The Effects of Anthropogenic Noise Playbacks on Marine Invertebrates

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I declare that the work in this thesis was carried out in accordance with the requirements of Edinburgh Napier University's Research Degrees Framework and that it has not been submitted for any other academic award or professional qualification. Except where indicated by specific reference in the text, the work is my own. Work done in collaboration with, or with the assistance of, others, is indicated as such at the start of each chapter. My own contributions to any collaborative work are here also highlighted. Any views expressed in this thesis are my own, and do not represent the views of Edinburgh Napier University.

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Abstract

Anthropogenic sound has profoundly changed the acoustic environment of aquatic habitats, with growing evidence that even a short exposure to man-made sound sources can negatively affect marine organisms. Marine invertebrates have received little attention regarding their responses to anthropogenic sound, despite their pivotal role in marine ecosystems, and preliminary evidence of their sensitivity. In this thesis, I critically review the methods used in studies investigating the effects of anthropogenic noise on marine invertebrates. I identify methodological trends that have developed along the timeline of this topic, and use this information to suggest three research strategies to further the development of research in this field. From this review, current knowledge gaps are identified, and two main routes to address them are taken in this thesis.

Firstly, to address the shortage of particle motion data in anthropogenic sound literature, two new low-cost and easily accessible particle motion sensors were developed and tested, one of them at 0.1% of the cost of currently commercially available models. These sensors will allow the measurement and reproduction of the sound fields experienced by marine invertebrates in bioacoustic research, even when research budgets are tight. Particle motion is the main sound component detected by invertebrates, yet neglected in many aquatic bioacoustical studies.

Secondly, to expand on the comparably small quantity of research investigating the effects of anthropogenic sound on marine invertebrates, a series of controlled laboratory experiments were conducted. Two commercially and ecologically important model species were chosen, the blue mussel *Mytilus edulis*, representing sessile benthic invertebrates, and the European lobster *Homarus gammarus*, representing mobile benthic invertebrates.

Experiments on *M. edulis* were conducted taking a mechanistic, integrative approach to investigate the effects of sound on multiple levels of biological organisation, including biochemistry, physiology, and behaviour. The ultimate aim was to understand the underlying drivers behind, and interactions between, responses. Comet assay analysis of haemocytes and gill cells demonstrated a significant six-fold higher single strand breakage in the DNA of cells of mussels exposed to ship-noise playback, compared to those kept

under ambient conditions. Superoxide dismutase analysis did not identify an excess of superoxide ions, and glutathione, and glutathione peroxidase assays showed no increase in either glutathione or glutathione peroxidase. TBAR assays however revealed 68% more thiobarbituric acid reactive substances, indicating lipid peroxidation in the gill epithelia of noise exposed specimens. Algal clearance rates and oxygen-consumption rates of noise-exposed mussels were significantly lower (84% reduction and 12% reduction respectively), than those of control animals, while valve gape was significantly (60%) wider. This seemingly converse reaction indicates a shock response in mussels with the onset of noise exposure. Finally, at the genetic level, heat shock protein 70 expression was investigated, but no change was identified during noise exposure.

Investigation into the noise induced behavioural responses of *H. gammarus* suggests seasonal differences in behaviour, using movement as a metric, in response to anthropogenic noise playbacks. During both summer and winter exposures, lobsters spent most time away from the highest noise area (98% of the observed time in summer and 78% in winter). The observed seasonal differences in the time spent in the highest noise area (2% in summer and 22% in winter) could be linked to the lobsters' requirement for shelter during winter. This requirement seems to have had a stronger influence over the animals' behaviour than any desire to avoid high noise levels.

The information generated in this thesis can be used by researchers working in the field of marine sound to develop well rounded studies exploring the effects of sound on not only marine invertebrates but other faunal groups as well. The construction details provided to produce low cost particle motion sensors, will allow bioacoustic researchers to easily include particle motion measurements in all future studies investigating the effects of sound on fish and invertebrates. The results of the conducted mussel and lobster experiments evidence how multiple aspects of invertebrate biology can be affected by noise. The observed impacts on both sessile and mobile life forms highlight that the effects of noise cannot be fully understood before a wide range of species with different biological and ecological traits have been studied. The integrative approach to noise research used here can serve as a model for other species, and the results pooled to inform governments and industry of the effects of anthropogenic noise in the marine environment.

Acknowledgements

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Glossary

Noise

Definitions for noise terms are adapted from www.acoustic-glossary.co.uk (Garacy and Associates, 2017).

Amplitude: the magnitude of an oscillating quantity, for example sound pressure or particle motion, here measured in Vpp.

dB: Decibel, a relative unit of measurement widely used in acoustics, electronics, and communications. The dB is a logarithmic unit used to describe a ratio between the measured level and a reference or threshold level of 0 dB. In this thesis, it is used to display sound intensity.

Frequency: the number of times that a periodic function or vibration occurs or repeats itself in a specified time, often 1 s - cycles per second. It is usually measured in Hertz (Hz).

Hz: Hertz, the unit of frequency or pitch of a sound. One hertz equals one cycle per second.

kHz: kilohertz, 1000 Hz.

Noise: any sound that is undesired by the recipient. Any sound not occurring in the natural environment.

Particle Motion: can describe the particle acceleration, particle displacement and particle velocity, terms used when discussing sound waves, where the particles 'assist' the transmission of the wave but then return to their 'original' state, no net movement. In this thesis, particle motion is measured according to the particle acceleration.

Peak Level: greatest instantaneous value of a standard frequency weighted sound pressure level, within a stated time interval.

PSD: Power Spectral Density, the spectral density of the wave, when multiplied by an appropriate factor, will give the power carried by the wave, per unit frequency.

RMS: Root Mean Squared, obtained by squaring the amplitude at each instant, obtaining the average of the squared values over the interval of interest, and then taking the square root of this average.

Sound: any pressure variation that the human ear can detect.

Sound Pressure: the difference between the pressure produced by a sound wave and the barometric (ambient) pressure at the same point in space and is a measure of the force per unit area. It is measured in Pascals - symbol Pa.

SPL: Sound Pressure Level, the RMS value of the Instantaneous sound pressures measured over a specified period of time. Because of the immense range of human hearing, the absolute sound pressure values are impractical for everyday use. So, the logarithmic sound level descriptor, dB, is used.

Vpp: Voltage Peak to Peak, the difference between the most positive and most negative value in a time waveform of amplitude.

Biochemistry

Biochemical definitions are adapted from the Oxford Dictionary of Biochemistry and Molecular Biology (Cammack and Attwood, 2008).

BHT: butylated hydroxytoluene, di-tert-butyl-p-cresol, an antioxidant.

BSA: bovine serum albumin - protein derived from cows. It is used as a protein concentration standard.

Comet Assay: single cell gel electrophoresis, a technique used to detect single strand breaks in the DNA of a eukaryotic cell.

ddH_2O: double distilled water.

dH_2O: distilled water.

DMSO: dimethylsulfoxide – a compound frequently used as a solvent for chemical reactions involving salts and an extractant in biochemistry and cell biology.

DTNB: 5,5′-dithio-bis(2-nitrobenzoic acid) - a reagent used for labelling thiol (SH) groups in protein side-chains used to oxidise GSH and form the yellow derivative.

EDTA: ethylenediaminetetraacetic acid - used for scavenging metal ions to deactivate metal-dependent enzymes to suppress damage to DNA or proteins.

Electrophoresis: the phenomenon of the movement of ions (including macromolecular ions) or charged particles through a fluid under the influence of an applied electric field.

GelRed: an intercalating nucleic acid stain used in molecular biology for agarose gel electrophoresis.

GPx: glutathione peroxidase - an enzyme that catalyses the reaction: 2 glutathione $+H_2O_2$ = oxidized glutathione $+_2H_2O$. Its main role is to protect haemoglobin from oxidative breakdown: the oxidized glutathione is reduced by glutathione reductase, the system thus contributing to a reduction of peroxide levels in the cell.

GSH: glutathione - a tripeptide that is widely distributed in most if not all cells. It acts as a coenzyme for some enzymes and as an antioxidant in the protection of sulfhydryl groups in enzymes and other proteins; it has a specific role in the reduction of hydrogen peroxide and oxidized ascorbate.

HBSS: Hank's balanced salt solution - a balanced salt solution used in tissue culture to provide a suitable ionic and osmotic environment for cell growth and development.

K_2HPO_4: dipotassium phosphate – a highly water soluble salt used as a buffering agent.

LMA: low melting agarose - an aqueous gel suitable for the solidification of microbiological culture media and for use as a support medium in zone electrophoresis or (immuno)diffusion techniques. Melting temperature 40 °C.

Lysis: the disintegration of cells or cell organelles by rupture of their outer membranes.

MDA: malondialdehyde - a compound produced by oxidation of unsaturated fatty acids.

NaCl: sodium chloride – salt.

NADPH: nicotinamide adenine dinucleotide phosphate - protects against the toxicity of reactive oxygen species (ROS), allowing the regeneration of glutathione (GSH).

NaOH: sodium hydroxide – a strong base.

PBS: phosphate-buffered saline - a water-based buffer solution containing disodium hydrogen phosphate, sodium chloride and, in some formulations, potassium chloride and potassium dihydrogen phosphate.

ROS: reactive oxygen species - a highly active oxygen species, such as superoxide ion, hydroxyl radical, hydrogen peroxide (H_2O_2), or peroxynitrite. Most ROS are generated as toxic by-products of oxidative phosphorylation in mitochondria. Cells protect themselves through the superoxide dismutase, glutathione peroxidase, and catalase activities, and the antioxidants (e.g. glutathione, carotene) they contain. Chronic exposure to ROS causes oxidative damage to mitochondrial and cellular proteins, lipids, and nucleic acids.

SOD: superoxide dismutase- Any of a group of metal-containing enzymes that bring about the dismutation of superoxide radicals to form dioxygen and hydrogen peroxide as follows: $O_2 - \bullet + O_2 - \bullet + 2_{H^+} = O_2 + H_2O_2$. It is important in removing the highly toxic superoxide radical.

TBA: Thiobarbituric acid (4,6-dihydroxy-2-mercaptopyrimidine) - reacts with aldehydes, particularly malondialdehyde, produced by oxidation of unsaturated fatty acids, to form a fluorescent red adduct.

TBARS: thiobarbituric acid-reactive substances - compounds of low-molecular-mass, particularly malondialdehyde, that are formed during the decomposition of lipid peroxidation products. They are measured in order to estimate the degree of cellular damage caused by oxygen radicals.

TCA: trichloroacetic acid - an organic acid widely used for the precipitation of protein from tissue and other cell extracts.

TEP: 1,1,3,3-tetraethoxypropane – a chemical they hydrolysis of which produced malondialdehyde (MDA).

Tris: tris(hydroxymethyl)aminomethane - a compound widely used as a biological buffer substance in the pH range 7–9; pKa (20°C) = 8.3; pKa (37°C) = 7.82.

Tris-HCL: tris hydrochloride - a product for simplifying the process of making Tris buffer solutions with more reproducibly.

Triton X-100: the proprietary name for any of a series of polyoxyethylene ethers of certain alkylphenols that are surfactants and classed as non-ionic detergents. based on the formula tert-octyl-C_6H_4- $OCH_2CH_2]_xOH$, x =9 or 10.

Trypsin: a serine protease used to digest proteins in the creation of cell suspensions.

WST: (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium) a monosodium salt), which produces a water-soluble formazan dye upon reduction with a superoxide anion.

Genetics

Genetic definitions are adapted from the Oxford Dictionary of Biochemistry and Molecular Biology (Cammack and Attwood, 2008), A Dictionary of Genetics (King et al., 2013), and www.qiagen.com (Qiagen, 2017).

Bromophenol blue: a standard tracking dye for electrophoresis. It migrates at approximately 300 base pairs on a standard 1% TBE agarose gel.

cDNA: copy DNA – single-stranded, complementary DNA produced from an RNA template by the action of RNA-dependant reverse transcriptase. The single-stranded, cDNA molecule may subsequently serve as a template for a DNA polymerase.

C_T: the spot where the reaction curve intersects a set threshold level above background fluorescence in qPCR.

DNA ladder: a molecular-weight size marker - used to identify the approximate size of a molecule run on a gel during electrophoresis, using the principle that molecular weight is inversely proportional to migration rate through a gel matrix.

DNase: an enzyme that catalyses the endonucleolytic cleavage of DNA to 5'-phosphodinucleotide and 5'-phosphooligonucleotide end products.

DNase/RNase free: a solution free of DNase and RNase.

dNTP: any artificial nucleoside triphosphate in which both of the hydroxyl groups on C- 2' and C-3' of the pentose moiety have been replaced by hydrogen atoms. They are used in sequencing DNA molecules by the chain-termination method.

Hairpins: any part of a linear molecular structure in which two adjacent segments of the molecule are folded back one on the other and are held in that conformation. When denatured it renatures extremely rapidly by intrachain base-pairing between complementary sequences of the inverted repeat.

Master Mix: a single solution containing most of the reagents required for qPCR made to cover all required reactions on a microtitreplate.

$MgCl_2$: magnesium chloride – the magnesium ion is necessary for DNA synthesis.

mRNA: messenger RNA - RNA molecules that convey genetic information from DNA to the ribosome, where they specify the amino acid sequence of the protein products of gene expression.

NTC: no/non template control - monitors contamination and primer-dimer formation that could produce false positive results. Contains all solutions for a reaction minus the test sample.

Primer: short strand of RNA or DNA (generally about 18-22 bases) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyse this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA.

qPCR: quantitative polymerase chain reaction is a commonly used genetic technique to monitor and quantify the amplification of targeted DNA molecules, in real time, during thermocycling and enzyme driven DNA replication.

RDD: buffer that provides efficient on-column digestion of DNA ensuring RNA remains bound to the column.

Reverse Transcriptase: a DNA polymerase enzyme that uses either DNA or RNA as a template. It catalyses DNA synthesis from an RNA template.

RLT: a lysis buffer for lysing cells and tissues prior to RNA isolation and simultaneous RNA/DNA/Protein isolation.

RNase: any of a group of nuclease enzymes that cleave phosphodiester bonds in RNA.

RPE: a concentrated buffer for washing membrane-bound RNA.

RW1: a buffer for washing membrane-bound RNA.

self-annealing: when a primer anneals to another matching primer rather than the target sequence creating a primer dimer that is amplified in the PCR.

self-complementarity: the tendency of primers to anneal to each other without necessarily causing self-priming in the PCR.

SYBR green: a proprietary name for one of two asymmetrical cyanine dyes whose fluorescence is greatly increased by binding to DNA. SYBR Green I is used for the measurement of low concentrations of DNA and in monitoring DNA amplification in real-time polymerase chain reaction.

Taq DNA polymerase: a heat-stable DNA polymerase from the thermophilic bacterium Thermus aquaticus. It is widely used in the polymerase chain reaction (PCR).

TBE: tris·borate–EDTA – an electrophoresis running buffer for the separation of nucleic acids.

$\Delta\Delta C_T$: delta delta C_T – relative quantification of gene expression based on the target gene being compared to a housekeeping gene.

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Chapter 1

General Introduction

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General Introduction

The world's oceans, although often thought of as silent, are far from quiet. Sound is abundant underwater, including biotic noise from whales to snapping shrimp, the sounds of tidal cycles and crashing waves, and the diverse sound and vibration of geological movements. Many marine organisms use sound for long distance transmission of information, due to the sound transmission characteristics of water, an especially important function in an optically poor environment (Hawkins and Popper, 2016). It is now known that both vertebrate and invertebrate marine animals use sound as a vector for communication (Dreher, 1964; Patek, 2001; Tyack, 1998), orientation within their habitat (Simpson *et al.*, 2004, 2011), predator detection (Robertis and Handegard, 2013), and prey acquisition (Barrett-Lennard *et al.*, 1996; Gannon *et al.*, 2005). With the rise in human activity in the oceans, there is growing concern over the levels of anthropogenic noise in this environment (Hildebrand 2009; Frisk 2012; Merchant *et al.*, 2015)

The underwater soundscape was once dominated by natural sound. However, since the dawn of the industrial era, anthropogenic activity in the oceans has been on the rise. Increases in shipping, oil and gas exploration, and more recently the installation of renewable energy devices have led to a sharp increase in the oceanic noise floor on a global scale (Hildebrand, 2009; Ross, 2005). Low frequency noise (20 - 200 Hz) produced by sonar, shipping, and marine construction, propagates over large distances and remains in the environment for a long period of time. Over the last century this has led to a 10 – 100 fold increase in oceanic background noise (Tyack, 2008), and with changing ocean pH increasing the transparency of the ocean to low frequency noise (Brewer and Hester, 2009) this increase could double over what remains of the twenty-first century (Ilyina *et al.*, 2010).

Anthropogenic noise has now been widely recognised as a pollutant in the marine environment, with legislation around the world being created (e.g. Marine Strategy Framework Directive 2012, Descriptor 11 and IMO MEPC.1/Circ.833) to address the observed rise, and to identify the effects that this noise has on marine organisms. Despite this, the growing reliance on maritime transport (Institute of Shipping Economics and

Logistics, 2016), and the push for renewable energy (Marine Board, 2010) will continue to increase the noise levels experienced by marine life.

To comprehend the effects of anthropogenic sound, we must first understand the sound itself. However, the physics of sound is complex, and even more so underwater. In acoustics, energy in the form of molecular vibrations propagates away from the sound source in waves. These waves are longitudinal in elastic media, such as water and air, or transverse in inelastic media, such as solid substrates (Au and Hastings, 2008). This sound is then presented in the form of two interlinked components, the sound pressure - the difference between the pressure produced by the sound wave and the ambient pressure, and the particle motion – the displacement of water particles to facilitate the movement of the pressure wave (Figure 1.1). These two sound components behave differently and propagate at different rates, as such, in the near-field (close to the sound source) the particle motion is typically far higher in relation to the sound pressure. The high particle motion will quickly drop off as it moves away from the noise source, until it becomes proportional to the sound pressure in the far-field (Mann *et al*., 2007). Particle motion is the component of sound detected by most fish, and all marine invertebrates (Chapman & Hawkins 1973; Fay 1984; Popper *et al* 2001), yet despite this is often neglected in bioacoustic studies (see chapter 3.1, p52).

The speed at which sound travels is directly correlated to the density and elasticity of the medium, thus sound under water travels approximately four times faster than in air (Urick, 1983), allowing it to propagate for longer periods of time, attenuating less rapidly. The distance that sound travels through a medium is related to the frequency, with the attenuation rate increasing as the frequency increases. This is of special importance in the marine environment where sound from geological movement, waves, and human activity lie mainly in the low frequencies, and hence can travel far from their sources.

Due to the properties of sound underwater, studying its effects is a complex task. These complexities are most noticeable in tank based studies where the size, material, and shape of the exposure tank will affect the generated sound field (the distribution of sound in the environment). Specifically, in many exposure tanks sound will be reflected from the tank walls (Rogers *et al*, 2016) creating a higher overall sound intensity, especially concerning the particle motion component of sound, than would be seen in the field.

Experimental aquaria can however be built to specifications (large size, non-reflective materials, calibrated sound fields) that allow these complexities to be considered and minimised. Tank based studies also present a number of benefits, such as increased control over the exposure conditions (further discussed in Chapter 2 p38), that make them more practical than field work.

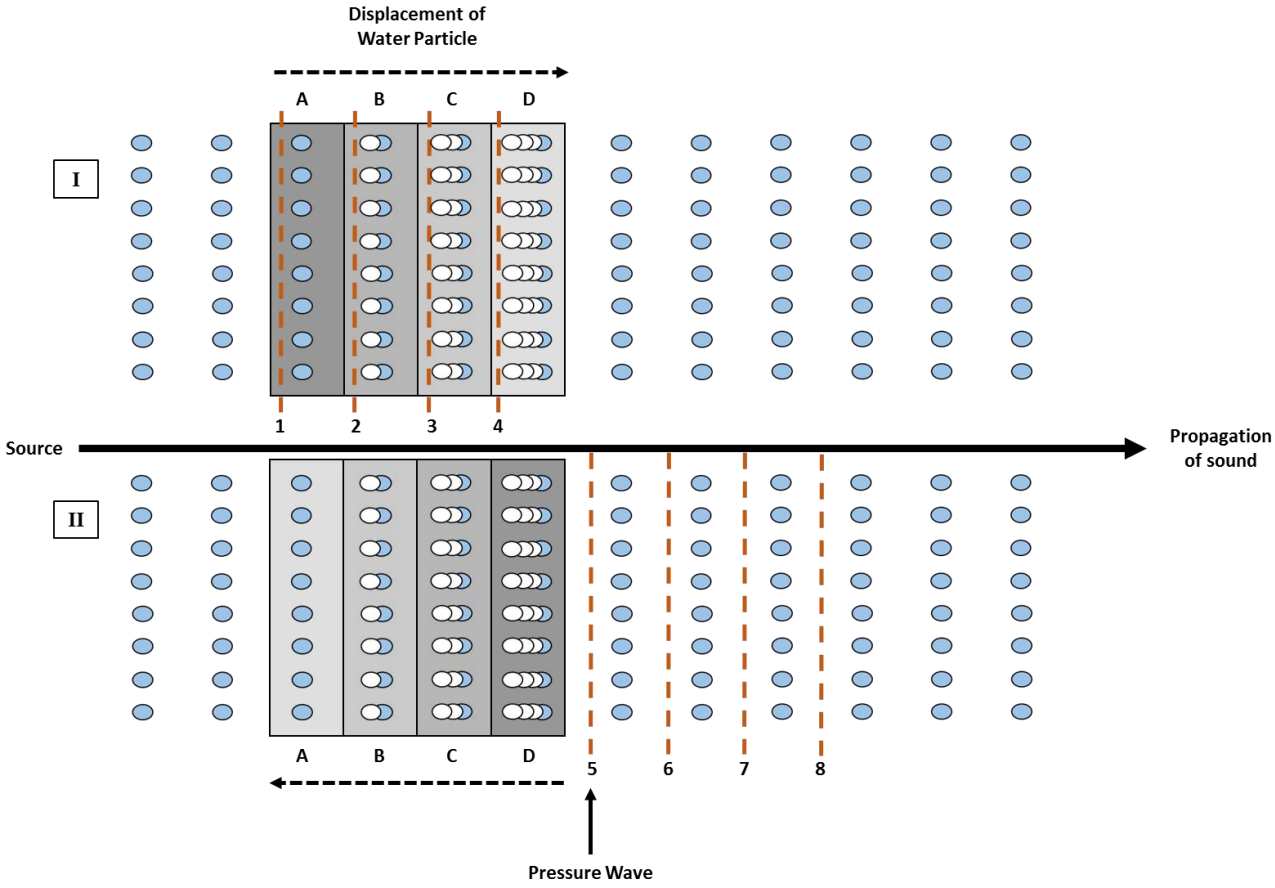

Figure 1.1| Particle motion. Movement of a sound wave through a medium facilitated by particle displacement. In this example (I) the water particle moves from position A through to position D allowing the pressure wave to travel in the direction of propagation (position 1 to 4). (II) Once the wave has passed (position 5 to 8) the particle reverts back to its original location, moving from D to A. Shading indicates movement of the water particle in focus, moving from darkest to lightest shade. The white dots indicate the shift in water particle position as the pressure wave moves through the medium.

A moving representation of particle motion, created by Dan Russel of Pennsylvania State University can be viewed with the following QR code:

When concerns over the effects of anthropogenic noise in the oceans first arose, the research focus lay solely on marine mammals (Malme and Thomson, 1973; Myrberg, 1978), organisms known to utilise sound in their environment. Due to the restrictive nature of marine mammal research, where few animals can be sampled and collection of biochemical and genetic data are impractical or impossible, the majority of these studies focused almost exclusively on behaviour (e.g. Nishiwaki and Sasao, 1977). As time progressed physiology was also considered (e.g. Turl, 1982), but this remained a comparatively minor component of the literature. The majority of these studies identified noise induced negative effects such as changes in heading patterns, diving and surfacing, and vocalizations (Nowacek *et al.* 2007). The effects of anthropogenic noise on marine mammals has been thoroughly reviewed over the years and more information can be found in Richardson *et al.*, (2013) and Gomez *et al.*, (2016).

