect the relationship between abalone and relaxants can be explored. Stress was a potential major compounding factor during the entire project. Farmed abalone require handling and exposure to air. The project required handling of the abalone for analysis and all handling needed to be done by technicians familiar with abalone behaviour. This requirement for suitable technicians placed limitations on how many people we had to undertake in-facility experiments. Kirsten et al 2011 and 2014 have undertaken some handling stress analysis of abalone during husbandry processes. While the addition of understanding stress factors to our work was beyond the time-frame and scope of the project, future stress response work could be incorporated into field trials to potentially assess a range of factors that could impact chemical relaxation on farms. Heat stress research is currently underway through the university faculty and on farm observations. Heat stress is a large area of research and potential observations from this could also be used to further individually adapt relaxant use on farms. Farm trials could also provide site specific stress issues. Some farms may experience heat stress at certain times of the year and low dissolved oxygen levels (Venter et al. 2018) could also be site-specific. Chemical and stress effects on product taste is carried out by farmers and industry bodies and can give further insight into this relationship.

7.5 Conclusions

The objectives of this study have been achieved. Candidate relaxants for commercial application for abalone farming have been identified and doses and durations have been optimised for 2-phenoxyethanol and $MgCl_2$ including assessing size dose and temperature dose relationships. $MgCl_2$ requires no WHP and is suitable for rested harvest. Margin of safety data provides an understanding of the relationship between optimised doses and exposures with toxic effects. Taxon-specific effects are common in relaxants; the abalone industry is developing data relevant to abalone because data from other mollusc and finfish species are often not transferable.

This project has improved coordination and increased collaboration between producers and researchers, informed improved abalone husbandry and abalone health management. Consultation through AAGA provided direct industry involvement in this project and ongoing R&D commitments are established with a number of farms to continue health and biosecurity research. All AAGA members will benefit from the research produced by this project. The outputs have contributed to production innovation by increasing control during handling and supporting consumer demands for safe, high-quality, welfare-friendly seafood products.

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