As the research field developed, its scope broadened to include other taxa, with work encompassing fish (e.g. Schwarz and Greer, 1984), marine reptiles (O'Hara and Wilcox, 1990) and invertebrates (e.g. Lagardère, 1982; Pearson *et al.*, 1994). Following suit, these studies mainly focused on the behavioural and physiological responses of marine animals in response to anthropogenic sound, or for fish and invertebrates, on fisheries success (La Bella *et al.*, 1996). Recently, there have been a number of studies also investigating biochemical responses to noise (Celi *et al.*, 2015; Filiciotto *et al.*, 2014). As with the research conducted on marine mammals, the majority of this work suggests that anthropogenic noise has a negative effect on both fish and invertebrates. The negative behavioural and physiological responses of fishes to anthropogenic noise has been reviewed a number of times and more information can be found in Popper and Hastings (2009), Radford *et al.*, (2014), and Juanes *et al.*, (2017). More detail about the history and state of the art of invertebrate noise research is presented in Chapter 2. The responses of marine invertebrates and reptiles to anthropogenic noise are still poorly studied compared to those of marine mammals (Williams *et al.*, 2015). As such, this thesis aims to propose improvements in methods used when researching the effects of noise on marine invertebrates, and add to their representation in the field (see section 1.1, p6).

Invertebrates are the most taxonomically diverse form of eukaryotic marine life (Mora *et al.*, 2011), constituting approximately 60% of all species (Ausubel *et al.*, 2010).

Throughout the oceans invertebrates are of major ecological value (New and Yen, 1995), forming important links in most food webs and performing essential ecosystem services such as bioturbation (Mermillod-Blondin and Rosenberg, 2006), water purification (Jorgsensen, 1955), and scavenging.

The commercial importance of invertebrates is also growing with a rapid expansion of invertebrate fisheries globally (Anderson *et al.*, 2011), and an increase in marine invertebrate aquaculture (Pauly *et al.*, 2002). This reliance on marine invertebrates for food has increased their value globally in both aquaculture (Marine Scotland Science, 2016; Pauly *et al.*, 2002), and fisheries (Fisheries F. A. O., 2016), accounting for 33% by value of fisheries stocks in 2013.

The large diversity of invertebrate physiologies, morphologies, and life-histories leads to unpredictable responses to anthropogenic noise exposure. For example, noise induced changes in larval movement speeds and settlement times are often highly variable and species specific (Branscomb and Rittschof, 1984; Pine *et al.*, 2012; Stocks *et al.*, 2012; Wilkens *et al.*, 2012). These variations demonstrate the need to study a large range of species, and their respective responses to sound. Despite this need, and the call for more research in this area from the Convention of Biological Diversity (CBD, 2012) and OSPAR (Gotz *et al.*, 2009), invertebrates are still understudied in their responses to anthropogenic noise in the oceans.

1.1 - Thesis Aim and Outline

In this thesis, I will begin to address the current lack of knowledge concerning the effects of anthropogenic noise on marine invertebrates, following two distinct but complimentary avenues of research. Firstly, by developing new accessible sensors to measure the particle motion component of underwater noise, I aim to provide a tool to characterise the noise experienced by marine invertebrates more accurately. This will allow me to more reliably reproduce these levels in all current and future experiments. Secondly, to investigate the effects of noise on invertebrate biology, I will consider behavioural, physiological, biochemical, and genetic responses to anthropogenic noise, with the overall aim of understanding firstly if noise affects the chosen model species, and secondly how and why the observed changes occurred.

Two model species, the blue mussel *Mytilus edulis*, and the European lobster *Homarus gammarus*, were chosen to represent bivalve molluscs and decapod crustaceans respectively. Both of these species are commercially and ecologically important on a national (Marine Scotland Science, 2016) and international scale (Fisheries F. A. O., 2016). The work in this thesis utilises playbacks of anthropogenic noise in controlled laboratory experiments to allow greater and more fine-scale control over the exposure conditions of each experiment. I will follow a mechanistic integrative approach, as outlined in detail below, to garner a more complete picture of how anthropogenic noise can affect these animals.

First suggested by Kight and Swaddle (2011), the mechanistic, integrative approach to noise research used throughout this thesis (Figure 1.2) assesses noise effects on multiple levels of biological organisation, from genetics through to population ecology. This multi-method approach allows the characterisation of (often subtle) responses on the level of the individual, their underlying drivers and interactions, and effects at the population and ecosystem level. By conducting research in such a way, a more complete picture of the effects of anthropogenic noise on marine invertebrates, and indeed on the environment as a whole, can be generated. The methods developed throughout this thesis can be utilised for the study of anthropogenic noise effects in other invertebrate species.

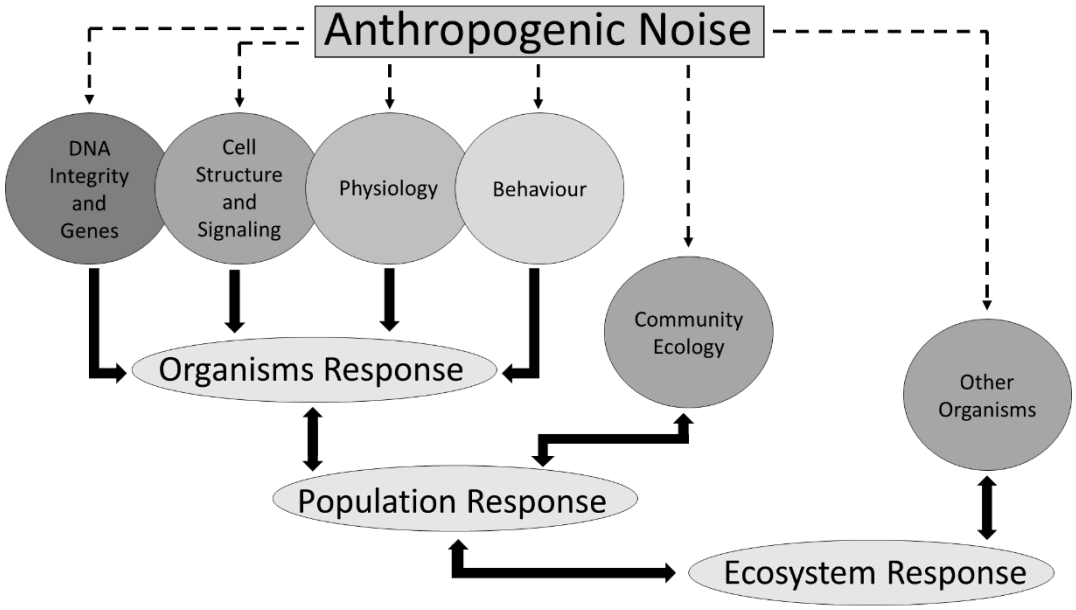

Figure 1.2| Integrative approach to noise research. How anthropogenic noise can affect an organism on multiple levels of biological organisation, leading to individual, population, and ecosystem scale responses - adapted from Kight and Swaddle (2011).

1.2 – Objectives

This thesis has the following three main objectives:

- To develop strategies for the assessment of anthropogenic noise effects in marine invertebrates.

- To develop accessible particle motion sensors for use in bioacoustic studies

- To investigate and identify anthropogenic noise induced biological changes in bivalve molluscs and decapod crustaceans

1.3 - Thesis Structure

- **Chapter 1 - General Introduction**

- **Chapter 2 - The Evolving Field of Anthropogenic Noise and Marine Invertebrates – Developing Methodological Trends and Proposed Future Directions**

The inclusion of marine invertebrates in anthropogenic noise research has increased over time as the topic has progressed and expanded. In this chapter, I detail how the study of anthropogenic noise effects on marine invertebrates has evolved throughout its 35 year development. I outline the history of publications and critically analyse the methods used in these studies, highlighting common benefits and limitations. The frequency at which these limitations and benefits occur through time is mapped to visually illustrate the evolving nature of the field, to give an accurate representation of the state of current research, and to produce research strategies that can be easily followed to further the development of this field.

- **Chapter 3 - New Accessible Devices for Aquatic Particle Motion Measurements in Bioacoustic Studies**

To accurately describe the noise field experienced by marine organisms, two components need to be measured, the sound pressure and the particle motion. Particle motion is often neglected in studies investigating the effect of anthropogenic noise. In this chapter I cover the creation of new accessible sensors for measuring particle motion from both waterborne and sediment-borne vibration, comparing a number of prototypes and assessing their abilities to perform well in bioacoustic research.

- **Chapter 4 - From DNA to Ecological Performance: Effects of Anthropogenic Noise on a Reef-Building Mussel**

This chapter is split into five sub chapters covering the genetic, biochemical, behavioural, and physiological responses of *Mytilus edulis* to anthropogenic noise playbacks.

- **Sub-chapter 4.1 - Effects of Anthropogenic Noise on the Blue Mussel *Mytilus edulis* – General Introduction**

Serving as a general introduction to the study species of chapters 4.2, 4.3, and 4.4, *Mytilus edulis*, this chapter explores the biology of this animal and its use as a model species investigating the effects of pollutants and stressors in the marine environment. In addition, I discuss the importance of *M. edulis* from both an ecological and commercial standpoint.

- **Sub-chapter 4.2 - Noise Induced Biochemical Stress Responses in *Mytilus edulis***

Although studies on the effects of noise on marine invertebrates are becoming more common, investigations to biochemical changes are still scarce. In this chapter, I begin to address this gap of knowledge, conducting experiments

on the effects of ship noise playbacks on the DNA integrity and oxidative stress responses of *M. edulis*. I take established ecotoxicological techniques and for the first time employ them in the context of anthropogenic noise research.

- **Sub-chapter 4.3 - Noise Induced Physiological and Behavioural Stress Responses in *Mytilus edulis***

Continuing the work reported in chapter 4.2, and following the integrative approach discussed in section 1.5.2.1, here I undertake experiments on noise induced physiological and behavioural changes in *M. edulis*. Calculations of the oxygen consumption rate, algal clearance rate, and valve movement are made, and links between the observed changes discussed.

- **Sub-chapter 4.4 - Noise Induced Molecular Stress Responses in *Mytilus edulis***

To date, only a single study has investigated the genetic effects of anthropogenic noise in the marine environment. In this chapter I begin to address this knowledge gap and, for the first time in marine noise research, look at the expression of the stress protein Hsp70, in *M. edulis* exposed to anthropogenic noise. *Hsp70* expression is calculated and changes in expression over time are mapped to identify suitable exposure lengths and procedures for future genetic studies.

- **Sub-chapter 4.5 - From DNA to Ecological Performance: Effects of Anthropogenic Noise on a Reef-Building Mussel – A Synthesis**

This final chapter on *M. edulis* acts as a general discussion for chapters 4.2, 4.3, and 4.4, bringing together the results of all three chapters to generate a more complete picture of how anthropogenic noise is affecting these animals. I draw links between the observed responses and create a response map to show how

each individual change can contribute to a much larger response from the animal as a whole. I also discuss the methods developed in the three previous chapters, and how they can serve as a model for future research on invertebrate responses to noise.

- **Chapter 5 - Behavioural Responses of the European Lobster *Homarus gammarus* to Anthropogenic Noise Playbacks**

Previous studies on the responses of decapod crustaceans to anthropogenic noise have found a number of often differing effects, ranging from potentially positive changes to extreme negatives. I cover the general biology of *Homarus gammarus*, and its selection as the model to represent decapod crustaceans, highlighting its commercial and ecological importance on a national, and international scale. Here, using movement as a metric, I assess the behavioural effects of noise on *H. gammarus* and identify any preference shown to differing noise intensities. I conduct experiments in both summer and winter, to disentangle any seasonal changes to the noise induced responses.

- **Chapter 6 – Synthesis**

The final chapter is a culmination of all the data and methods generated throughout this thesis. It serves as a synthesis of the conducted work, discussing its place within the literature and the importance of the study. I highlight the implications of the results, and applications of the newly-developed equipment and methods. Finally, I suggest improvements to the conducted research and future directions to effectively expand the field of marine anthropogenic noise research.

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Chapter 2

The Evolving Field of Anthropogenic Noise Research on Marine Invertebrates – Trends in Methods and Future Directions

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The Evolving Field of Anthropogenic Noise Research on Marine Invertebrates – Trends in Methods and Future Directions

Interest in the effects of anthropogenic noise on marine invertebrates began at a similar time to that of other groups (Lagardère, 1982). However, it was not until 30 years later that the concern over human generated disturbances caused a proliferation of research focusing on the effects this noise has on marine invertebrates. The recent advances in this field have shown man-made noise can alter the biology of some marine invertebrates in a number of ways, from behaviour (Mooney *et al.*, 2016; Roberts *et al.*, 2016), through to physiology (Langhammer *et al.*, 2016; Solan *et al.*, 2016), biochemistry (Steffano *et al.*, 2016; Vazzanna *et al.*, 2016), and genetics (Peng *et al.*, 2016). Yet despite the current interest in how marine invertebrates react to man-made noise, the full extent of these effects, and the number of species affected, are still poorly understood. To date, invertebrates represent only a small proportion of the literature, highlighted in an ISI Web of Science search where 153 results were generated for marine mammals, and only 21 were found in the same search for marine invertebrates (Basic Search, Topic, Search terms "Marine, Mammal [replaced with Invertebrate in second search], Anthropogenic, Noise", 23/03/2017).

Recent reviews (e.g. de Soto, 2016; Hawkins, 2014; Hawkins and Popper, 2017) have summarised the findings, and highlighted the current limitations when investigating marine invertebrates in the field of anthropogenic noise research. However, these reviews have not shown how recent studies are attempting to address these shortcomings, such as the drive to include particle motion data. As such, generalisations have been made about the current state of the field as a whole that are not truly representative of all recent work. To address this lack of evaluation, a timeline of the methods used in the study of anthropogenic noise and marine invertebrates is presented, highlighting how recent studies have begun to address methodological limitations, and providing a more accurate representation of the state of the art in this research field.

In this methodological review, the approaches taken to study the effects of anthropogenic noise on marine invertebrates are critically evaluated. The focus is not on the results of each study, which have largely been reviewed elsewhere (see, de Soto 2016), but on the methods used to generate said results, with the aim of identifying trends in the methods chosen by studies that have developed over the 35 year history of this field, and not to downplay the importance of findings of any reviewed study. The aim of this review is to highlight how the field of marine invertebrate noise research has evolved over time, and to develop strategies for the assessment of anthropogenic noise effects in marine invertebrates. This structured review is targeted at those starting out in the field of anthropogenic noise research, and provides three research strategies that can be easily followed to produce well rounded studies and help expand the knowledge of this field. These strategies can also be followed by established researchers to help identify the most appropriate methods of study in respect to their specific questions. This is an adaptive process and as such will be improved further as the field progresses.

2.1 – Methods

Literature searches were conducted multiple times from August 2011 to March 2017, the final search taking place on 25 March 2017. Searches were performed in the ISI Web of Science database (Basic Search, Topic) covering all available indexes and utilising an adapted version of the search terms outline by Williams *et al*. (2015). An additional set of invertebrate specific search terms were used in combination with the above to reduce the number of irrelevant results (Table 2.1). This led to a total of 2407 results (including duplicates), with 44 unique results returned when the second search term was included. All results were manually sorted and assessed for validity leaving 22 relevant papers. The reference lists of all identified papers were searched for additional relevant studies and these were added to the literature found in the Web of Science search. Further to this, Google Scholar searches, personal communication, and citation alerts set up in Google Scholar, Mendeley, and ResearchGate since 2011 have led to a final count of 48 studies for assessment in this review. Of the 48 studies assessed the majority (36) are peer-reviewed

literature, the remaining are technical reports produced for government agencies (4), conference proceedings (6), university published research (1), and industry reports (1).

Each study has been assigned a number of descriptors to characterise its method. These descriptors comprise three neutral categories focussing on the type and length of sound exposure used, and the area of biology investigated. In addition to these, six pairs of opposing descriptors focus on specific components of the method. The six descriptor pairs have binary outcomes, with one outcome indicating a benefit in the study of anthropogenic noise impacts on marine invertebrates, and the other outcome indicating a limitation. These outcomes should not however be considered positive or negative judgements on the quality of the research, as many of the "limitations" have their own advantages and practicalities. For example, in the lab/field descriptor pair (P4 below), aquarium studies provide a level of control over exposure conditions that would be unattainable in the field, however a field component would allow a better representation of the noise field experienced in situ and provide potential validation of any laboratory findings. Therefore, it is seen as the benefit in this pair.

Table 2.1 | Terms used in the ISI search. Adapted after Williams *et al*. (2015) with the addition of invertebrate specific terms. Terms enclosed in quotation marks to omit unrelated publications

	Term 2							
Term 1	**Crab**	**Lobster**	**Clam**	**Mussel**	**Scallop**	**Squid**	**Invertebrate**	**Without Term 2**
Airgun Noise	-	-	-	-	-	1	-	63
"Anthropogenic Noise"	7	4	-	1	-	2	21	516
Marine Anthropogenic Noise Impact	3	4	1	1	2	4	13	160
"Marine Noise"	-	-	-	-	-	-	-	17
"Noise Playbacks"	-	-	-	-	-	-	1	12
"Ocean Noise"	-	-	-	-	-	1	2	201
"Pile Driving" Noise	2	2	-	1	-	-	4	144
"Seismic Survey" Noise	-	1	-	-	-	1	2	277
"Shipping Noise"	-	1	-	-	-	-	-	104
Sonar Anthropogenic Noise	-	-	-	-	-	-	1	66
"Tidal Turbine" Noise	1	-	-	-	-	-	1	14
"Underwater Noise"	2	4	-	2	1	1	7	522
"Wind Farm" Noise	2	1	-	-	-	1	3	311
Total (Duplicates not removed)							109	2407
Total (Duplicates removed)							44	

The chosen descriptors are as follows, with descriptions given where necessary. N = Neutral Descriptor, P = Descriptor Pairs:

2.1.1 - Neutral Descriptors

N1 - Exposure Length

There is no exact definition of the length of an acute exposure. It is defined by Hawkins and Popper (2017) as occurring "for a brief period, usually from a particular source". Chronic exposure, both repeated and continuous, occurs over a longer period, although there is no consensus over what constitutes long or short. Here a maximum exposure length of 12 h was chosen for an acute exposure to accommodate studies where a single airgun exposure was used.

Papers can contain more than one of these exposure types.

- **Acute**: A single short-term exposure to noise of less than or equal to 12 h.
- **Continuous**: A long-term noise exposure of longer than 12 h.
- **Repeated**: An exposure where animals receive an acute noise exposure on multiple occasions, resulting in a longer exposure period.
- **Modelled**: Noise responses modelled from previous data, this noise can fall under any of the below.

N2 - Exposure Type

Playback of noise through underwater or in-air speakers is a common technique for noise exposure and can occur in both laboratory and field studies. Exposure to the original noise source is less common in laboratory studies, but can occur when the authors are investigating aquarium or aquaculture noise, as well as pile driving noise created by actively piling.

Semi-field experiments are those where sea cages or other holding systems are used in the field, or where experimental tanks are so large that the effects of captivity are significantly reduced.

A single paper will contain at least two of these descriptors, if not more. In the analysis, lab/field are analysed together, and playbacks/source are analysed together as although related, these pairs act independently of each other.

- **Lab**: Exposure to noise in aquarium tanks, both large and small.
- **Field**: Exposure to noise in the field, or in a semi-field environment.

- **Playbacks**: Exposure to noise either through underwater speakers, in air speakers, shaker tables, or a combination of these.
- **Source**: Exposure to noise directly from the original noise source.

N3 - Area of Biology

A single study can contain multiple descriptors.

- **DNA Integrity and Genetics**
- **Biochemistry**
- **Physiology**: Including changes to morphology and physical injury.
- **Behaviour**
- **Ecology**: Studies investigating population or ecosystem scale abundance or distribution.
- **Fisheries**: Studies that use catch rates as a metric for noise effects.

2.1.2 - Descriptor Pairs

In all pairs the first descriptor is considered the limitation and the second the benefit.

P1 - Particle Motion

This descriptor covers the inclusion of particle motion measurements in the study. In some instances where the noise stimulus is present via shaker tables, but the exact particle motion has not been measured, the study was ranked as having included particle motion measurements, as the methods of exposure can be replicated to produce the same particle motion levels.

- **No Particle Motion Measurements**
- **Particle Motion Measurements**

P2 - Number of Species

- **Single Species**
- **Multiple Species**

P3 - Number of Areas of Biology

These areas refer to those identified in descriptor N3.

- **Single Area of Biology**
- **Multiple Areas of Biology**

P4 - Lab/Field

- **Aquarium Only**
- **Field Aspect**: This applies to studies that are wholly based in the field, semi-field, or those with a combined lab/field approach.

P5 - Tank Size

The size of exposure tanks applies only to laboratory based studies, and is most relevant to studies using noise playbacks from speakers. This descriptor focuses specifically on the tank in which the animals are exposed rather than any vials or chambers used to hold the animals (often important for larval studies). A large tank was defined as a tank in which the animal can be exposed to noise at a distance greater than 1m (Gray *et al.*, 2016), or where the noise field produced would have limited reflection and refraction.

- **Small Tanks**: Aquaria where the animal is exposed at a distance closer than 1m.
- **Large Tanks**: Aquaria where the animal can be exposed at 1m or more from the noise source.

P6 - Sample Size

Sample size will always vary in response to the expected magnitude of the response, the statistical tests chosen to answer the research question, and the experiments performed. A smaller sample size, although sometimes necessary, can create large confidence intervals (Ennos, 2000), with variability around the mean decreasing as sample size increases. Small sample sizes in biochemical and genetic studies are common and should not be considered a negative. Here, 10 replicates were chosen to represent a relatively large sample size.

- **Small Sample Size**: Less than 10 replicates per treatment
- **Large Sample Size**: More than or equal to 10 replicates per treatment

A full breakdown of the descriptors assigned each study is presented in Table 2.2 later in the thesis.

2.2 - State of the Art

Mirroring the marine research conducted on vertebrates, the majority of studies on invertebrates focus mainly on behavioural responses. For example, Wilkens *et al*. (2012) found that the mussel *Perna canaliculus* larvae exposed to high intensity vessel noise displayed significantly faster, intensity correlated settlement times, and Wale *et al*. (2013a) observed changes in the foraging and antipredator behaviours of the shore crab *Carcinus maenas*. Stocks *et al*. (2012) showed species specific changes in the swimming speed of larval bryozoans *Bugula neritina*, echinoderms *Heliocidaris erythrogramma*, bivalves *Crassostrea gigas*, and gastropods *Bembicium nanum* exposed to both outboard motor noise and natural shallow reef noise.

Many studies have also investigated physiological responses, although these studies are less common than those focussing on behaviour. Wale *et al*. (2013b) found that both acute and repeated exposure to ship noise playbacks significantly increased the oxygen consumption of mature *C. maenas*. Physiological effects of noise are also apparent in larvae. de Soto *et al*. (2013) showed that playbacks of seismic pulses can cause body malformations and delayed development in New Zealand Scallops *Pecten novaezelandiae*.

Unlike behavioural and physiological responses, biochemical and genetic responses to noise are poorly studied in all taxa, with the latter only investigated in a single study. Peng *et al*. (2016) studied the expression of 10 genes in the rRNA of the Chinese razor clam *Sinonovacula constricta*, exposed to noise playbacks and found that gene expression was highest when exposed to natural intertidal noise, and lowest when exposed to anthropogenic noise. Celi *et al*. (2015) and Filiciotto *et al*. (2014) both investigated biochemical stress responses in *Palinurus elephas* exposed to ship noise playbacks, and identified changes in haemolymph chemistry as well as increases in heat shock protein (Hsp27 and Hsp70 respectively) concentrations.

The majority of studies focus on only a single noise source (for exceptions see Pine *et al.*, 2012; Stocks *et al.*, 2012) with 55% of studies using a single noise exposure (Day *et al.*, 2016; Payne *et al.*, 2007; Wale *et al.*, 2013a). A few studies have explored the effects of chronic (Lagardère, 1982; Nedelec *et al.*, 2014), or repeated exposures (Regnault and Lagardere, 1983; Wale *et al.*, 2013b), but these are far less abundant than acute exposure experiments, constituting 23% and 15% of studies respectively.

To date, 48 publications (gathered through search criteria above) have been produced covering 47 species from six phyla (Figure 2.1). The majority of studies focus on commercially important crustaceans, bivalves, and cephalopods, with animals from other groups receiving only scant attention. To create a broader, more accurate representation of the effects of noise on marine invertebrates, a larger number of species, response parameters, exposure lengths and noise sources need to be researched.

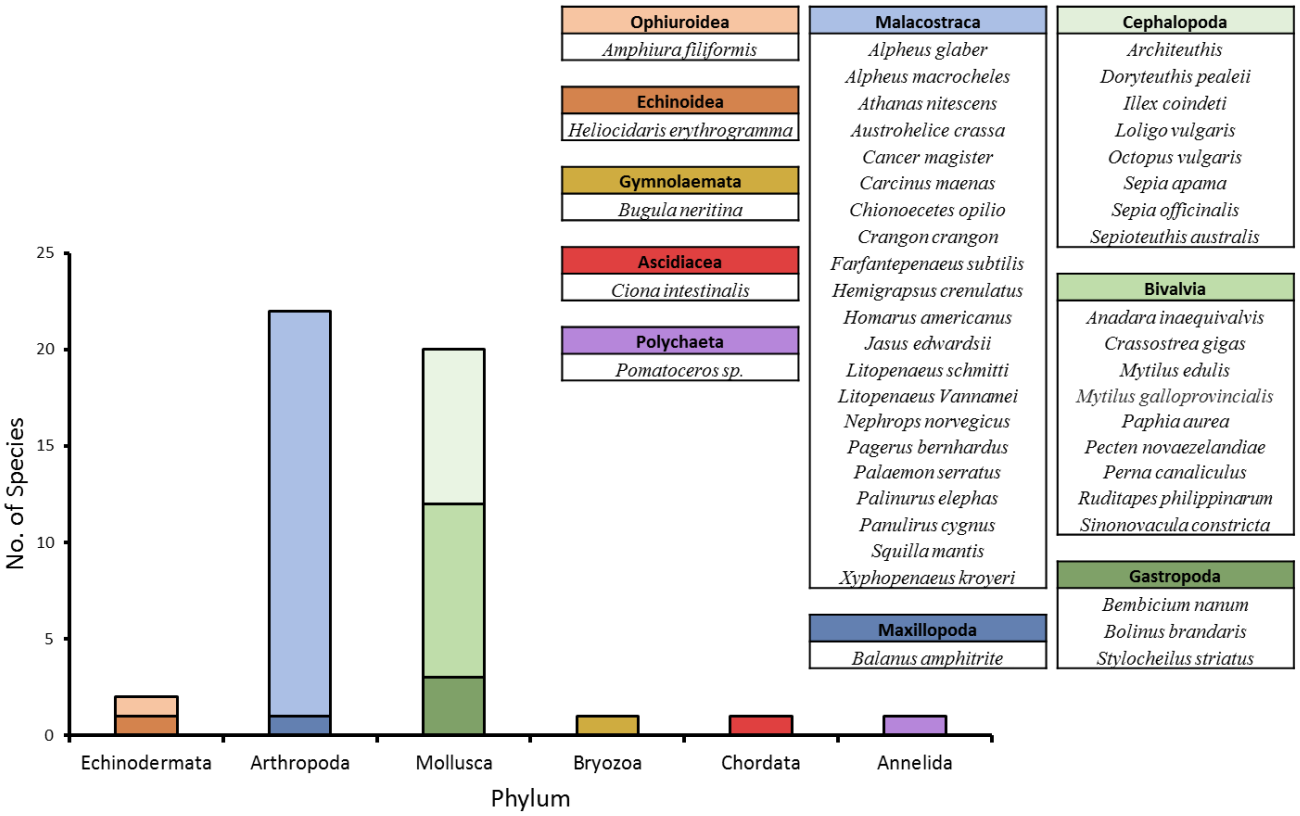

Figure 2.1| Phyletic distribution of invertebrates in anthropogenic noise research. Breakdown of the 47 species currently present in literature. Species cover 10 classes from six phyla.

A recent influx of studies focusing on the responses of marine invertebrates to anthropogenic noise (Figure 2.2) has begun to fill the gaps in our knowledge of both the effects on invertebrates individually and on the wider ecosystemic implications of man-made noise. These studies have occurred mainly over the last five years (2012-2016) and the ways this period has altered the field are discussed further below.

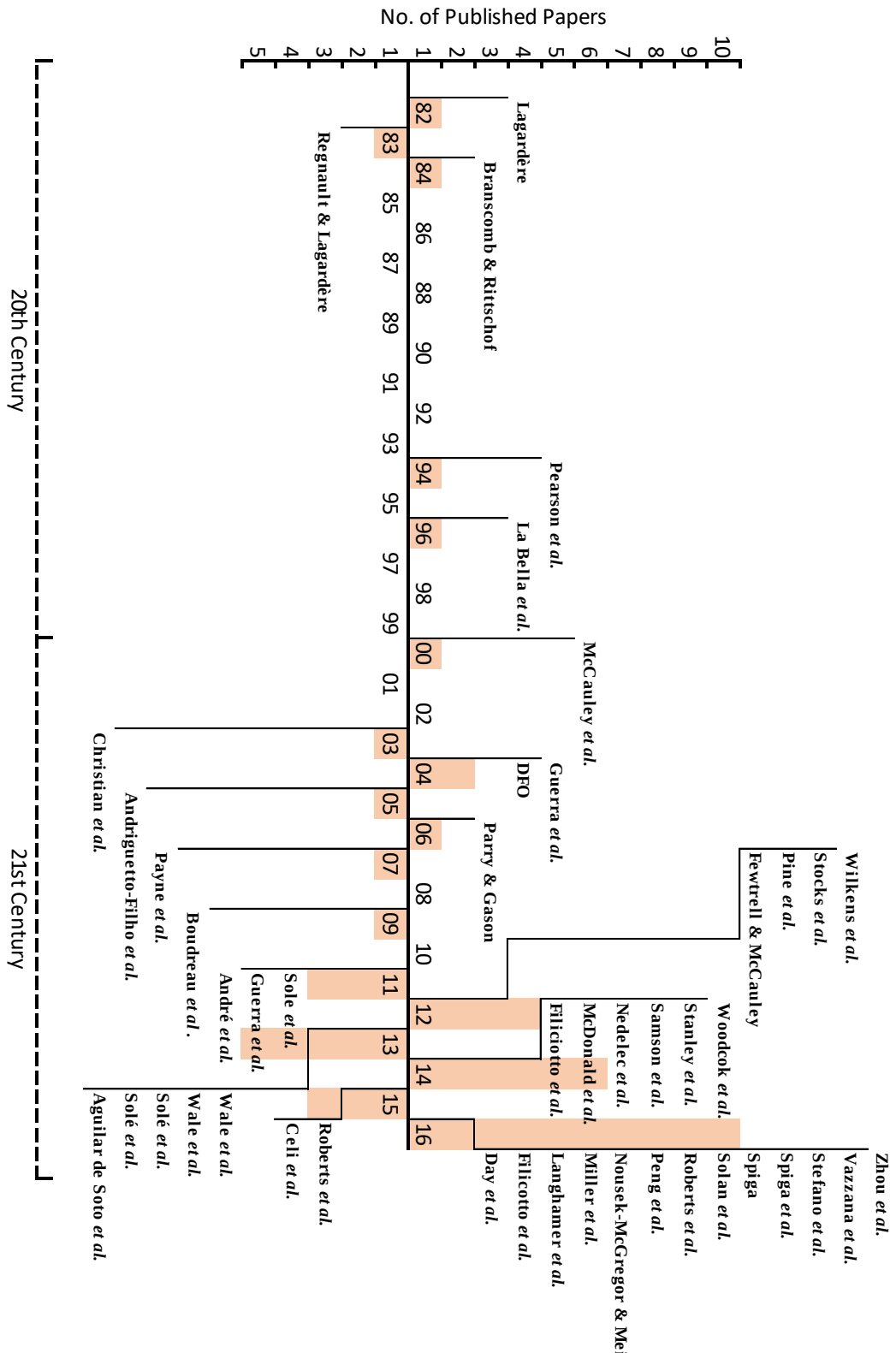

Figure 2.2| History of marine invertebrate anthropogenic noise studies. Publications on the effects of anthropogenic noise on marine invertebrates, occurrence over time.

2.3 - Trends through Time

2.3.1 - Exposure and Area of Biology (N1, N2, N3)

Research on the impact of anthropogenic noise on marine invertebrates has undergone substantial changes since its beginning (Figures 2.3 – 2.11), most of which have occurred over the last five years. Acute noise exposures have dominated the field throughout its history. However, the studies conducted from 2012 - 2016 have increasingly used continuous noise exposures (26% of the literature) and have started to include modelled noise exposures (3% of the literature) (Figure 2.3).

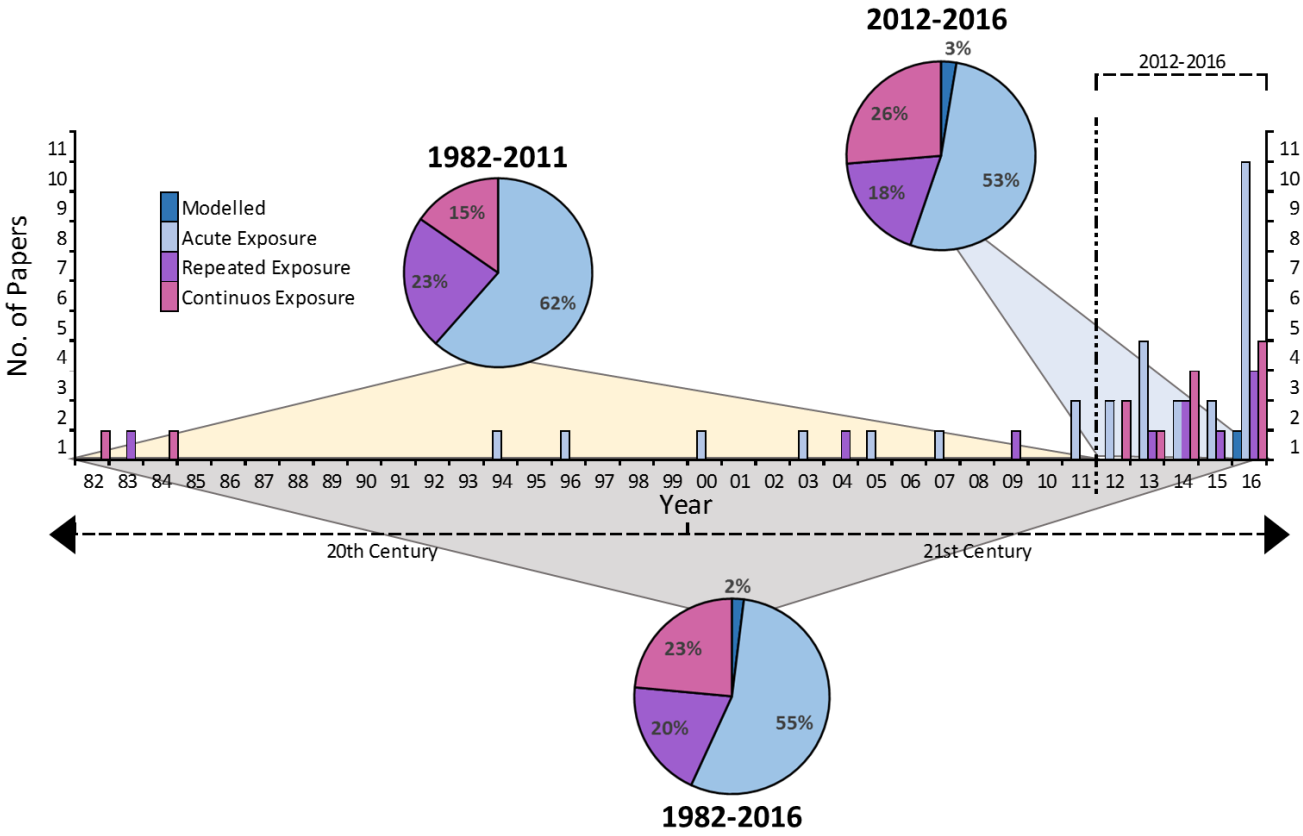

Figure 2.3 | Exposure length (N1). Exposure length used in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

One of the most dramatic changes is the increase in the use of noise playbacks, which has risen from 19% of exposures to 76% since 2012. Field exposures were the most abundant prior to 2012 (55% of publications) and, although their frequency has increased of late, their proportion within the literature has decreased (24%) (Figure 2.4).

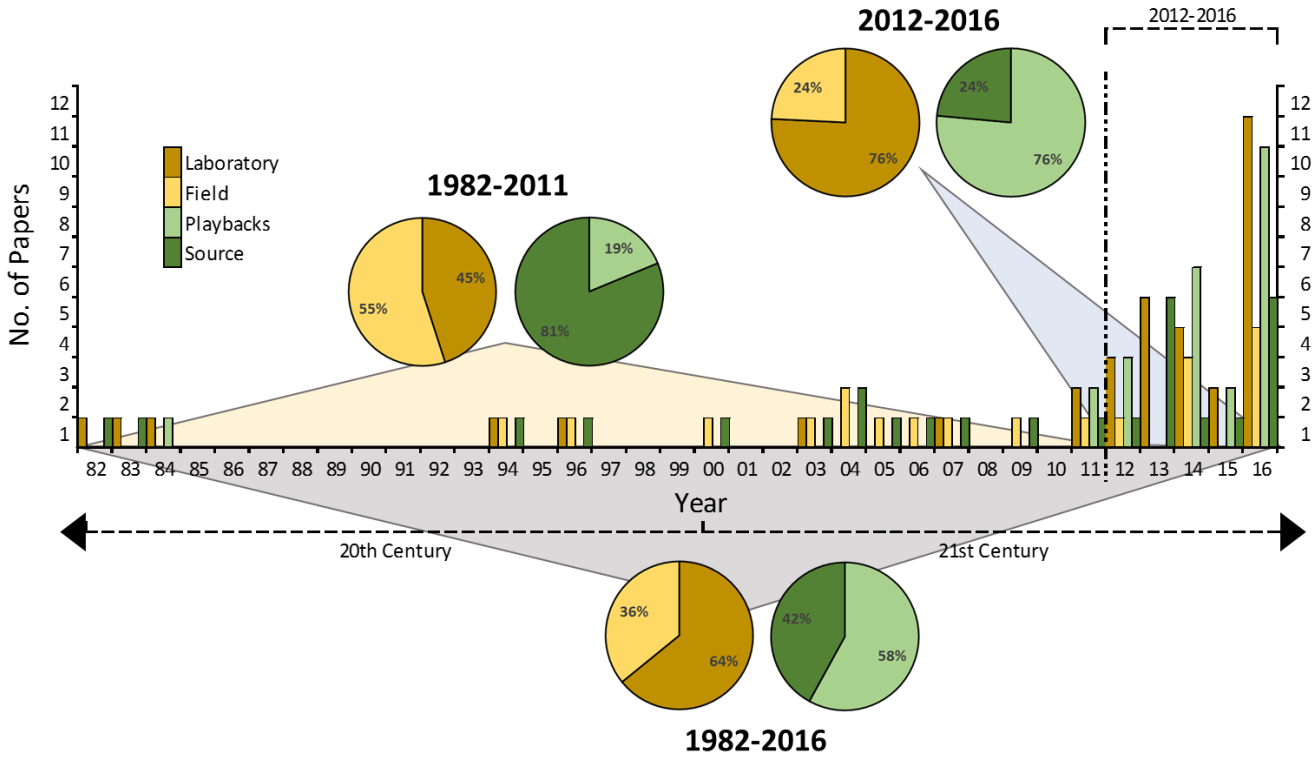

Figure 2.4 | Exposure Type (N2). Type of exposure used in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

The field has been dominated by behavioural and physiological studies from its outset, and this trend has continued until today. Behavioural studies made up 24% of the literature and physiological studies 48% prior to 2012, with this changing to 42% and 35% respectively over the last 5 years (Figure 2.5). There has been a rise in the number of biochemical experiments performed (9% rising to 14%) and the first experiments on the effects of noise on ecology (Langhamer *et al.*, 2016) and genetics (Peng *et al.*, 2016) were performed last year (Figure 2.5). The study of the effects of noise on invertebrate fisheries has decreased in representation falling from 19% to 6% since 2012.

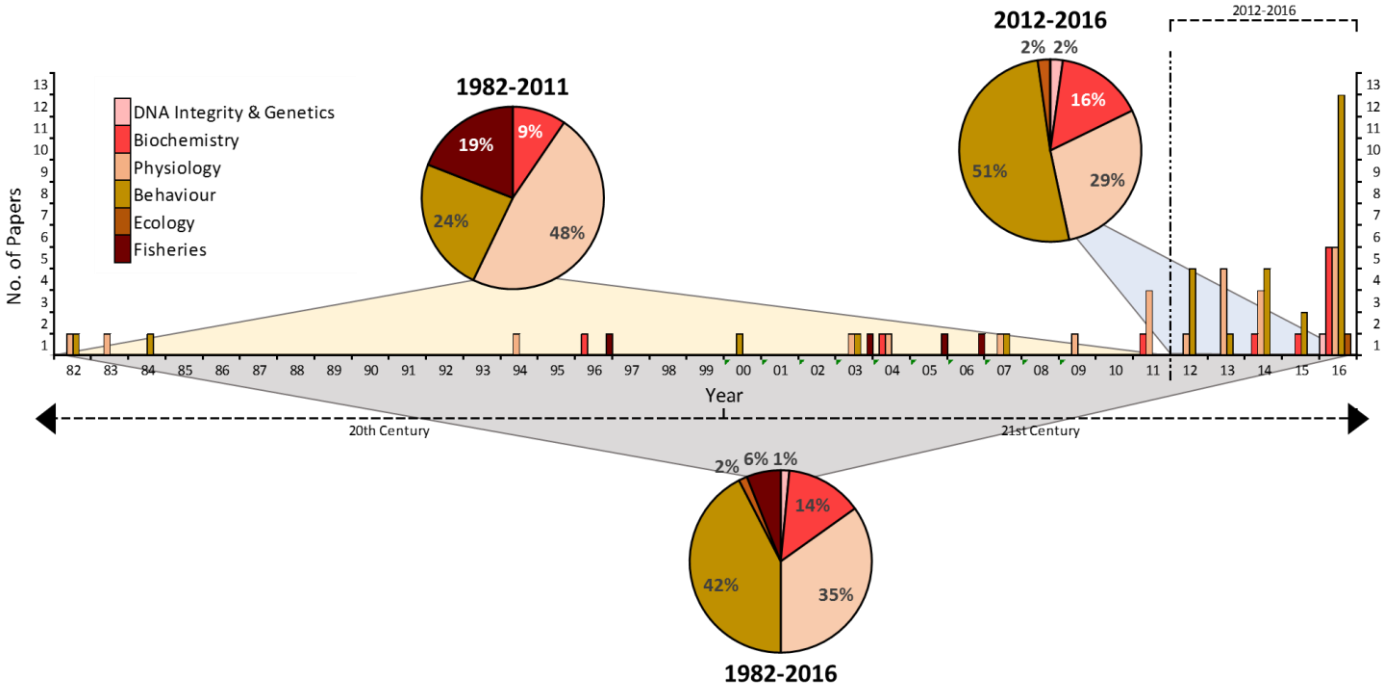

Figure 2.5| Area of Biology (N3). Areas of biology covered in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

2.3.2 - Particle Motion (P1)

Particle motion is the component of sound detected by most fish, and all marine invertebrates, yet despite this it is often neglected in bioacoustic studies. To truly characterise the sound field experienced by these animals in the field and to accurately reproduce it in aquarium experiments, the particle motion must be measured along with the sound pressure. Branscomb and Rittschof (1984) were the first to include particle motion in their analysis. Through the use of a shaker table, the authors were able to reasonably characterise the particle motion in their experiment. However, for the authors of studies using field exposures or speaker playbacks, including particle motion measurements has been a lot more difficult, as the available devices are too large, or too costly (see chapter 3.1, p52 / section 2.4). Consequently, particle motion measurements were absent from the field for 28 years until 2012. Over the last five years several studies have attempted to address this knowledge gap, and during this period 23% of studies have now included particle motion measurements (Figure 2.6).

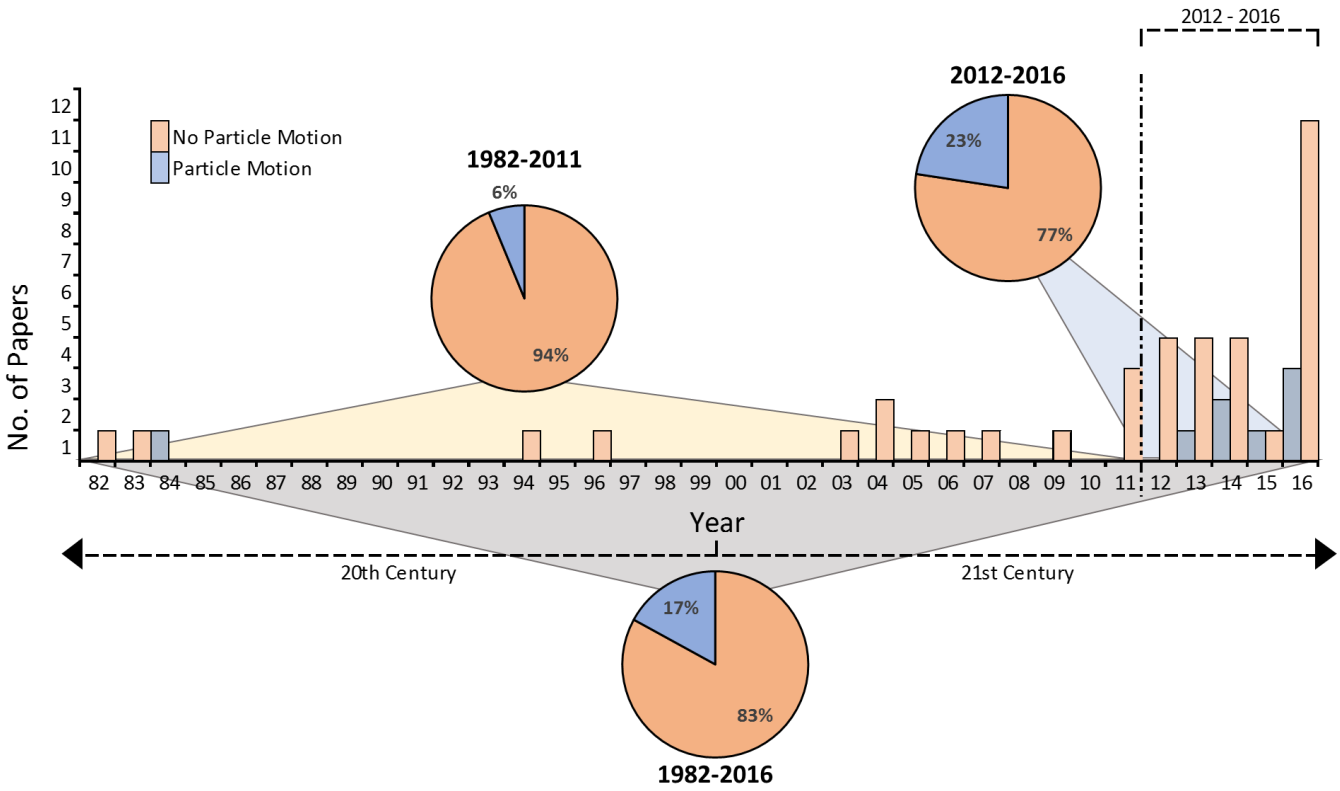

Figure 2.6| Particle motion (P1). Particle motion measurements in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

2.3.3 - Number of Species (P2)

The responses to anthropogenic noise in marine invertebrates is often species specific. Therefore, to uncover shared responses that can then be extrapolated to other organisms, ideally multiple species should be investigated in a single study. The number of such studies has been growing, accounting for 27% of assessed publications from the last five years (Figure 2.7). In contrast, when investigating a single species there is an opportunity to study multiple aspects of biology at the same time (Figure 2.8, Descriptor P3) and 73% of studies published in the last 5 years (74% of the 35 year history of the field) have focused on the responses of only a single species (Figure 2.7).

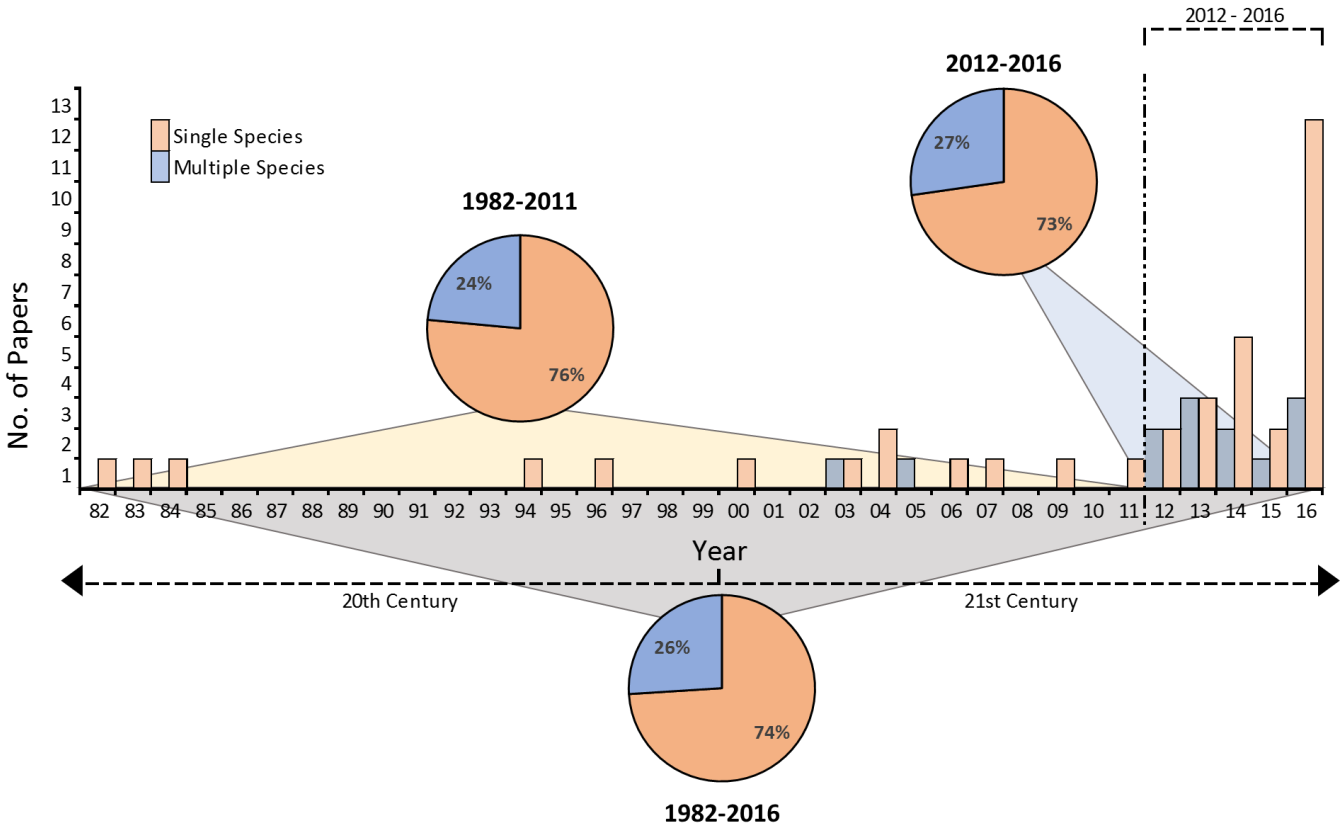

Figure 2.7| Number of species (P2). Studies focusing on single or multiple species in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

2.3.4 - Number of Areas of Biology (P3)

To get a more complete picture of the way anthropogenic noise affects an organism, responses must be investigated at multiple levels of biological organisation. This allows links to be determined between responses and the underlying drivers of more visual behavioural changes to be uncovered (see chapter 4.5, p148). It is becoming increasingly common for authors to explore multiple aspects of an animal's biology, with these studies now accounting for 34% of the literature (Figure 2.8), and 42% of studies in the last five years.

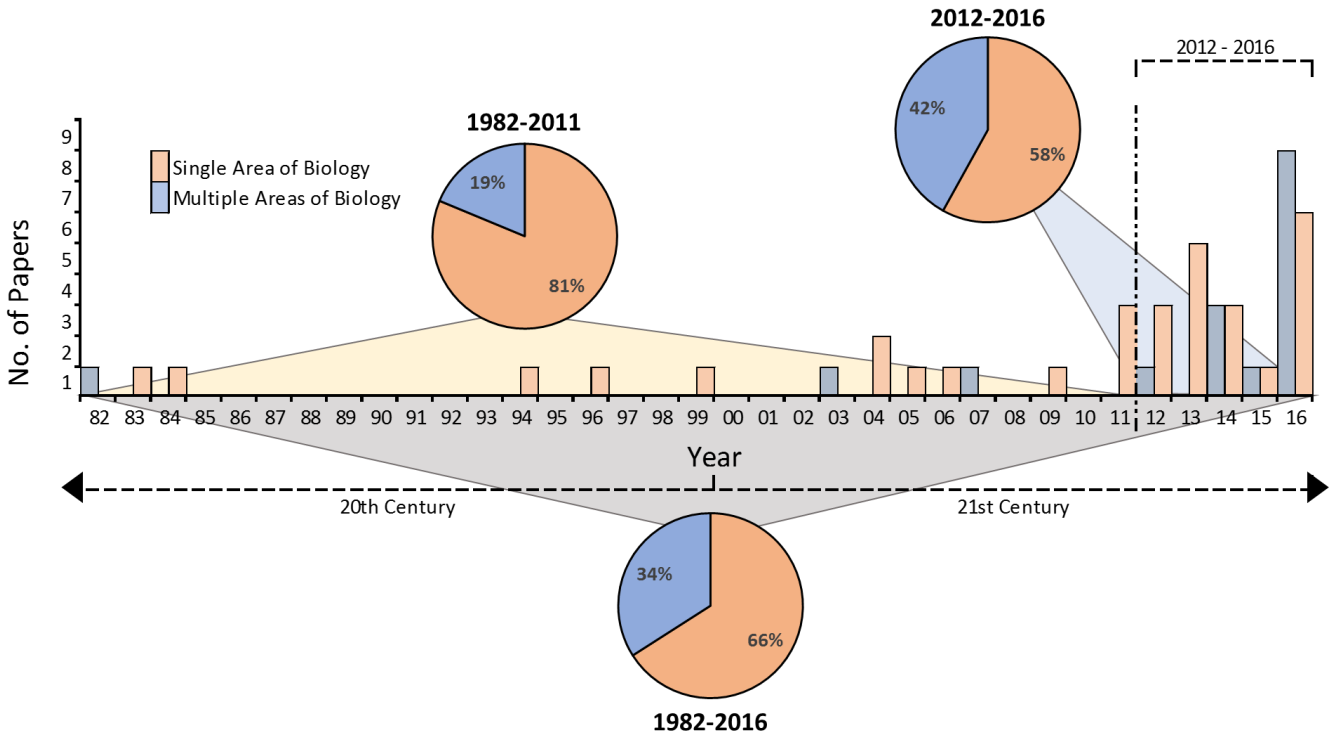

Figure 2.8| Number of Areas of biology (P3). Studies focusing on a single or multiple areas of biology in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

2.3.5 - Lab/Field (P4)

When deciding whether to conduct a study in aquaria or in the field, a number of factors must be considered. Laboratory based studies offer a fine degree of control unobtainable in the field where external factors may influence the exposures, and consequently the final results may be skewed. Conversely, the noise exposures presented in an aquarium will not precisely match those experienced in the field, and removing animals from their natural environment may artificially influence their responses to stress. The use of field investigations will help validate results found in the laboratory, and give results more representative of animals exposed in situ. Although early studies on the responses of marine invertebrates to anthropogenic noise were laboratory based (Branscomb and Rittschof, 1984; Lagardère, 1982; Regnault and Lagardere, 1983), the emphasis quickly switched to studies incorporating a field component (Figure 2.9). Recently however there has been a rise again in aquarium only investigations with their representation in the literature increasing from 33% to 61% between 2012 and 2016 (Figure 2.9).

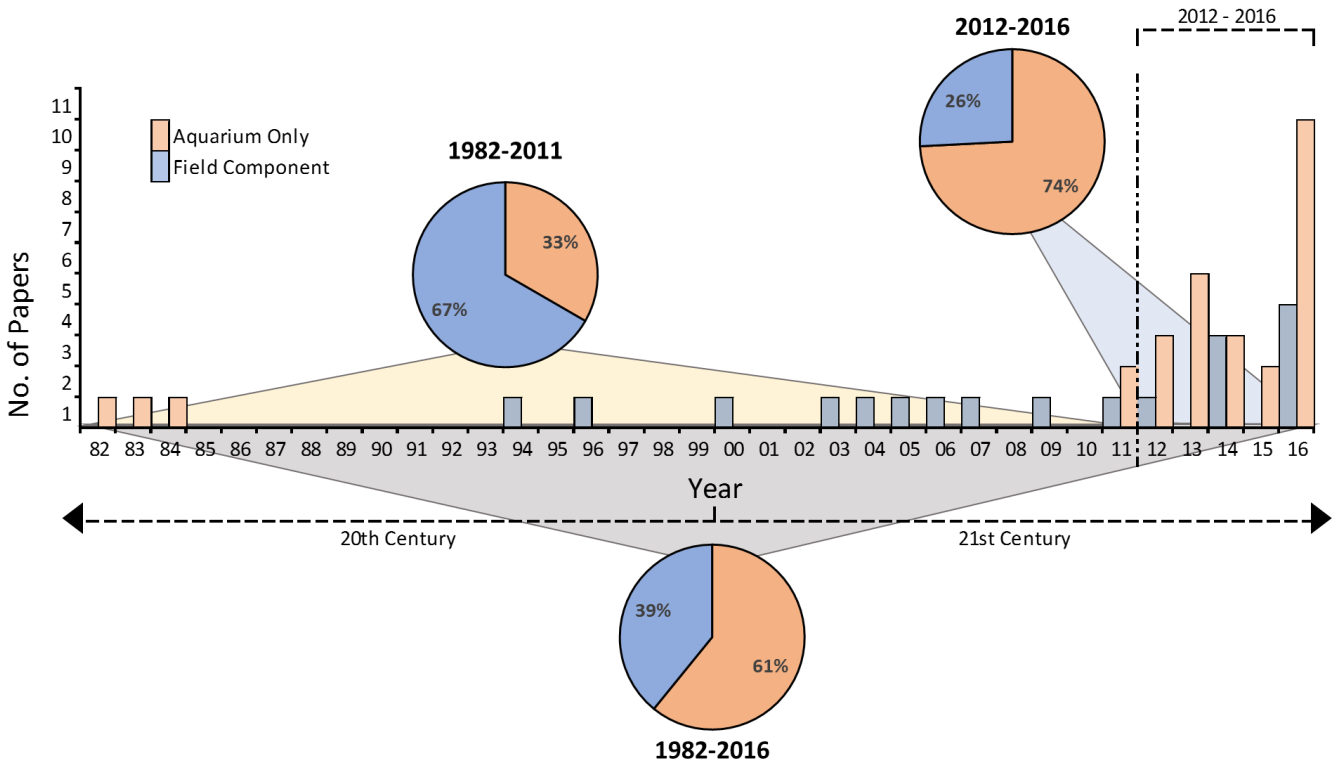

Figure 2.9| Lab/Field (P4). Studies with aquarium only experiments and those with a field component in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

2.3.6 - Tank Size (P5)

When investigating the effects of anthropogenic noise in aquaria it is important to present the noise in such a way as not to artificially increase the animals' response. When dealing with noise playbacks, a small tank will often increase the levels of particle motion, as this is greatest close to the noise source, and therefore potentially expose an animal to noise greater than intended. It is therefore important to use large tanks, where appropriate, in aquarium based experiments that use noise playbacks. The tank size is however less important when introducing vibration into the system to create the desired particle motion, as there is no pressure wave to be reflected. There has been a shift towards large tank studies over the last five years, moving from 17% of the literature before 2012 to 41% after this point (Figure 2.10).

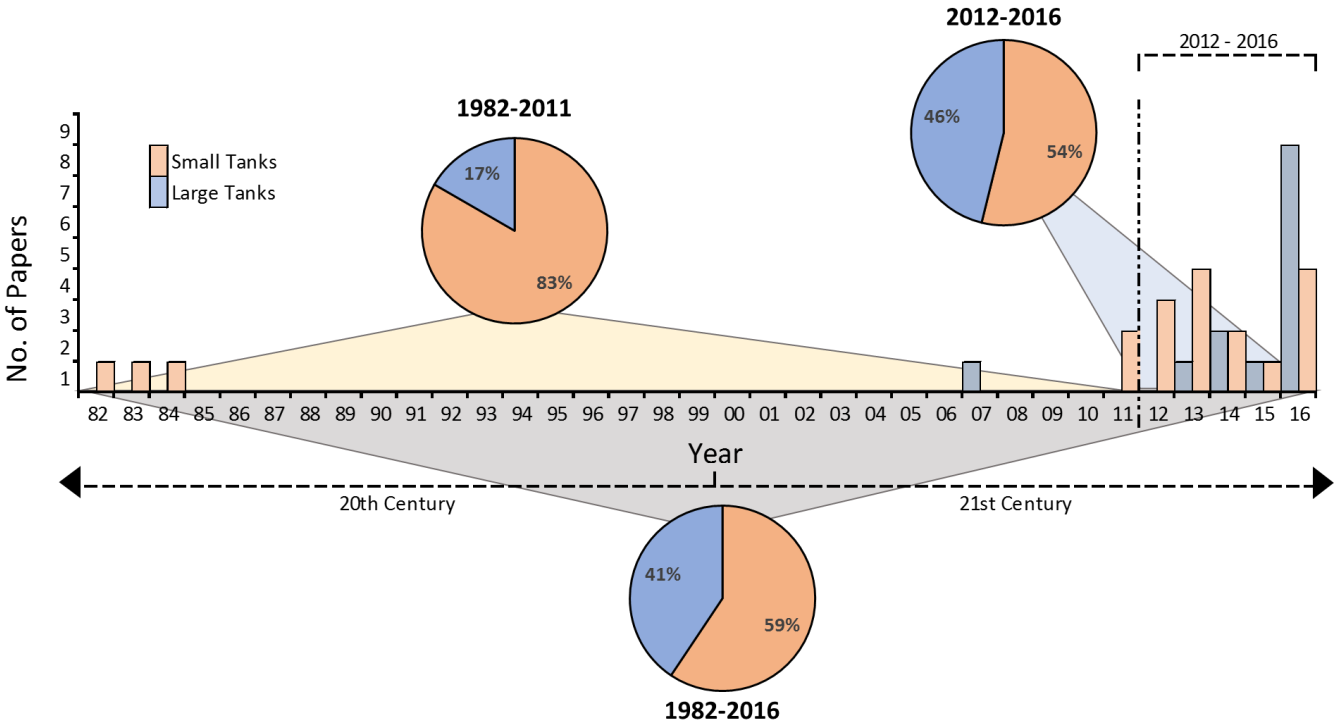

Figure 2.10| Tank size (P5). Studies exposing marine invertebrates to noise in small or large tanks over the 35 year history of the field. Information gained from 48 publications.

2.3.7 - Sample Size (P6)

In general, a larger sample size will allow more robust conclusions about the effects anthropogenic noise is having on the organisms in question, and should be strived for wherever possible without conflicting with ethics. However, this is not always feasible, especially when working with complex systems, vulnerable species, limited resources, or specific techniques. The majority of studies have endeavoured to use as large a sample size as was practical, and therefore the studies labelled here as having a small sample size do not have invalid results. Since 2012 the number of studies that use small sample size has increased to a relatively small extent, from 29% to 35% (Figure 2.11) and follows the increase in biochemical and genetic studies (Figure 2.4).

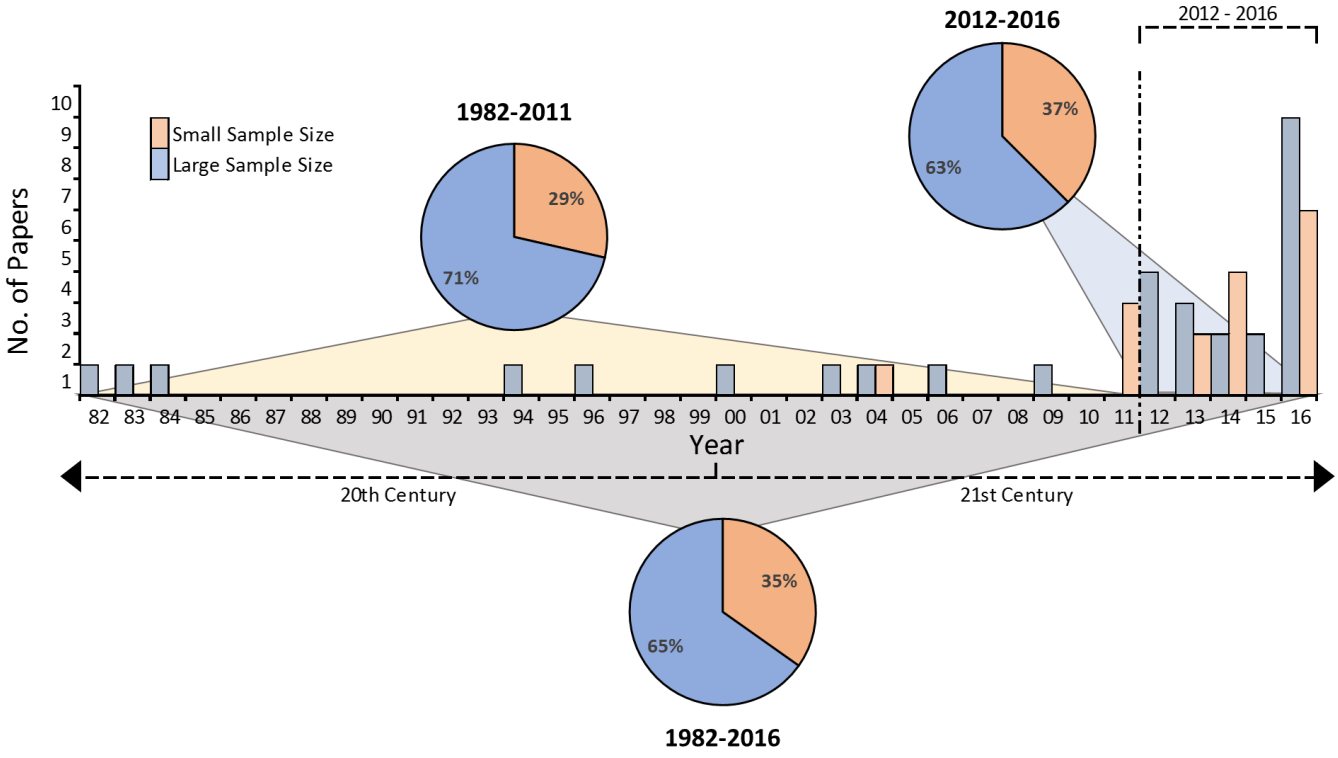

Figure 2.11| Sample size (P6). Studies with small and large sample sizes in marine invertebrate noise literature over the 35 year history of the field. Information gained from 48 publications.

Table 2.2| Categorical analysis of marine invertebrate noise literature. Full analysis of the 48 papers on the effects of anthropogenic noise on marine invertebrates. Assigned descriptors indicated by a filled cell. XXX indicates a study where the exposure length is unclear. * indicates a study where replicable vibration was used, but no particle motion measurements were made. Colours match those in figures 2.3 – 2.11.

Paper	Year	Modeled Exposure	Acute Exposure	Repeated Exposure	Continuous Exposure	DNA Integrity & Genetics	Biochemistry	Physiology	Behaviour	Ecology	Fisheries	Laboratory	Field	Playbacks	Source	No Particle Motion	Particle Motion	Single Species	Multiple Species	Single Area of Biology	Multiple Areas of Biology	Aquaium Only	Field Component	Small Tanks	Large Tanks	Small Sample Size	Large Sample Size
Lagardère	1982																										
Regnault & Lagardère	1983																										
Branscomb & Rittschof	1984																*										
Pearson *et al.*	1994																										
La Bella *et al.*	1996																										
McCauley *et al.*	2000																										
Christian *et al.*	2003																										
DFO	2004																										
Guerra *et al.*	2004		x	x	x																						
Andriguetto-Filho *et al.*	2005																										
Parry & Gason	2006		x	x	x																						
Payne *et al.*	2007																										
Boudreau *et al.*	2009																										
Sole *et al.*	2010																										
André *et al.*	2011																										
Guerra *et al.*	2011		x	x	x																						
Fewtrell & McCauley	2012																										
Pine *et al.*	2012																										
Stocks *et al.*	2012																										
Wilkens *et al.*	2012																										
Aguilar de Soto *et al.*	2013																										
Solé *et al.*	2013																										
Solé *et al.*	2013																										
Wale *et al.*	2013																										
Wale *et al.*	2013																										
Filiciotto *et al.*	2014																										
McDonald *et al.*	2014																										
Nedelec *et al.*	2014																										
Samson *et al.*	2014																										
Stanley *et al.*	2014																										
Woodcock *et al.*	2014																										
Celi *et al.*	2015																										
Roberts *et al.*	2015																										
Day *et al.*	2016																										
Filicotto *et al.*	2016																										
Jolivet *et al.*	2016																										
Langhamer *et al.*	2016																										
Miller *et al.*	2016																										
Mooney *et al.*	2016																										
Nousek-McGregor & Mei	2016																										
Peng *et al.*	2016																										
Roberts *et al.*	2016																										
Solan *et al.*	2016																										
Spiga *et al.*	2016																										
Spiga	2016																										
Stefano *et al.*	2016																										
Vazzana *et al.*	2016																										
Zhou *et al.*	2016																										
Number of Studies		1	28	10	12	1	10	22	28	1	4	33	19	29	21	39	8	36	12	30	17	28	19	19	12	17	29
Percentage of Studies		2	58	21	25	2	21	46	58	2	8	69	40	60	44	81	17	75	25	63	35	58	40	40	25	35	60

2.4 - Discussion

Since the fields' beginning 35 years ago, there have been a number of changes in the methods chosen to investigate the effects of anthropogenic noise on marine invertebrates. One of the most dramatic changes is the rise in both laboratory based studies and the use of noise playbacks. This rise is likely due to the increased level of control garnered from these styles of experimentation. As the field progressed there has been a move away from simply asking does sound affect these animals, towards investigations helping to understand the specific type of reactions exhibited during sound exposure. The use of both noise playbacks and laboratory/aquarium environments give researchers the opportunity to conduct a range of experiments under controlled conditions, and at relatively low costs compared to field studies, allowing more in-depth studies, exploring a range of responses, to be undertaken. Additionally, laboratory studies are often employed when the alternative field experiment would prove impractical.

As the number of laboratory studies has increased so too has the size of the tanks used within these studies, due to the increasing awareness of the acoustical properties of aquarium tanks, especially concerning the reflection of sound waves and subsequent increase in sound intensity when a small tank is used. The use of large tanks is especially prudent when dealing with marine invertebrates, where the particle motion component of underwater sound is most important. Large tanks allow animals to be exposed at a suitable distance from both the noise source and any tank walls, such that the received particle motion is not dramatically increased. Similarly, the measurement of said particle motion has increased in recent years. Particle motion has, until recently, been neglected in most studies. This was likely due to the high cost of commercially available particle motion sensors which are often covered under export laws due to their original military applications. As these sensors are becoming more readily available, both through the creation of new sensors (see Chapter 3, p51), and the increased collaboration, through equipment sharing, within the field, particle motion is increasingly measured.

The first studies conducted to investigate the effects of noise on marine invertebrates focused on behavioural and physiological responses, these have remained the dominate topics with behavioural studies having overtaken physiological studies in the last five years.

As more investigations are undertaken to uncover how anthropogenic noise affects the biology of marine invertebrates, the field has expanded to include biochemical and genetic studies. These follow on from previous behavioural and physiological work, and help to identify the underlying drivers behind and links between observed responses. In a similar manner, as more complex questions are asked the number of investigations that use multiple species within a single study has increased. Multiple species analysis allows more complex questions to be answered, such as how noise effects communities, and how different species respond to the same noise exposure, potentially uncovering varying levels of susceptibility to said noise.

2.4.1 - Research Strategies

Through the above analysis of the methods used in marine invertebrate noise studies, three research strategies are proposed here. These strategies are based on observations of the research topic's development and work currently being conducted in this area. They additionally follow the suggestions of Kight and Swaddle (2011) and Hebel *et al*. (1997) on conducting research in a holistic manner to assess the effects of pollutants in the environment. Although responses in behaviour and physiology are often explored together (Lagardère, 1982; Christian *et al*., 2003; Payne *et al*., 2007; Pine *et al*., 2012; McDonald *et al*., 2014; and Solan *et al*., 2016), only recently a small number of studies have included biochemical analysis alongside behavioural observation (Filiciotto *et al*., 2014; Celi *et al*; 2015 Filiciotto *et al*., 2016; Stefano *et al*., 2016; and Vazzana *et al*., 2016). To date, only two studies have adopted a thoroughly integrative approach (see Jolivet *et al*., 2016; and Peng *et al*., 2016) combining response parameters from multiple levels of biological organisation, and in doing so they have been able to uncover links between the visually obvious behavioural responses and more cryptic responses to noise exposure.

The three strategies proposed below form general guidelines to inform the development of future investigations into the effects of noise on marine invertebrates, with the aim to produce well rounded studies and help expand the knowledge in this field. These strategies can additionally be applied to other taxa or other disciplines. The strategies were

developed with the aid of a research development flow chart (Figure 2.12) that can be worked through to assess the method options of prospective studies.

All studies involving marine invertebrates should include particle motion measurements so that the noise field experienced by the experimental animals (Chapman & Hawkins 1973; Fay 1984; Popper *et al* 2001) can be accurately characterised and compared across studies. Therefore, this metric is not specifically mentioned in any of the below strategies.

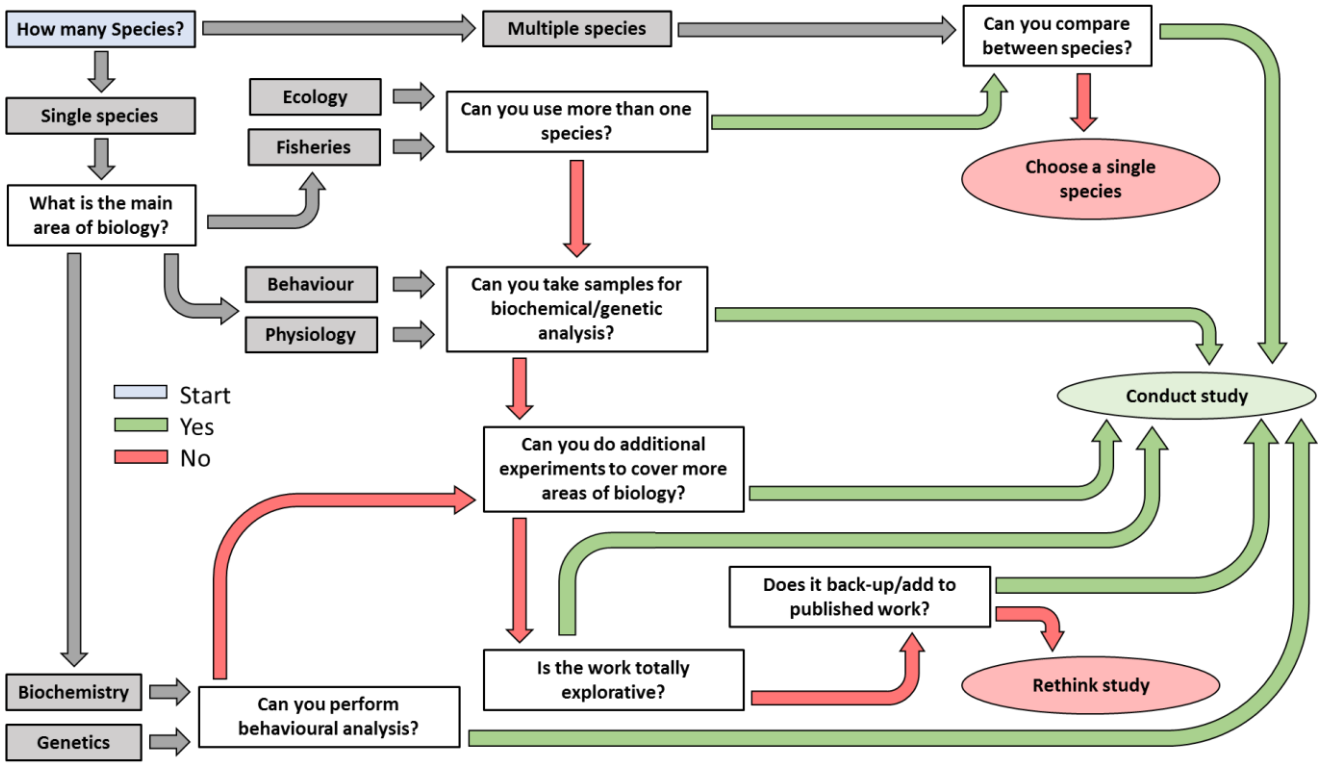

Figure 2.12| Research Strategy Flow Chart. Flow chart to assess the methods of prospective studies on the effects of noise on marine invertebrates, used to generate the suggested research strategies.

Research Strategy 1

Number of Species – Single

Main Area of Biology – Behaviour/Physiology/Biochemistry/Genetics

For single species, it is beneficial to cover as many areas of biology as possible in a single experiment (Kight and Swaddle, 2011), forming a more complete assessment of how noise affects that species. To this end, when the main area of biology investigated is either behaviour or physiology, there is added value in securing samples for later biochemical and/or genetic analysis. Conversely, when the work focuses on biochemical or genetic responses, then, where possible, changes in behaviour should be observed, or captured for later analysis, during the noise exposure. If neither of these options are possible, then additional experiments should be performed as part of the same or subsequent studies to allow multiple areas of biology to be examined.

Research Strategy 2

Number of Species – Single

Main Area of Biology – Ecology/Fisheries

Where possible, studies focusing on ecology or fisheries should examine the effects of noise on multiple species at once, and in a context where the response parameters allow easy comparability between species. If this is not possible and only a single species can be used, as above, additional samples should be taken for later biochemical and/or genetic analysis, or behavioural observations or recordings taken during the exposure.

Research Strategy 3

Number of Species – Multiple

Main Area of Biology – Any

When investigating the effects of noise on multiple species in a single study one must be sure that the parameters tested can be easily compared between species. With multiple species experiments, although beneficial to explore more than one area of biology during

the study, the identification of species specific responses in the same context may allow only a single area of biology to be explored.

Exceptions

The exceptions to the three above strategies come firstly when conducting work that is completely explorative and there is no initial expectation of the outcome, with the work in question acting as a pilot study or proof of concept. A second exception is when the aims of the study are to expand a previous data set, or confirm previously published data. These types of studies hold their own degree of validity, and form useful investigations in themselves.

2.5 – Conclusions and Future Directions

This review summarised and assessed the methods of studies investigating the effects of anthropogenic noise on marine invertebrates. It should be stressed that although much of the literature analysed have a large number of "limitations" (Table 2.2) the aim was not to undermine the results that these studies generated. Instead by conducting the analysis in this way evolving trends in the chosen research methods could be easily identified, and a more accurate evaluation of the current state of the art generated. This allows a more competent comparison of the techniques used in noise research, and the ways in which recent studies are attempting to address historic research limitations can be identified.

There have been many changes to the field of marine invertebrate noise research over its 35 year development (Figure 2.3-2.11), with most of these taking place in the last five years. Between 2012 and 2016 there has been a sharp rise in interest in the field, leading to 32 publications (67% of the literature) in this five year period, twice that of the preceding 30 years. This substantial increase in studies (Figure 2.2) has caused a number of trends to develop in the methods chosen to investigate the effects of noise on marine invertebrates. The majority of these trends move the field away from the highlighted "limitations" and towards the identified benefits (Figures 2.5-2.10).

Twenty three percent of papers published over the last five years have included particle motion measurements (through the use of costly and poorly accessible sensors) allowing more accurate characterisation of the noise fields experienced by marine invertebrates, and a higher degree of precision when reproducing these noises in aquarium trials. This ability to measure particle motion will soon be more readily available (see Wale *et al.*, in prep; Chapter 3) and these readings should continue to be included in future studies. The use of large tanks for playback experiments in combination with particle motion measurements will allow experiments where noises can be represented as accurately as possible in the laboratory. 26% of studies published over the last five years have also included a field component to help validate results in situ. There is however a renewed trend towards aquarium-only studies due to their fine scale control and ability to act as a starting point in noise investigations. This is especially useful when studying a species for the first time and the logistics and expense of a field study are high.

Conducting research in a multidisciplinary integrative way, although logistically challenging, allows a more thorough assessment of the effects of anthropogenic noise, and shows not only if noise affects an organism but potentially how and why these responses occur. There is a developing trend towards studies that focus on a single species in detail, with 42% of papers from 2012 to 2016 assessing the effects of noise on multiple levels of biological organisation. These studies create a more complete breakdown of how noise is affecting each species, both visually through behavioural studies and more cryptically through physiological and biochemical investigation (see Filiciotto *et al.*, 2016; Jolivet *et al.*, 2016; Peng *et al.*, 2016). This way of conducting research has been highlighted here in two of the three suggested strategies for conducting experiments on the effects of noise on marine invertebrates. The other strategy focuses on using multiple species in the same experiment, a strategy which 27% of studies have followed over the last five years. The three research strategies presented here can be easily followed by those entering into noise research, and will allow a more complete analysis of the ways noise affects marine invertebrates.

The analytical framework flowchart (Figure 2.12) presented in this review can be used as a guide for all future work on marine invertebrates and anthropogenic noise, allowing easy description of individual studies and their relationship to the rest of the field.

This way of assessing research methods can be adapted not only for other taxa in noise research, but also as an outline for methodological analysis in other research topics where descriptors can be specifically chosen for that particular field.

Looking to the future, there are a number of recommend directions for the field of marine invertebrate noise research. First and foremost is to include particle motion measurements in all future studies, and to investigate an organism's biology on multiple levels of biological organisation. Additionally, where possible, the use of a complimentary laboratory and field approach should be taken, where the fine scale control of aquarium experiments is coupled with field trials to validate the findings in situ.

With the current interest in the interactions between man and the marine environment, and in the field of anthropogenic noise research, there is now the potential to work across disciplines and integrate multiple stressors into studies to investigate any interactions that may occur. Similarly, cross taxa experiments are becoming a possibility, and will allow research to be conducted with a holistic approach to assess noise effects on species interactions, environmental dynamics, and larger ecosystemic effects of anthropogenic noise.

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Chapter 3

New Accessible Devices for Aquatic Particle Motion Measurements in Bioacoustic Studies

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The following chapter forms part of the manuscript "Wale, M.A., Diele, K., Swift, R., Johnson, M. New Accessible Devices for Aquatic Particle Motion Measurements in Bioacoustic Studies. *In Prep.* The manuscript contains a condensed version of the work in this chapter.

K.D., M.J., and M.A.W. conceived the research; M.A.W. designed the devices with input from all authors; R.S., M.J., and M.A.W. constructed the sensors; M.A.W. analysed the acoustics and calibrated the sensors with input from M.J.; M.A.W. wrote the manuscript and K.D. contributed revisions.

3.1 – Introduction

When attempting to understand the effects anthropogenic noise has on marine organisms, it is first important to accurately measure and describe the noise field they experience. To do this, two interlinked components of the noise need to be characterised. One component is the sound pressure, detected by marine mammals and many fishes. It can easily be measured, through the use of hydrophones, and is the most common metric included in studies investigating the effects of noise, as well as reports on noise levels in the field (see, Hildebrand, 2009; McDonald *et al.*, 2006; Merchant *et al.*, 2016), and legislation limiting noise levels (see, Marine Strategy Framework Directive 2012, Descriptor 11 and IMO MEPC.1/Circ.833). The second component, detected by many fishes and all marine invertebrates, is the particle motion. The term particle motion describes the displacement of water particles that facilitate the movement of the sound pressure wave (Figure 1.1). The particle motion component of underwater noise is far more difficult to accurately measure (Hawkins *et al.*, 2014) and existing, commercially available particle motions sensors have a number of constraints that have prevented their use. As such particle motion measurements

are missing in most aquatic bioacoustical studies to date (see chapter 2.4, p38 / 2.4.1, p39). The lack of particle motion data in most publications has recently been highlighted as a major shortcoming in aquatic sound research (see reviews by Hawkins *et al.*, 2014; Nedelec *et al.*, 2016). To further advance our insights into how specific noise sources and intensities affect marine invertebrates and fishes, and to correctly evaluate the obtained data, this parameter should now be included in future work.

To measure the particle motion component of underwater sound, a vibration sensor needs to be inserted into the water column. This sensor usually consists of a geophone (which senses the velocity component of seismic movements) or an accelerometer calibrated inside a waterproof housing. While pressure is a scalar field and so can be characterized at a given point with a single sensor, particle displacement is a 3-dimensional vector field requiring 3 orthogonal single axis sensors. Vibration sensors therefore may consist of a single triaxial sensor or a combination of three uniaxial sensors mounted so as to measure vibration in all three spatial dimensions. There are a few commercially available particle motion sensors, and all of these possess a number of limitations. These shortcomings include their size, with currently available sensors such as the Hydroflown system (Microflown Maritime B. V., The Netherlands) being too large for deployment in most aquarium based trials; their cost, commercially available sensors cost a minimum of £10k when this PhD project began; their availability, a number of commercially available sensors are restricted by national regulations and export laws, often as a result of their original military applications; or a combination of the above.

The aim of this work was to produce an instrument for use in bioacoustic research that is devoid of these limitations and can be easily reproduced by other scientists within the field of underwater noise research. In doing so it is possible for future studies to produce adequate particle motion readings, addressing the associated knowledge gap. Three compact and low cost particle motion sensors were developed from commercially available accelerometers and tested for deployment in bioacoustic research. Sensors were fashioned to allow measurement of both water borne particle motion, to allow characterisation of the noise field, and sediment borne particle motion, to detect sediment vibration when dealing with benthic organisms, during laboratory and field studies. The produced sensors were calibrated, and their performance (accuracy, precision, noise floor, frequency response)

assessed. Finally, the sensors were compared in terms of cost-benefit, and recommendations for the most suitable sensor for bioacoustic research made.

3.2 – Methods

Throughout the following methods section the term "sensor" is used to describe the particle motion sensors in production. If an individual accelerometer is discussed as a component in the construction of the sensors it is described as an accelerometer.

3.2.1 - Sensor creation

Three commercially available triaxial accelerometers (instruments where a voltage is generated by stress on an internal crystal [Piezoelectric] or movement of a known "proof" mass within the instrument [MEMS] in response to movement) were chosen for comparison spanning a range of price and performance. These were: PCB Piezotronics YTLB356A12 (PCB Piezotronics, Hertfordshire, UK), Measurement Specialities 832M1 (Measurement Specialities, Hampton, New Hampshire, USA), and STMicroelectronics LIS344ALH (STMicroelectronics, Geneva, Switzerland). The technical specifications of these accelerometers are given in Table 3.1 and the full data sheets in Appendix A.

Table 3.1| Technical specifications of chosen accelerometers for building particle motion sensors.

Accelerometer	Weight g	Sensitivity mV/(m/s^2)	Spectral Noise (µm/s2)/√Hz @100Hz	Cost £
PCB Peizotronics YTLB356A12	5.4	10.2	49.0	1200
Meas Spec 832M1	3.0	5.1	624.0	125
STMicroelectronics LIS344ALH	0.04	6.6	490.0	3.50

The PCB Piezotronics YTLB356A12 is a piezoelectric accelerometer with phantom powered (power supplied down cable attached to recorder rather than an external supply) preamplifier, supplied in a module with a cable connector (Figure 3.1). This device was

used with the manufacturer-supplied cable (3 mm ø) and phantom power supply. The Measurement Specialities 832M1 is also a piezoelectric accelerometer with built-in preamplifier and was supplied in a surface mount module. This was wired to an external power pack (three AA batteries) to provide power for the accelerometer (Figure 3.1). The STMicroelectronics LIS344ALH is a MEMS (Micro-Electro-Mechanical System) based accelerometer with built-in signal conditioning and preamplifier supplied in a 4 x 4 mm surface mount integrated circuit. This required mounting to a custom circuit board, and attachment to a power pack (two AA batteries) (Figure 3.1). Miniature screened cable (4 core, 1.2 mm diameter, Micronmetres Inc., Saint George, Utah, USA) was used to connect power and bring signals from the 832M1 and LIS344ALH. All accelerometers had outputs wired to three BNC connectors, one for each axis, to allow attachment to data acquisition devices, such as hand held recorders or data capture cards.

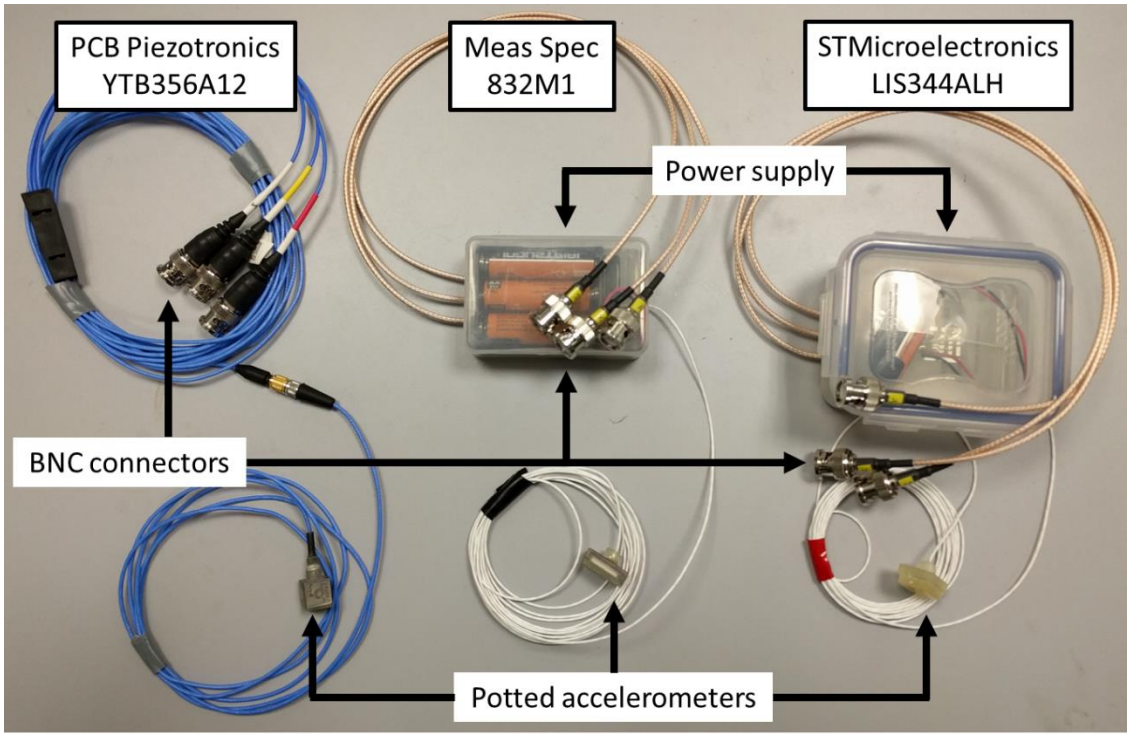

Figure 3.1| Potted accelerometers. Shown here are the three sensor types potted in epoxy resin for the detection of sediment borne vibration. Highlighted here are the power supply systems, The PCB Piezotronics YTLB356A12 utilises phantom power, while the Measurement Specialities 832M1 and the STMicroelectronics LIS344ALH are powered by power packs. The sensors connect to data acquisition systems through three BNC connectors. The bottom side of the potted accelerometer (opposite the exiting wire) is placed on the substrate to measure sediment borne vibration.

None of the sensors were waterproof as supplied and so required casting in plastic i.e. potting. To achieve this, a mould was produced using Lego bricks as a support, and a small layer of two-part silicone rubber (Flexil-S RTV-40c, Jacobsen Chemicals, Alton, UK). Once the silicone had dried, an appropriately sized blank (Figure 3.2 - 3.3) was placed on the initial silicone layer and covered completely with silicone rubber (Figure 3.2). Blanks consisted of a 3D printed nylon plastic cube (created in Autodesk Inventor Professional 2015 Student version, Autodesk, San Rafael, California, United States, Figure 3.3) for the sediment borne vibration sensors, and a glass marble large enough to hold the accelerometer and associated wiring for the water borne vibration sensors. To remove air bubbles from the silicone created in the mixing and pouring process, the liquid silicone was placed in a vacuum chamber both after mixing, and after pouring. Once fully cured, the Lego bricks were removed and the blank freed from the mould. A thin layer of clear epoxy resin (Epotec, Rüsselsheim Germany) was poured into the mould and allowed to cure to provide a stable platform for mounting the accelerometers. Standoffs, consisting of small sections of epoxy, were placed on the epoxy resin to hold the accelerometers in place (Figure 3.2) and the accelerometers themselves added to the mould. Finally, further epoxy resin was poured into the mould to cover the accelerometer, and the mould placed into a vacuum chamber to remove any created air bubbles. Once cured, the potted accelerometers were removed.

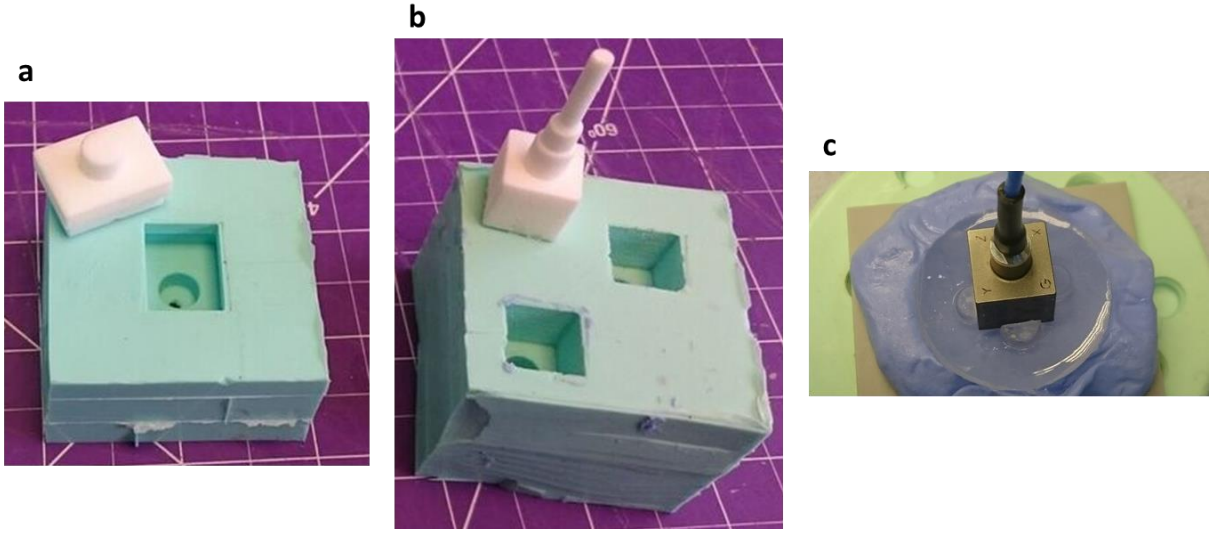

Figure 3.2| Silicone moulds. Silicone mould for potting the accelerometers. (a) silicone mould for the sediment borne vibration sensors created from the PCB YTB356A12 accelerometer. (b) silicone mould for the STMicroeletronics LIS344ALH and Measurement Specialities 832M1 accelerometers. (c) accelerometer held by standoffs to keep it in the centre of the sphere created by the blank for the water borne vibration sensor.

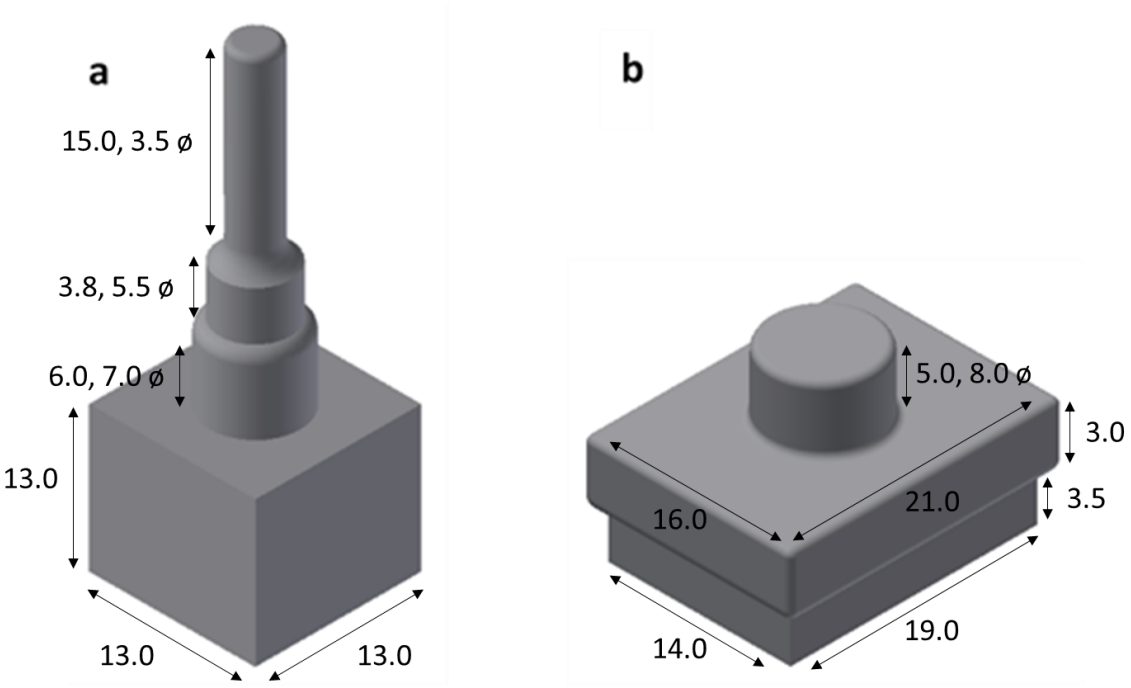

Figure 3.3| Sensor blanks. 3D models of sensor blanks created in Autodesk Inventor Professional 2015 (Student version, Autodesk, San Rafael, California, United States). Blanks were printed in white nylon plastic before moulding. (a) Blank for PCB Piezotronics YTLB356A12 accelerometer. (b) Blank for MeasurementSpecialities823M1 and STMicroelectronics LIS344ALH accelerometers. Dimensions in mm.

The finished sensors all had a density greater than water. Although this is desirable in a sediment sensor to maintain the sensor on the substrate, neutral buoyancy is preferable for a water column sensor to avoid vibration coupling from the surface through the cable. Neutral buoyancy could be achieved by adding buoyancy material (e.g., syntactic foam) to the package but this increases the volume of the sensor impacting its high frequency sensitivity. Instead, the potted accelerometers were suspended via a 1.0 mm diameter elastic chord (Beadalon Valley Township, Pennsylvania) and elastic netting, to two interlocking 3D printed nylon rings (Figure 3.4). This mounting system allows the accelerometers to detect the particle motion, but remove any dampening effect through the downward pull of the accelerometer. The accelerometer wire was spiralled through the mount (Figure 3.4) and coiled around a supporting 3.0 mm diameter elastic cord to prevent both surface movement, and the effect of water movement on the wire, from transferring

to the accelerometer and reducing the accuracy of readings. Lastly, a 100 g fishing weight was suspended below the accelerometer in the z direction to keep the housing level within the water column, and help alleviate any large sideways movements caused by the surrounding water. A 100 g weight was chosen as it was greater than the weight of the constructed sensor but small enough as not to damage the nylon rings.

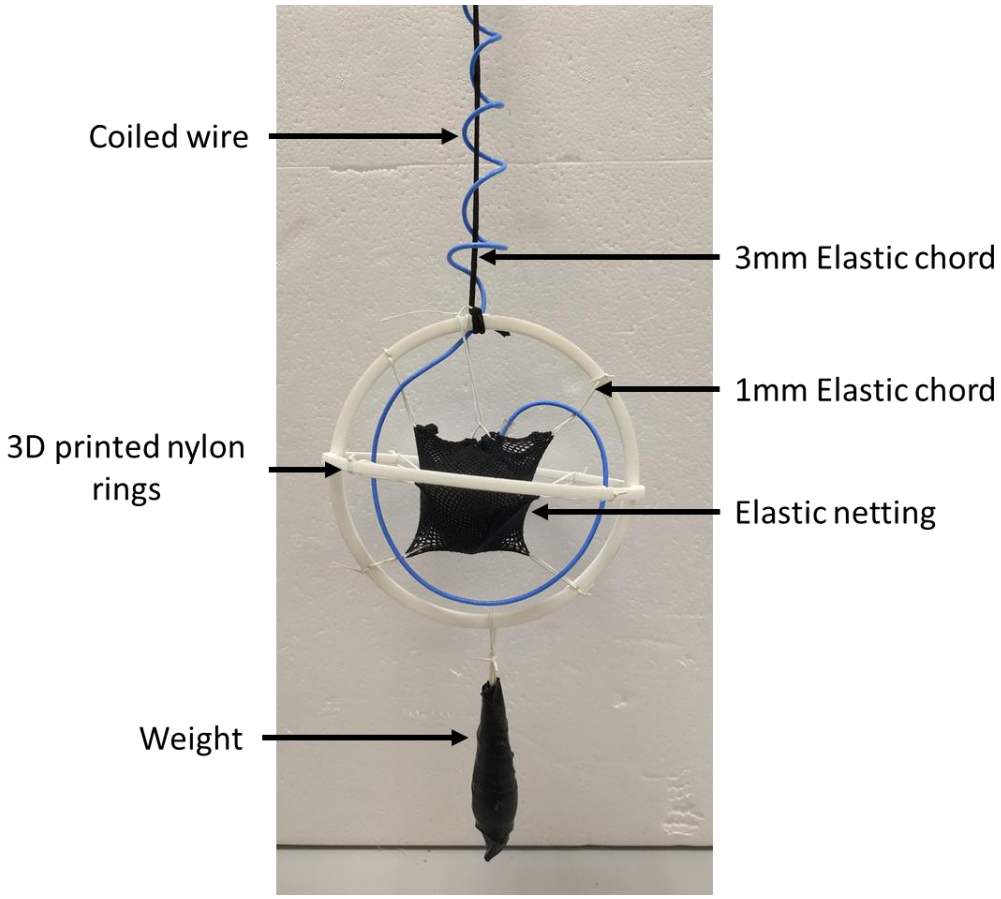

Figure 3.4| Water borne particle motion sensor housing. Housing for the water borne vibration sensors consisting of two interlocking 3D printed nylon rings suspending the accelerometer inside elastic netting by 1 mm elastic chord. The accelerometer wire is wound through the supports but does not touch the nylon rings, and coiled around a 3 mm elastic chord used to deploy the sensor.

3.2.2 - Calibration

All sensors were calibrated by comparing their responses to that of a reference accelerometer (Brüel & Kjær 4508B, Brüel & Kjær, Nærum, Denmark). Sensors were exposed to vibrations of known frequency and amplitude (50, 60, 70, 80, 90, 100 Hz at 1 and 2 Vpp, and 50 Hz at 3, 4, 5, 6, 7, 8, 9, 10 Vpp) on a Controlled Vibration EDP-2424 24 Inch Platform shaker (Crowson Technology, Santa Barbara, California, USA) (Figure 3.5). Tones were presented in order from lowest to highest frequency (1 and 2 Vpp) and lowest to highest amplitude (50 Hz), with measurements taken one minute after the vibration began. The above frequencies were chosen as although there are currently no known measurements of the particle motion produced by anthropogenic noise sources, the low frequency vibrations presented here fall in line with those detected by marine invertebrates (Roberts *et* al., 2015; 2016). The shaker and sensors were in air and the sensors were mounted to the platform with beeswax. The platform shaker is driven by two Crown DSI 1000 amplifiers (Crown Audio, Elkhart, Indiana, USA) and a Siglent SDG1020 Arbitary Waveform Generator (1 µHz – 20 MHz range, 125 MSa/s, Siglent Technologies, Shenzhen, China) set to generate sine waves. This system allows programmable frequencies and voltages to be sent through the platform shaker and the resulting velocity and acceleration to be measured. These measurements were then compared against a standard created from reference accelerometers (Brüel & Kjær 4508B, Sensitivity 10 mV ms^{-2}, Spectral Noise <343 (µm s^{-2})$\sqrt{Hz^{-1}}$ @100Hz, Brüel & Kjær, Nærum, Denmark) to create a calibration curve and establish the accuracy of each sensor. This sensor is used within industry and was itself calibrated externally prior to its use. All calibration measurements were made using a Brüel & Kjær 5 Channel Pulse 3560-B-140 front end, and the attached LabShop Pro (Type 7700, Brüel & Kjær, Nærum, Denmark) analysis software.

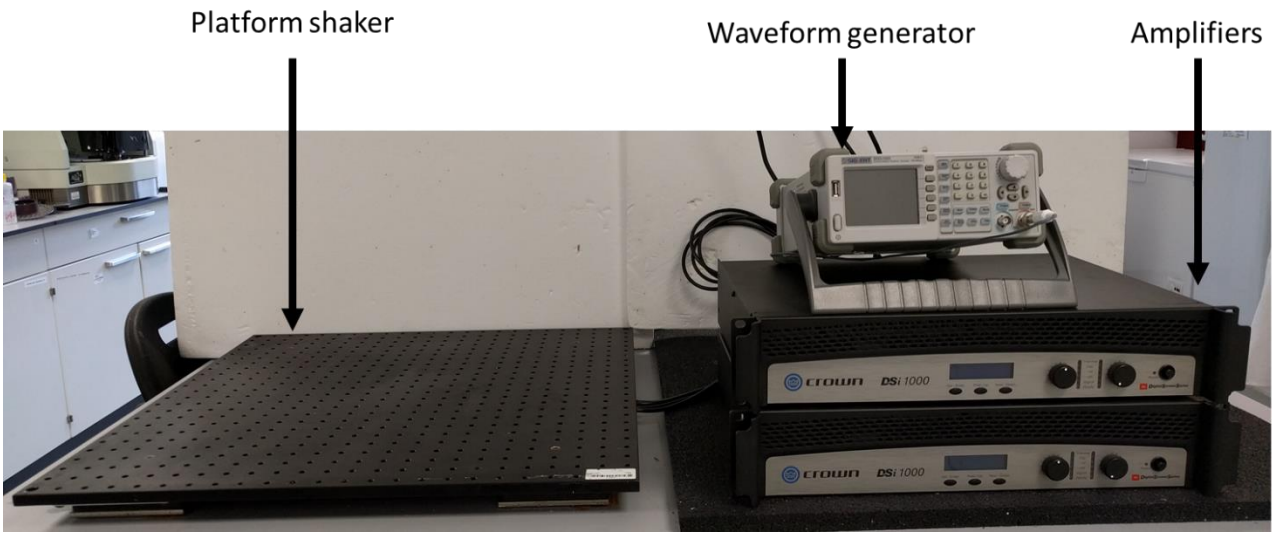

Figure 3.5| Calibration set-up. Set up for sensor calibration. 24" platform shaker driven by 2 amplifiers. Signals produced through an arbitrary waveform generator.

To further assess the accuracy of the sensors, the STMicroelectronics LIS344ALH, chosen as the sensor most easily replicable and employable by others due to its low construction cost and minimal requirement for additional equipment, was deployed alongside a twin hydrophone system where two Brüel & Kjær Type 8103 Miniature Hydrophones are spaced a known distance apart. This system provides a measure of the particle velocity from the pressure gradient between the two hydrophones (Bai *et al.*, 2013), but requires that the hydrophones are closely matched for amplitude and phase response, and can only measure velocity in a single axis at a time, making the system both expensive and impractical for field use. Both the particle motion sensor and the hydrophones were mounted in a tank (7 m long, 2 m wide, 1 m deep) and exposed to a step-up series of tones (10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 1000 Hz) produced in Audacity 2.0.5 and to broadband pre-recorded ship noise. Exposures were played back through a laptop (Acer E5-571 series, Acer Inc., Xizhi, New Taipei, Taiwan); amplifier (Pioneer A-10-K, 50W, frequency response: 20-20,000 Hz, Pioneer Corporation, Tokyo, Japan); and Clark Synthesis AQ339 underwater speaker (effective frequency range 20-17,000 Hz, Clark Synthesis Inc., Littleton, CO, U.S.A). In all positions recordings were taken from the x, y, and z channels of the accelerometer and from both hydrophones. Recordings were repeated with the axis of the hydrophone array aligned with the accelerometer's x, y, and z axis in

turn. These recordings were then analysed in paPAM (Nedelec *et al.*, 2016) for the particle motion sensors, and in MATLAB (version 2015B) using custom code for the twin hydrophone measurements. At the time of writing, an inconsistency in the gain setting for the charge amplifiers used with the hydrophones (B&K Type 2635) prevented a meaningful result from this validation method. This will be rectified in future work and the readings will then be compared to gauge the accuracy of the developed sensors.

3.2.3 - Statistical analysis

All statistics were performed in R version 3.3.1 (The R foundation for Statistical Computing). Regression analysis was performed between each axis of the created sensors (dependent variable) and the corresponding axis of the reference accelerometers (independent variable). The results of all regressions are presented in Table 3.2.

3.3 – Results

The Measurement Specialities 832M1 accelerometer failed to measure the particle motion of underwater noise. After initial calibration tests and troubleshooting it was found that the accelerometer was unfit for purpose. This accelerometer was originally designed to work with large forces, rather than the smaller particle motion movements presented here. Consequently, when the accelerometer sensitivity was amplified to detect these small movements the internal 50 Hz noise of the power supply was amplified to the same degree, drowning out any other readings. Therefore, this accelerometer is not presented in any of the forthcoming calibration data.

Table 3.2| Results of regression analysis for the created sensors, including the equation used for calibration, R^2 value, Std. Error, and P-Value.

Accelerometer	Vibration Type		X	Y	Z
PCB Peizotronics YTLB356A12	**Sediment**	**Equation**	y=1.0217x	y=1.192x	y=0.9506x
		R^2	0.999	0.998	0.992
		Std. Error	0.007	0.010	0.021
		P-Value	2.20E-16	2.20E-16	2.20E-16
	Water	**Equation**	y=1.0424x	y=1.215x	y=0.9745x
		R^2	0.996	0.995	0.997
		Std. Error	0.014	0.013	0.013
		P-Value	2.2E-16	2.2E-16	2.2E-16
STMicroelectronics LIS344ALH	**Sediment**	**Equation**	y=10.247x	y=17.001x	y=5.4691x
		R^2	0.999	0.663	0.991
		Std. Error	0.002	0.006	0.003
		P-Value	2.20E-16	1.33E-04	9.18E-15
	Water	**Equation**	y=9.3845x	y=17.035x	y=5.2929x
		R^2	0.995	0.617	0.989
		Std. Error	0.003	0.007	0.003
		P-Value	2.20E-16	3.18E-04	2.47E-14

The most accurate accelerometer for measuring the particle motion was the PCB Piezotronics YTLB356A12, which, when calibrated against the reference accelerometers, was consistent in its measurement across all three axes (Figure 3.6, Table 3.2). Both the sensor for measuring sediment borne vibration (Figure 3.6a) and the sensor for water borne vibration (Figure 3.6b) had high accuracy and high precision (R^2 sediment borne vibration sensor X= 0.999, Y= 0.998, Z = 0.992; water borne vibration sensor X= 0.996, Y=0.995, Z=0.997).

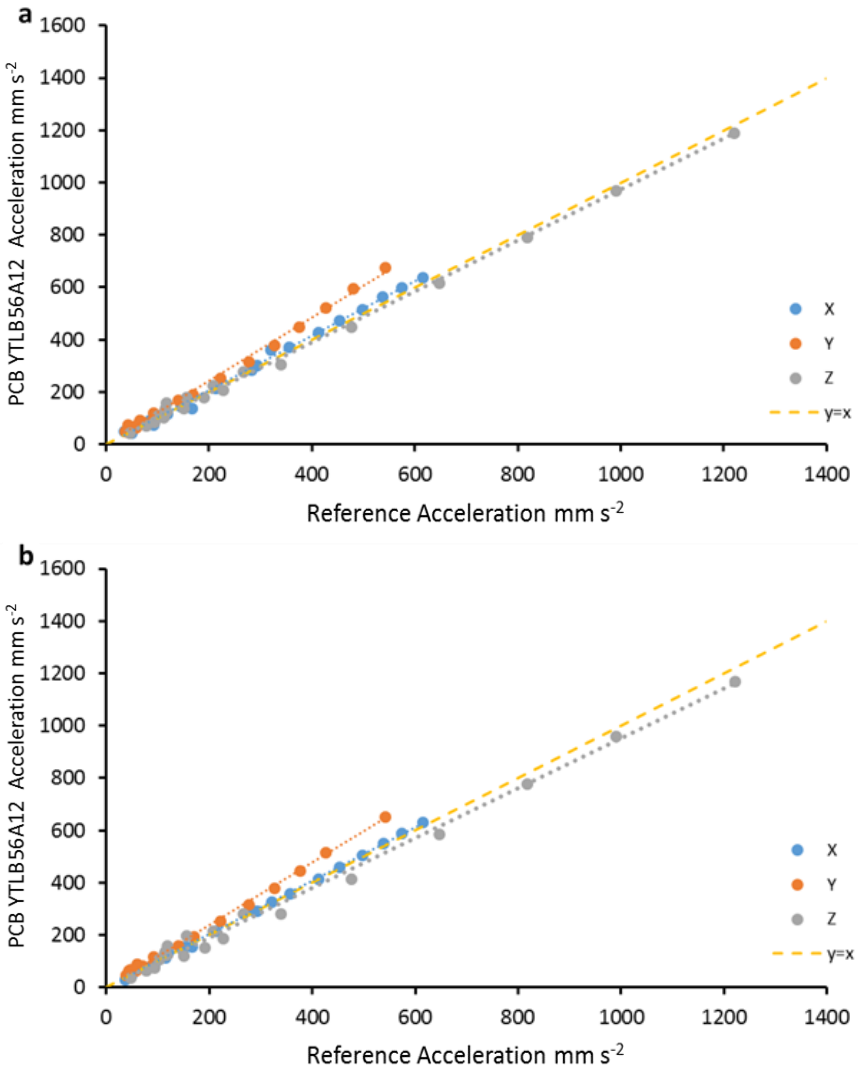

Figure 3.6| Calibration of PCB Piezotronics YTLBB356A12. Sensor calibration of (a) sediment borne vibration sensor and (b) water borne vibration sensor. Acceleration measured in mm s^{-2} for both the developed sensors and the reference accelerometers.

The STMicroelectronics LIS344ALH had distinctly poorer accuracy and precision. On the sediment borne vibration sensor both the X and Z axis calibrated well against the reference accelerometers (R^2 sediment borne vibration sensor X= 0.999, Z= 0.991; water borne vibration sensor X= 0.995, Z=0.989, Figure 3.7a, Table 3.2) showing high precision despite the poor accuracy. The precision of the Y axis was however dramatically lower than that of the X and Z axis (R^2 sediment borne vibration sensor Y = 0.663; water borne vibration sensor XY= 0.617, Figure 3.7b, Table 3.2). For both sensors however, an accurate calibration could nonetheless be made.

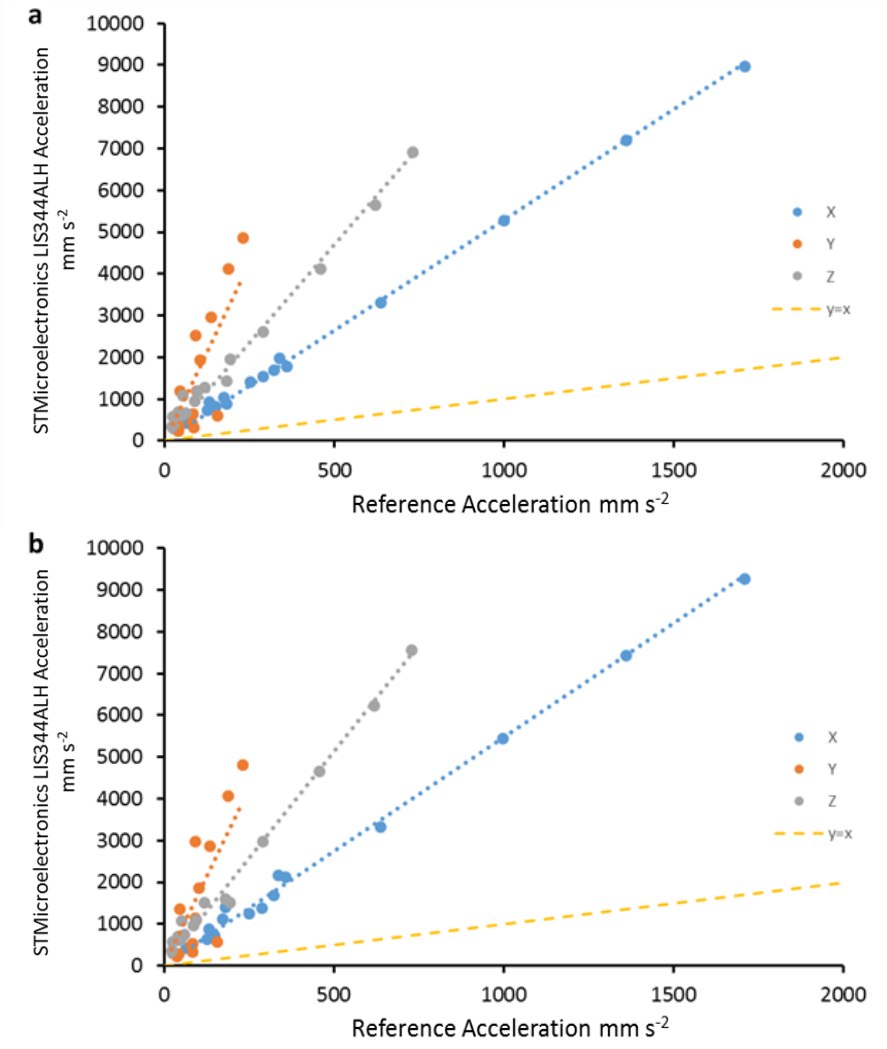

Figure 3.7| Calibration of STMicroelectronics LIS344ALH. Sensor calibration of (a) sediment borne vibration sensor and (b) water borne vibration sensor. Acceleration measured in mm s^{-2} for both the developed sensors and the reference accelerometers.

3.4 – Discussion

Here three accelerometers models representing a range of price points were chosen to be adapted into aquatic particle motion sensors. It was shown that two (the PCB Piezotronics YTLB356A12 and the STMicroelectronics LIS344ALH, compared in Tables 3.2 and 3.3) of these accelerometers, representing the high and low price points respectively (see Table 3.3), could be suitably converted for aquatic deployment in bioacoustic studies. The third chosen accelerometer (the Measurement Specialities 832M1), representing the middle price point was unsuitable for this task, due to its low sensitivity, which when amplified to detect small particle motion movements caused the internal 50 Hz noise of the power supply to be equivalently amplified, masking all other signals. It should, however, be noted that other models of this type of accelerometer, with different sensitivities, may perform better, and could be potentially also be used for the production of particle motion sensors.

Table 3.3 Comparison of accelerometers for building particle motion sensors, the orange box denotes catagories where the sensors can be calibrated to overcome any shortcomings.

Accelerometer	Weight g	Sensitivity mV/(m/s^2)	Spectral Noise (µm/s2)/√Hz @100Hz	Cost £
PCB Peizotronics YTLB356A12	5.4	10.2	49.0	1200
Meas Spec 832M1	3.0	5.1	624.0	125
STMicroelectronics LIS344ALH	0.04	6.6	490.0	3.50

Accelerometer	Precison	Accuracy	Noise Floor	Mobility	System Cost £
PCB Peizotronics YTLB356A12	High	High	Low	Low	≈ 7000
Meas Spec 832M1	-----	-----	-----	-----	-----
STMicroelectronics LIS344ALH	Poor	Poor	High	High	≈ 80 - 100

Of the two suitable accelerometers further tested here, the PCB Piezotronics YTLB356A12 was the costliest model (£1200). Aside from the higher price, this accelerometer presented an ease of use constraint. To allow effective data acquisition it must be coupled to a computer through costly and less readily available systems, such as the Brüel & Kjær Pulse (minimum of £9000), or National Instruments capture cards (~£5600). This increases the total cost of the deployable sensor assembly and adds to the complexity of at-sea measurements. Despite this, the YTLB356A12 accelerometer is far more precise than the lower price accelerometer tested, and as such would provide more reliable and comparable information. The STMicroelectronics LIS344ALH had poorer precision, especially in the Y axis. This is likely due to the potting of the sensor, with the Y axis having an increased distance from the edge of the housing, and the larger layer of epoxy resin between the housing edge and the accelerometer in this axis. This difference however can be calibrated for in future readings, allowing the production of accurate measurements of particle motion levels in studies.

Despite the lower accuracy of the LIS344ALH compared to the YTLB356A12, the considerably lower cost (£3.50) of this accelerometer makes it far more accessible. Additionally, this low cost sensor can easily be attached to a hand held recorder and the signals analysed in readily accessible computer software. Due to the more than 300-fold difference in price between these two sensors, at a trade-off of only 5 times increased accuracy (something that can be calibrated for), the LIS344ALH is likely to be the most suitable for the majority of researchers in bioacoustics, particularly when multiple spatial measurements are required. The mobility of a system using the LIS344ALH and a hand held recorder additionally makes it far more suited for field studies or situations where mains electricity is inaccessible. An important drawback of the cheaper LIS344ALH compared to the more expensive YTLB356A12 is the increased noise floor of the former. According to their data sheets, the noise floor of the LIS344ALH at 100 Hz is about 40 dB greater than for the YTLB356A12. This restricts its ability to measure low signal levels making it most suitable for situations in which high noise levels are of interest as is often the case in sound exposure studies on fish and invertebrates.

Following the construction details presented here (see section 3.2, and figures 3.1-3.4), both the high- and low-cost sensors can be replicated easily by anyone working in the

field of aquatic noise. It should be noted that for any sensor created following these sets there will be a cost associated with the calibration of the sensor. There are a number of ways this can be achieved, either as done here with a shaker table and reference accelerometers, or through calibration devices such as those available through Brüel & Kjær that emit a constant frequency for sensor calibration. Any researcher wishing to recreate these sensors should however be aware of this additional cost.

The particle motion sensors created here will allow inclusion of this important noise metric in investigations of the effects of noise on marine invertebrates and fishes, as well as in studies quantifying environmental noise levels. These readings will enable more accurate presentations of sound fields in playback experiments, the accurate quantification of the source levels of noise exposures (see chapter 2.4.1, p39), and the ability to compare noise exposures across studies. The sediment borne vibration sensors will permit more representative readings of the vibration received by benthic animals. For these reasons, the developed sensors could help fill the current knowledge gap regarding the lack of particle motion measurements in noise literature, enabling significant advancement in this field of research.

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Chapter 4

From DNA to Ecological Performance: Effects of Anthropogenic Noise on a Reef-Building Mussel

Chapter 4

From DNA to Ecological Performance: Effects of Anthropogenic Noise on a Reef-Building Mussel

The following chapter is broken into five sub-chapters expanding upon the work included in the manuscript "Wale, M.A., Briers, R.A., Bryson, D., Hartl, M.G.J., Diele, K., From DNA to Ecological Performance: Effects of Man-Made Noise on a Reef-Building Mussel. *In Prep.*" This manuscript is a condensed version of this chapter. Here, additional information on preliminary work, method development, and the use of *Mytilus edulis* as a study species is added, as well as genetic experiments not considered in the above manuscripts. The results of all experiments are then collectively discussed to allow a more complete understanding of the way noise affects *M. edulis*.

Sub-chapter 4.1

Effects of Man-Made Noise on the Blue Mussel *Mytilus edulis* – General Introduction

Sections of the following sub-chapter are included in the manuscript "Wale, M.A., Briers, R.A., Bryson, D., Hartl, M.G.J., Diele, K., From DNA to Ecological Performance: Effects of Man-Made Noise on a Reef-Building Mussel. *In Prep*." The manuscript contains a condensed version of the work outlined in this sub-chapter.

K.D. and M.A.W. conceived the research; M.A.W. designed the experiments with input from all authors; M.A.W. analysed the acoustics; M.A.W. wrote the manuscript and K.D., M.G.J.H., and R.B. contributed revisions.

4.1.1 – Introduction

Mytilus edulis is widespread throughout cold and temperate waters in the North East Atlantic from the White Sea to southern France, the Western Atlantic from the Canadian Maritimes along the western coast of the U.S.A (Tyler-Walters, 2008), and the coast of Iceland (Varvio *et al.*, 1988). *M. edulis* also occurs along both the east and west coasts of South America, and the Falkland Islands (Seed, 1992). Primarily an intertidal species, the upper limit of the distribution of *M. edulis* is limited by the specific temperature and desiccation experienced at any one site, with mass mortalities occurring during prolonged periods of low rainfall (Seed and Suchanek, 1992). The lower distribution limit is controlled mainly by predation from subtidal predators, such as *Asterias rubens*, *Nucella lapillus*, and *Carcinus* sp. (Kitching and Ebling, 1967; Seed, 1969). The lack of predation experienced on sea mounts, dock pilings, and renewable energy devices often results in increased growth of subtidal populations (Seed and Suchanek, 1992).

Individual *M. edulis* can reach upwards of 20 years of age (Theisen, 1972), however longevity is dependent predominately on predation, which causes lower shore individuals to survive no more than 2-3 years (Seed, 1969). Spawning occurs in spring and summer

depending on environmental conditions (Newell *et al.*, 1982), with larvae developing planktonically over a 1 month period (Strathmann, 1987) after which settlement occurs. Settlement can occur as either a two stage process, with the pediveliger settling on filamentous substrates and drifting to more suitable hard substrates, or in a single stage process where they settle directly on established mussel beds (King *et al.*, 1990). The latter single stage settlement is however less desirable for the post larvae due to the risk of ingestion by adults (Tyler-Walters, 2008), something that would not occur in the two stage settlement. *M. edulis* is highly gregarious, and the final settlement often occurs around established beds of adult mussels, where competition for resources is high. Unless adults are lost from the established beds this competition can lead to a density depended reduction in the growth of juveniles, or to outward expansion of the mussel beds as animals on the fringes have lower competition (Seed and Suchanek, 1992). Stable *M. edulis* populations can survive with relatively little recruitment, allowing mussel beds to remain persistent through long time periods (McGrorty *et al.*, 1990).

M. edulis has been a commercially important species for centuries (Howard *et al.*, 1987; McKay and Fowler, 1997). In Scotland 7,270 tonnes of *M. edulis* was produced for consumption in 2015, accounting for £8.8million, 87% by value of all Scottish shellfish production (Marine Scotland Science, 2016). As the global reliance on marine invertebrate fisheries and aquaculture rises (Pauly *et al.*, 2002, 1998), their commercial importance, including that of *M. edulis,* will rise alongside this demand. *M. edulis* is not only commercially important, but also ecologically, and has a two-fold role. Firstly, as a biogenic reef builder, these animals produce essential habitat for other organisms (Borthagaray and Carranza, 2007; Wootton *et al.*, 2003) with large stable mussel beds developing over many years of repeated recruitment (McGrorty *et al.*, 1990). Secondly, as a filter feeder, *M. edulis* removes large quantities of particulates from the water surrounding mussel beds, consequently improving the water quality for other organisms in the system (Officer *et al.*, 1982). In addition to these main ecosystem functions, *M. edulis* is a common prey item for many echinoderm (Dare, 1982; Wong and Barbeau 2005), crustacean (Elner 1978; Wong and Barbeau 2005), and bird species (Bustnes and Erikstad, 1990; Zwarts and Drent, 1981), forming an important link in many marine food webs.

As a sessile benthic invertebrate, *M. edulis* is ideally suited for the study of anthropogenic noise effects. Not only does it experience noise transmitted through the surrounding waters, but due to its coupling with the substrate it is also sensitive to sediment vibration (Roberts *et al.*, 2015). Due to their sessile nature, *M. edulis* is unable to move away from unwanted sources of noise, a common behaviour in fish, mammals, and mobile invertebrates (see chapter 5, p158). This behaviour is often mentioned as a mitigation strategy for marine mammals and fish (Halvorsen *et al.*, 2011; Lloyd *et al.*, 2011), lowering or dismissing the potentially negative effects of noise exposure. Any potential negatives said noise has on the mussels can therefore be compounded, as they will experience noise from fixed sources for longer periods of time than animals which choose to move away.

Previous work conducted on noise exposed bivalve molluscs has highlighted a number of negative effects. Both larvae and mature bivalves can be affected by anthropogenic noise. De Soto *et al*. (2013) exposed larval New Zealand scallop *Pecten novaezelandiae* to seismic air-gun noise (161 to 165 dB RMS re 1 mPa^2s) through their development. Noise exposed larvae displayed significantly higher levels of mortality and malformation (abnormal growth and bulges in the soft body) than control animals. Vazzana *et al*. (2016) identified a number of biochemical changes in the Mediterranean mussel *Mytilus galoprovencialis* when exposed to noise playbacks. Noise exposed mussels showed changes in glucose, total protein, total haemocyte number and osmolarity of the haemolymph, and increased acetylcholinesterase activity in the gills and mantle. Additionally, heat shock protein 70 was investigated at the protein level using non-quantitative techniques, showing increased concentrations in the gills and mantle of noise exposed specimens. The only previous study to explore the effects of noise on *M. edulis*, Roberts *et al*. (2015), exposed adult animals to a sequence of pure tones presented as sediment vibration. The authors measured valve closure as a behavioural response, and showed that *M. edulis* can perceive noise in their environment.

The aim of the work presented in the following sub-chapters (4.2 - 4.4) was to establish whether anthropogenic noise playbacks affect *M. edulis*. Taking an integrative approach to answering this question (see chapter 1, p6) a range of laboratory experiments were conducted to explore potential noise effects. Responses were chosen to represent biochemical (DNA integrity and oxidative stress), physiological (oxygen consumption),

behavioural (valve movement and algal filtration), and genetic (heat shock protein expression) processes, and to generate a more complete picture of how noise affects the biology of these animals. The individual responses tested, and their associated hypotheses, are explained further in their respective sub-chapters: biochemistry – sub-chapter 4.2, physiology and behaviour – sub-chapter 4.3, and genetics – sub-chapter 4.4.

4.1.2 – Methods

The methods of animal husbandry, noise recordings and noise playbacks discussed here are used across all experiments in sub-chapters 4.2, 4.3, and 4.4. These methods are therefore presented here to avoid repetition across these sub-chapters.

4.1.2.1 - Permits and Ethical Approval

The work conducted required no specific permits but was conducted following the ethical guidelines of Edinburgh Napier University.

4.1.2.2 - Animals and Husbandry

Individual *M. edulis* were manually collected at low tide two weeks prior to noise exposure (12 October 2015, 9 November 2015, 1 March 2016, 10 October 2016, and 27 October 2016) from Fisherrow Sands, Musselburgh, UK (55.94° N, 3.07° W) (Figure 4.1.1). This area has historically been rich in *M. edulis* beds (Howard *et al.*, 1987), today estimated (through manual analysis of aerial photographs) as covering 1 km^2, which occur along the coast at the mouth of the River Esk. To lower any potential variability in response across size and maturity of the mussels, individuals were taken from mature sections of the mussel bed where average mussel size exceeded 49 mm, and any individual mussel collected exceeded 47 mm. A minimum distance of 200 mm was left between each collected mussel to reduce the impact on the mussel bed community. The Firth of Forth water body in which Fisherrow sands resides regularly experiences anthropogenic noise caused through sporadic watercraft and the regular traffic of larger ships further afield. The mussel beds themselves

are intertidal, and as such regularly receive high levels of natural noise from waves and tidal movements.

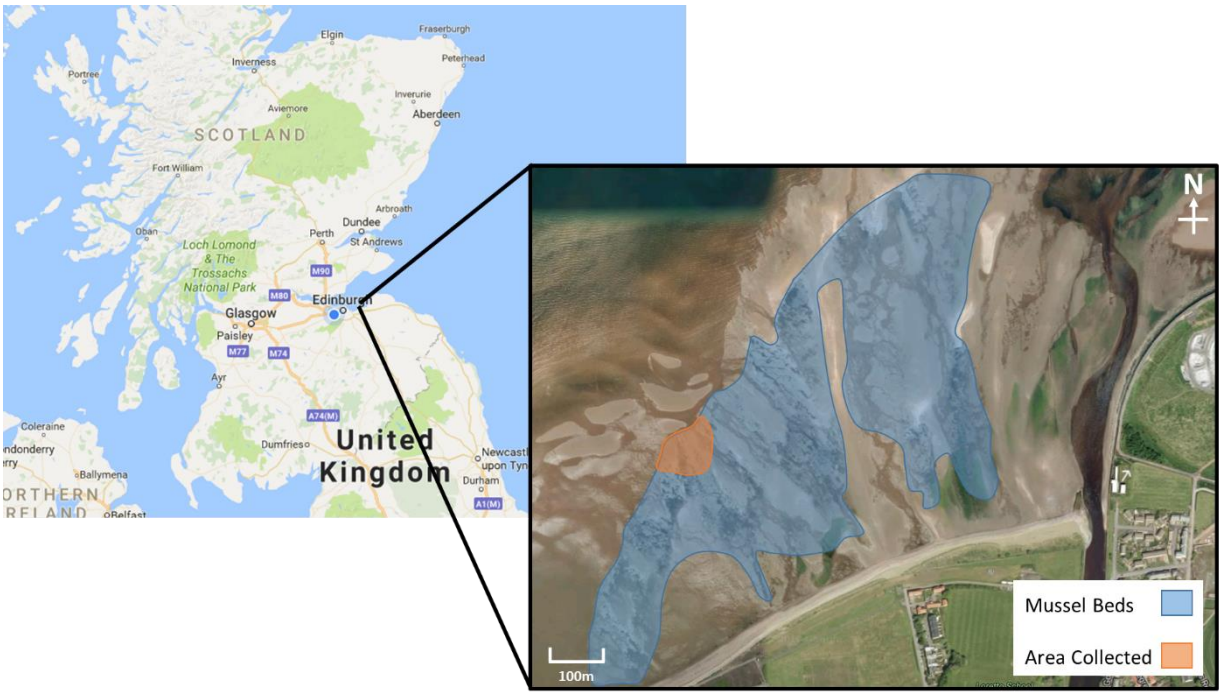

Figure 4.1.1| Musselburgh mussel beds. Location of mussel beds at Fisherrow Sands, Musselburgh, UK. The area where experimental mussels were collected is highlighted in orange. Adapted from Google Maps, 2016.

Following collection, the mussels were transported to St Abbs Marine Station (St Abbs, Berwickshire, UK) for biochemical and genetic experiments (sub-chapter 4.2 and 4.4) or to the AquaLab at Edinburgh Napier University for behavioural and physiological experiments (sub-chapter 4.3). Once on site the animals were cleaned of all epibiota, predominately consisting of *Elminius modestus* and *Semibalanus balanoides* barnacles, by carefully scrapping the edge of an oyster knife along the shell of the mussel.

At St Abbs Marine Station the mussels were subsequently housed in small groups of 40 animals suspended in mesh bags inside a 675 L holding tank with flow-through natural seawater. The mesh bags were suspended into the tanks on twine, with neoprene matting between the twine and the suspension point to prevent transferred vibration. Both the holding tank and the experimental tanks were fitted with a subsurface inflow pipe to prevent noise from falling water or collision with the tank floor, minimizing the ambient sound

levels (see below for sound levels), and isolated from the surrounding surfaces with neoprene rubber to prevent vibration transmission. The flow rate of this system varied over the course of acclimation depending on tides, due to the raw seawater draw design of the Marine Station. Salinity and temperature in the tanks matched the surrounding coastal waters and varied depending on flow rate (10 - 14 °C, salinity 32 - 35 ppt; NO_2: < 0.3 mg L^{-1}; NO_3^-: 0 mg L^{-1}; NH_3^+: ≤ 0.25 mg L^{-1}; pH: 7.8 - 7.9).

At Edinburgh Napier University, the mussels were housed in 120 L flow-through tanks within a closed recirculation system of natural seawater (12 - 13 °C, salinity 32 - 35 ppt; NO_2: < 0.3 mg L^{-1}; NO_3^-: 0 mg L^{-1}; NH_3^+: ≤ 0.25 mg L^{-1}; pH: 7.8 - 7.9). Due to the nature of the recirculation system there was constant water flow in the holding tanks. The holding tanks were kept in an insulated temperature controlled room, with soundproofing to remove noise from other laboratories. The holding tanks were separated from the surrounding surfaces by anti-vibrational matting (such as in the experimental tanks shown in Figures 4.3.1, 4.3.3, and 4.3.6) to prevent the transfer of vibration from any surrounding activity, and covered by acrylic lids to minimize noise from outside the tanks. All animals were acclimated in holding tanks for two weeks prior to the experiments, and allowed an additional 24 h acclimation to the experimental set-up prior to any noise exposure. All possible effort was made to keep the sound levels in the holding tanks as low as possible and matching those of the control treatments (Figure 4.1.2).

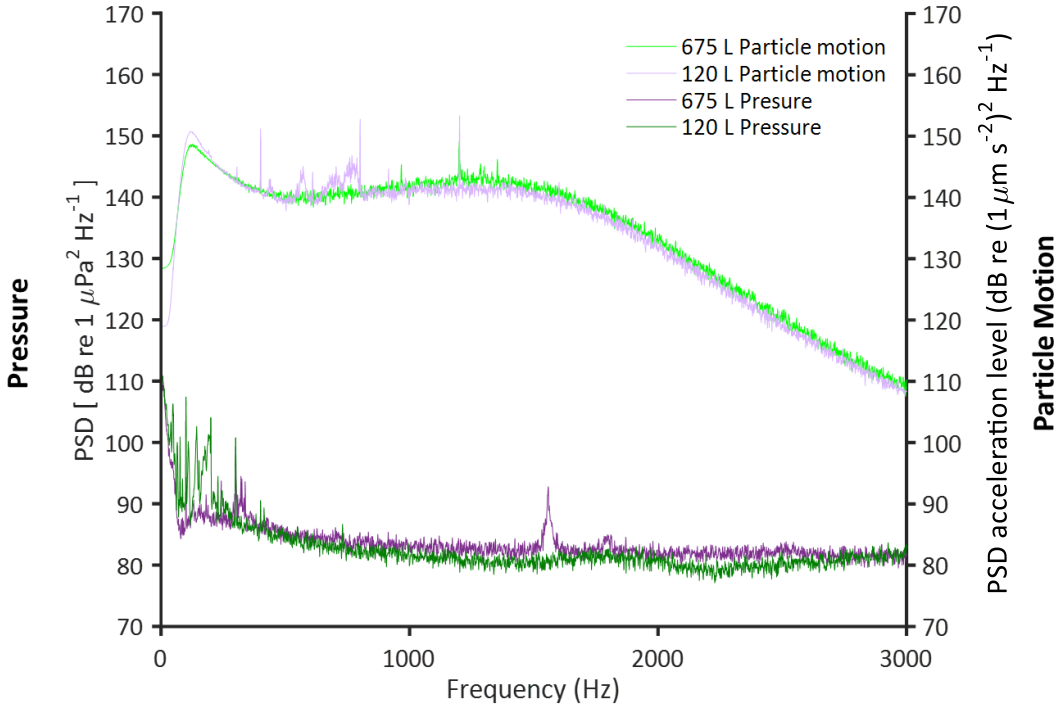

Figure 4.1.2| Acoustic analysis of holding tanks. Mean power spectral density (PSD) of 30 s of each sound condition of acoustic pressure and particle acceleration, for holding conditions in both the 675 L (DNA integrity and oxidative stress) and 120 L (Oxygen consumption, algal filtration, and valve movement) tanks. Analysis performed in MATLAB R2015b (pressure) and MATLAB Compiler Runtime R2013a (particle acceleration). fft lengths = 48 kHz (pressure) and 44.1 kHz (particle acceleration), both resulting in 1 Hz bands.

4.1.2.3 - Sound Recordings and Playback

Ship noise playbacks produced by Wale *et al*. (2013a, 2013b) were used in all experiments. Recordings of large vessels were collected from three major U.K. ports (Gravesend, Plymouth, and Portsmouth). In each location, recordings were made of a single passing ship at approximately 200 m distance (Gravesend: Rio de la Plata – IMO 9357951, a 286 m long, 73,899 tonne container ship; Plymouth: Bro Distributor – IMO 9313113, a 147 m long, 11,344 tonne LPG tanker; Portsmouth: Commodore Goodwill - 9117985, a 126 m long, 11,116 tonne ferry). Ships were travelling at constant speeds (<10 knots), as enforced by port authorities for vessels entering and leaving estuarine areas. All recordings were made with a calibrated omnidirectional hydrophone (HiTech HTI-96-MIN with inbuilt preamplifier, High Tech Inc., Gulfport, MS, U.S.A.) and an Edirol R09- HR 24-Bit

recorder (44.1 kHz sampling rate, Roland Systems Group, Bellingham, WA, U.S.A.). The recording level was calibrated for the R09-HR using pure sine wave signals produced by a function generator, measured in line with an oscilloscope. Intensity (root mean square, RMS) and power spectral density (units normalized to 1 Hz, Hann evaluation, 50% overlap, FFT size 1024; averaged from 2 min of recording) were calculated for each recording in Avisoft SASLab Pro v4.5.2 (Avisoft Bioacoustics, Berlin, Germany). This analysis evidenced some variation in average sound levels between 126 and 136 dB RMS re 1 µPa. Sound samples of 60-140 s, incorporating the highest amplitude of the ship passes where amplitude did not vary by more than one quarter in magnitude, were used to create noise tracks in Audacity 1.3.13 (http://audacity.sourceforge.net/).

Experimental tracks were compiled in Audacity 2.0.5 and included a 30 s fade in, 6.5 min of ship noise and a 30 s fade out for each of the recorded vessels. A random selection of these tracks was compiled to create a six h playback track of continuous ship passes (Figure 4.1.4). Experimental tracks were played back as WAV files. The set-up consisted of an Mp3 player (SanDisk sansa clip+ 8GB, frequency range 10-20,000 Hz, Western Digital Technologies Inc., Irvine, CA, U.S.A); amplifier (Pioneer A-10-K, 50W, frequency response: 20-20,000 Hz, Pioneer Corporation, Tokyo, Japan); and Clark Synthesis AQ339 underwater speaker (effective frequency range 20-17,000 Hz, Clark Synthesis Inc., Littleton, CO, U.S.A). To ensure consistency between sound levels of replicate tanks in each treatment, tracks were re-recorded in the centre of the experimental tank (HiTech HTI-94-SSQ with inbuilt preamplifier, Roland R-26 24-bit recorder – calibrated with the methods above and PAMGuide; Merchant *et al.*, 2015) and modified (uniform amplification or attenuation) to give the desired exposure pressures. Particle acceleration was measured using a custom-built calibrated sensor (Wale *et al.*, in prep; Chapter 3), consisting of a STMicroelectronics LIS344ALH triaxial accelerometer (STMicroelectronics, Geneva, Switzerland) potted in clear epoxy resin and suspended via 1.0mm diameter elastic cord to two interlocking 3D printed nylon rings (see Chapter 3, Figure 3.4, p58). The accelerometer was linked to a Roland R-26 24-bit recorder for recording, and chosen due to its mobility allowing consistency across all experiments. Particle acceleration was recorded separately for all three axes and combined internally within paPAM during the analysis process.

Received sound pressure levels at the position of the exposed mussels in the 670 L tank (DNA integrity and oxidative stress) peaked at 150-155 dB re 1 μPa^2Hz^{-1} for ship noise playbacks and 85-95 dB re 1 μPa^2Hz^{-1} for control conditions (Figure 4.1.3a), as measured in PAMGuide (Merchant *et al.*, 2015). Particle acceleration peaked at 160-165 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for ship noise playbacks, and 140-148 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for control conditions (Figure 4.1.3b) as measured in paPAM (Nedelec *et al.*, 2016). For the 120 L tank (algal filtration, oxygen consumption, and valve movement) the noise peaked at 140-145 dB re 1 μPa^2Hz^{-1} for ship noise and 85-100 dB re 1 μPa^2Hz^{-1} for ambient tank noise (Figure 4.1.3a). Particle acceleration peaked at 165-175 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for ship noise playbacks, and 150-155 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for control conditions (Figure 4.1.3b). Exact sound levels will have differed throughout the tanks. This, however, did not affect the interpretation of results since the aim of this study was to determine the potential impact of additional anthropogenic noise in the environment, rather than establishing the precise links between given sound levels and responses.

For all experiments a control was created using a track of silence presented in the same way as the ship noise tracks. These silent controls consisted of six h of silence generated in Audacity 2.0.5, and played through the sound equipment to control for potential electromagnetic effects, and any internal noise generated by the equipment. Acute (<12 h, see chapter 2.1.1, p21) noise exposure was presented. Acute exposure was presented in all experiments and chosen so the initial reaction to noise, and any negatives associated with it, could be established. The aim of this work was not to uncover any habituation or tolerance that may have occurred during a chronic or repeated noise exposure.

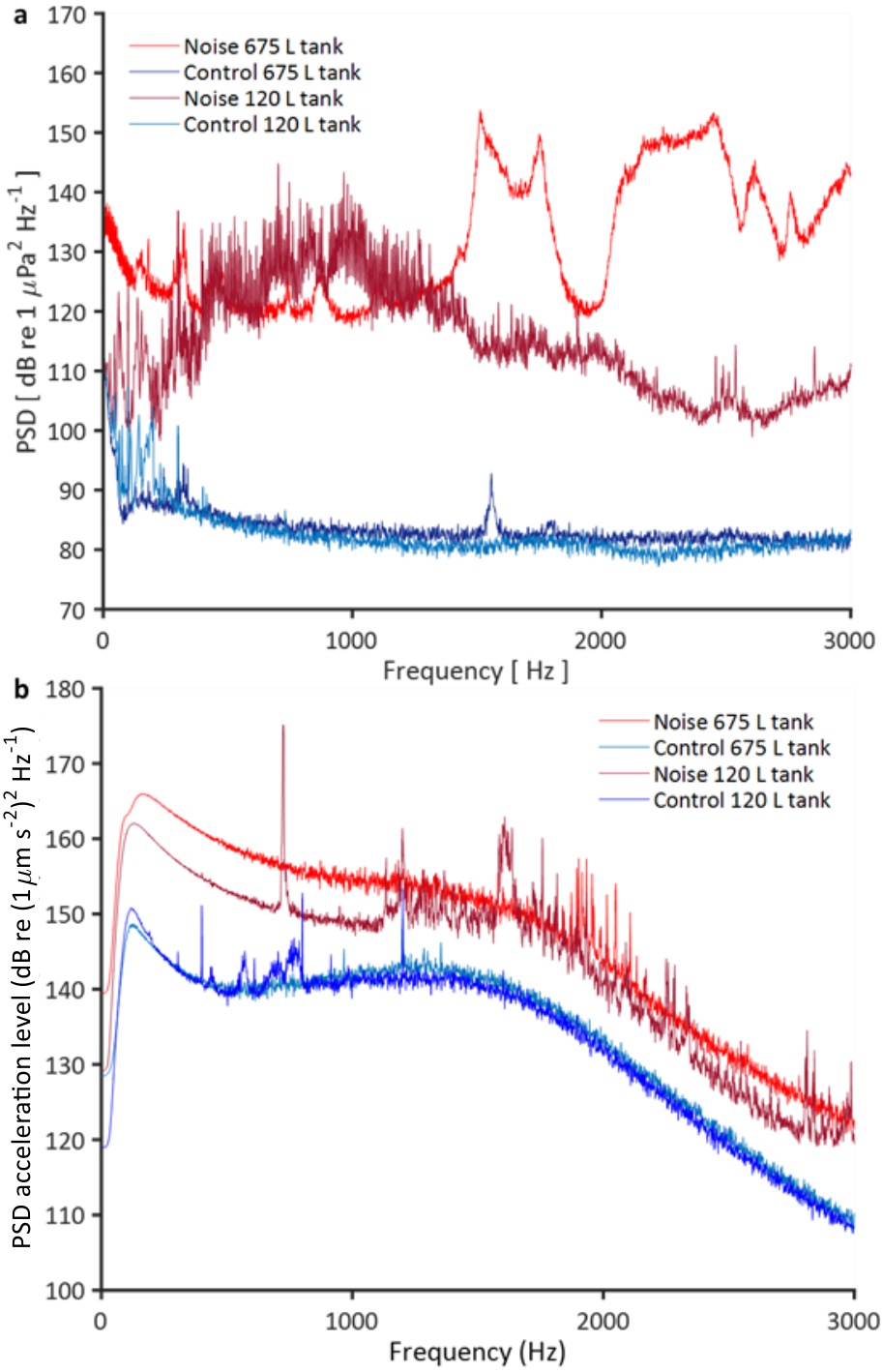

Figure 4.1.3| Analysis of acoustic stimuli and sound playback conditions. Mean power spectral density of 30 s of each sound condition of (a) acoustic pressure and (b) particle acceleration, for control and exposure conditions in both the 675 l (DNA integrity and oxidative stress) and 120 l (Oxygen consumption, algal filtration, and valve movement) tanks. In both the pressure and particle motion domains there was a clear difference between the noise exposure and the control conditions. Analysis performed in MATLAB R2015b (pressure) and MATLAB Compiler Runtime R2013a (particle acceleration). fft lengths = 48 kHz (pressure) and 44.1 kHz (particle acceleration), both resulting in 1 Hz bands. Noise levels may differ from those in the field.

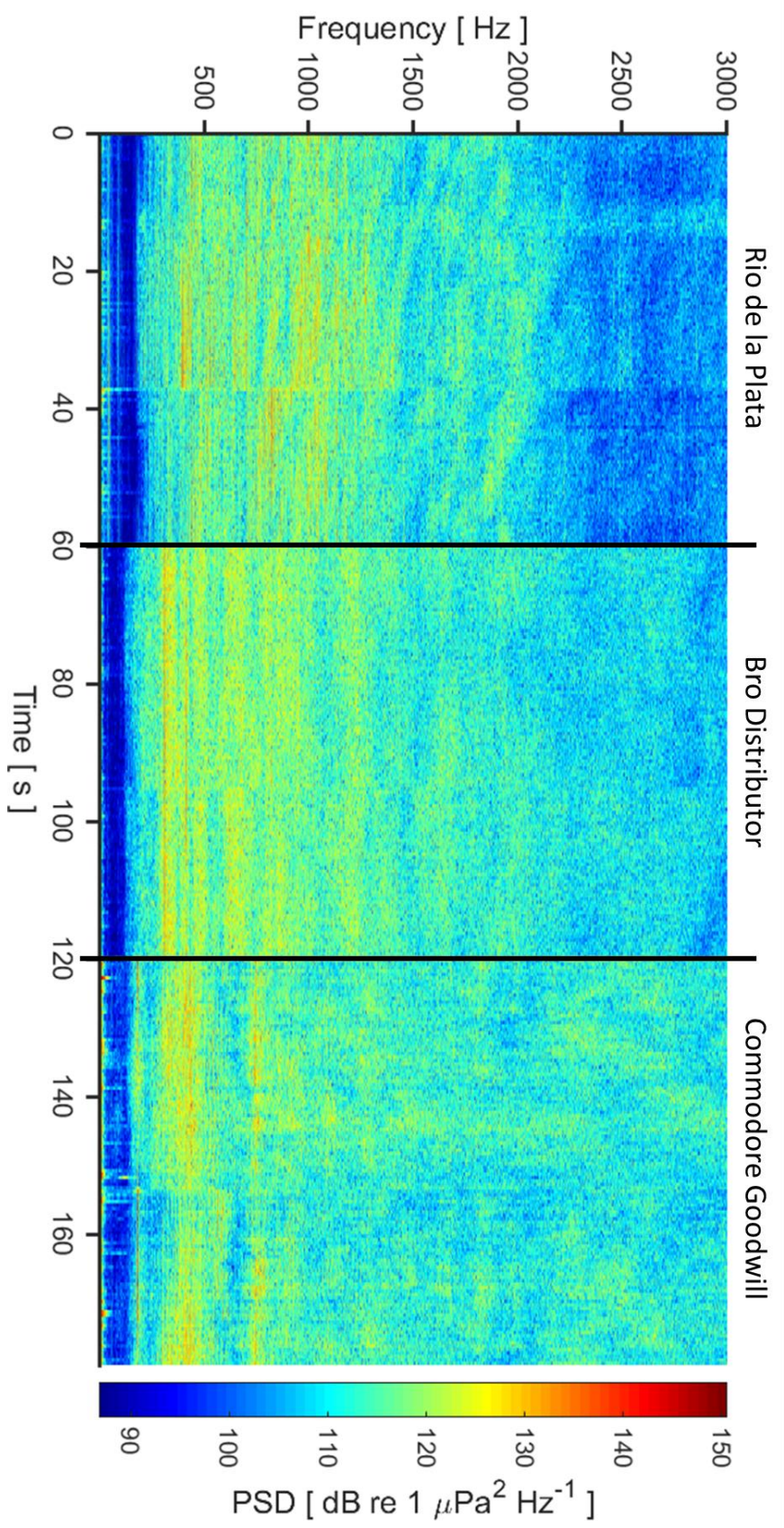

Figure 4.1.4| Spectral analysis of acoustic stimuli. Power spectral density of 1 min of each ship passed used in the generation of the playback tracks for all exposures in both the 675 L and 120 L tanks. Analysis performed in MATLAB R2015b, Hann window with 1 s length and 50 % overlap, fft length = 48 kHz resulting in 1 Hz bands.

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Sub-Chapter 4.2

Noise Induced Biochemical Stress Responses in *Mytilus edulis*

The following sub-chapter forms part of the manuscript "Wale, M.A., Briers, R.A., Bryson, D., Hartl, M.G.J., Diele, K., From DNA to Ecological Performance: Effects of Man-Made Noise on a Reef-Building Mussel. *In Prep.*" The manuscript contains a condensed version of the work presented in this sub-chapter.

K.D. and M.A.W. conceived the research; M.A.W. designed the experiments with input from all other co-authors; M.A.W. conducted the biochemical assays with instruction from M.G.J.H.; M.A.W. analysed the acoustics and performed the statistical analysis with input from R.A.B. and K.D.; M.A.W. wrote the manuscript and K.D., M.G.J.H., and R.B. contributed revisions.

4.2.1 – Introduction

As a genus, *Mytilus*, and consequently *Mytilus edulis*, is frequently used as a model to study biochemical responses to marine pollutants (Wootton and Pipe, 2003) and adaptations to different environmental conditions (Philipp *et al*., 2012). To date only a small number of studies have included biochemical analysis in the study of anthropogenic noise effects on marine invertebrates (see chapter 2.3.1, Fig. 2.5, p30), and none for *M. edulis*. However, when working with animals that show little visually obvious responses to stressors, such as bivalves and other sessile molluscs, there is a need to assess more cryptic biochemical and genetic responses to ascertain stressor impacts. Identifying biochemical or genetic changes can also reveal the underlying drivers of any physiological or behavioural responses that may be observed in the study species.

Recently, biochemical responses to noise were studied by Vazzana *et al*., (2016) in a related species to *M. edulis*, the Mediterranean mussel *Mytilus galloprovincialis*. When

exposed to a range of tonal sweeps, mussels expressed higher glucose, total protein, total haemocyte number and osmolarity of the haemolymph, as well as increased acetylcholinesterase activity in the gills and mantle. The identification of biochemical changes in response to noise within this genus highlights the potential for other species, such as *M. edulis*, to react to noise exposure. In the here presented study, responses in DNA integrity and the concentration oxidative stress endpoints were investigated to determine whether and how underwater noise affects *M. edulis* on a biochemical level. For the first time in noise research, established ecotoxicological techniques were employed, namely the Comet Assay and oxidative stress tests.

Single cell gel electrophoresis, or Comet Assay, is a technique to study DNA damage in individual cells, expressed as single strand breaks, without prior knowledge of the karyotype and cell turnover rate (Hartl *et al.*, 2010). In the Comet Assay the target cells' membranes are stripped away and the DNA unwound. Exposing these lysed cells to electrophoresis separates the damaged cells causing the relaxed and broken fragments of the negatively charged DNA to move away from the central body and form a "tail". The size of this tail in proportion to the body represents the degree of DNA damage in the cell. A build up to single strand breaks within cells can, if unrepaired, lead to mutation and further degradation and atrophy in tissues and organs (as reviewed in Kurelec, 1993 and De Flora *et al.*, 1991). This in turn can result in an impairment of the organism's fitness through inhibited growth and reduced "scope for growth".

Oxidative stress is a term used to describe the build-up of reactive oxygen species (ROS), brought about by an organism's inability to regulate and detoxify these free radicals and their harmful effects. A build-up of ROS can in turn lead to DNA damage and cell necrosis by promoting microsatellite instability, inhibiting methylation and accelerating telomere shortening (reviewed in Evans and Cooke, 2004). Direct measurements of ROS levels are difficult due to their short half-lives (Pryor, 1991). As such, indirect measurements through the quantification of the end products of oxidative stress are more common. Four of these end products are here measured, superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), total glutathione (GSH), and glutathione peroxidase (GPx). SOD and GPx are enzymes that regulate ROS, with SOD converting the superoxide radical to oxygen (Hayyan *et al.*, 2016), and GPx converting lipid

hydroperoxides to alcohols and hydrogen peroxide to water (Bhabak and Mugesh, 2010). The TBARS assay measures the presence of malondialdehyde (MDA) formed as primary and secondary ROS, generated in lipid peroxidation as fatty acids decompose (Marnett, 1999). GSH is an antioxidant that reacts with ROS to reduce or prevent cellular damage (Pompella *et al.*, 2003). The levels at which these end products are present can then be used to determine the presence or absence of oxidative stress.

The aim of these analyses was to provide information on the biochemical responses of *M. edulis* to anthropogenic noise playbacks. The identified responses in combination with the physiological, behavioural, and genetic responses investigated in sub-chapters 4.3 and 4.4 create a more complete picture of how noise affects these animals.

4.2.1.1 - Hypotheses

H_1: Acute exposure to ship noise playbacks will cause increased single strand breaks in the DNA of *M. edulis* haemolymph and gill epithelia when compared to those from a silent control.

H_2: Acute exposure to ship noise playbacks will produce evidence of an oxidative stress response in *M. edulis* gill epithelia when compared to those from a silent control.

4.2.2 – Methods

Methods of animal collection and husbandry, along with the sound exposure conditions are covered in sub-chapter 4.1. A total of four exposures were performed over four months from August to November 2015. The first of these trials acted as a proof of concept test of the methods, the second exposure was used to further perfect the chosen methods. Neither of these trials (August and September) are included in the analysis, as during protocol refinement human error in the Comat assay process caused increased damage of all samples, including the controls. Two final exposures (October 26th and November 23rd 2015) were performed to provide an increased sample size and show replicability of the findings. These exposures were free of any known error and their results are analysed and discussed below.

4.2.2.1 - DNA Integrity

Following acclimation (see sub-chapter 4.1.2.2, p74), the mussels were suspended on a tray (30 x 15 cm) midwater in a 675 L natural seawater tank in the same system as used for the holding tanks (Figure 4.2.1). Exposing the mussels midwater allowed more accurate control over the sound exposure conditions, and eliminated potential bias and variability created from varying substrate vibration. The supporting tray consisted of a plastic base with holes cut into it to hold the mussels in place throughout the acclimation period, and the exposure proper. The tray was suspended with nylon twine into the centre of the exposure tank in line with the subsurface speaker. The twine was attached to the walls of this tank via metal hooks that were insulated from vibration originating in the walls of the tank using neoprene rubber (Figure 4.2.1). Two groups of 12 mussels were selected, one for noise exposure and one for the control treatment. Each group was further split into two replicate groups of six animals to give two exposures per treatment (Figure 4.2.2). A size deviation of no more than 5 mm between the largest and smallest animal across all treatments (size range and mean [mm], October: N1, 48.2 – 54.4, 51.5; N2, 48.0 – 52.6, 50.3; C1, 49.6 – 55.1, 52.4; C2, 49.2 – 50.5, 49.7; November: N1, 41.4 – 52.7, 48.3; N2, 47.7 – 53.7, 49.1, C1, 47.1 – 51.2, 48.8, C2, 48.3 – 53.6, 50.3). The mussels were given 24 h to acclimate to the exposure tank followed by exposure to either ship noise playback or a silent control (see sub-chapter 4.1.2.3, p77) for six h.

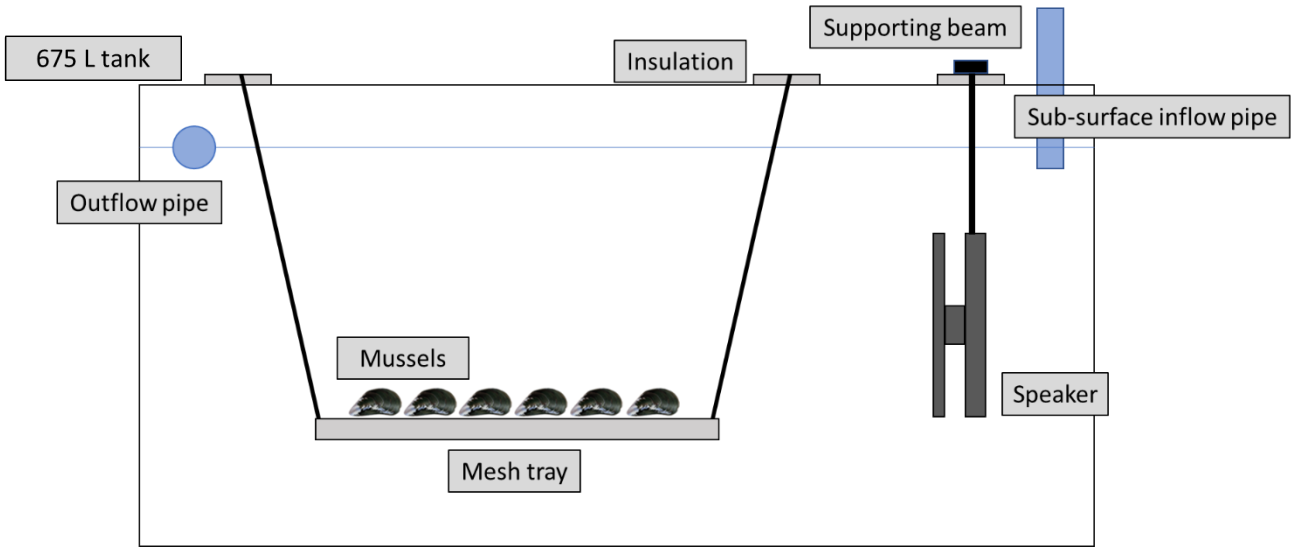

Figure 4.2.1| Exposure tank. Layout of the exposure tanks for all experiments. The layout was used in all four tanks, with two tanks used for noise exposure and two tanks for control animals. Tank dimensions: 1040 x 1000 x 650 mm, speaker placed 200 mm above tank floor and 150 mm from the tank wall, tray positioned 200 mm above the tank floor and 500 mm from the speaker. Mussels were distributed in two rows in the centre of the tray, a minimum of 550 mm and maximum of 580 mm from the speaker.

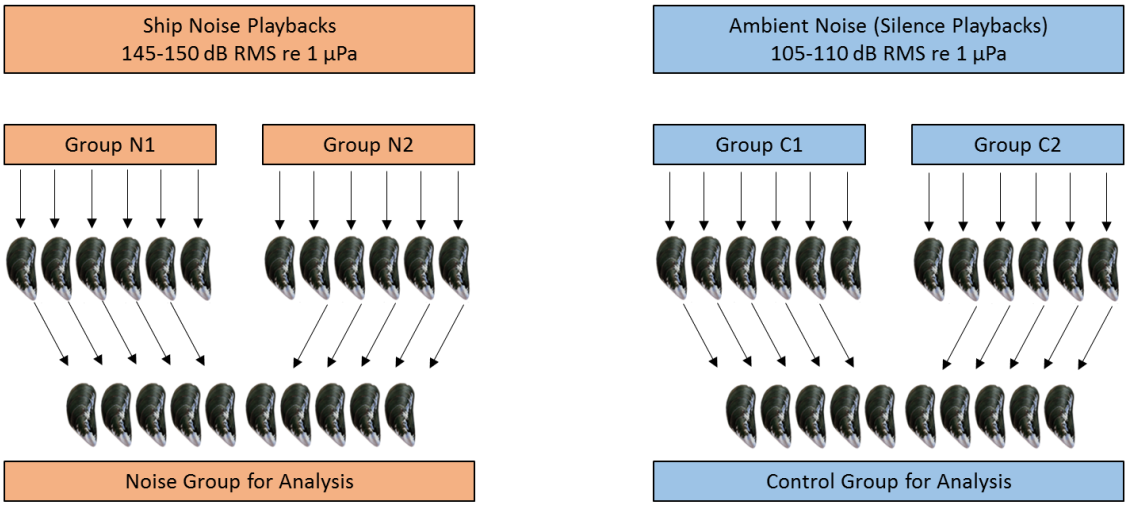

Figure 4.2.2| Experimental set-up. Mussels exposed as two replicates of six animals. Five animals from each replicate were combined into the final group for analysis.

Following exposure, haemocytes and gills cells (Figure 4.2.3) were isolated as per Hartl *et al*. (2010). Scissors were inserted into the ventral side of the mussel around the area where byssus threads protruded from the shell, wedging the shell open and exposing the posterior adductor muscle (Figure 4.2.3). Any sea water within the valves was subsequently emptied, and a haemolymph sample was drawn from the exposed posterior adductor muscle by inserting a 21-gauge needle on a 1 ml syringe. The needle was removed and the haemolymph transferred into an Eppendorf tube with an equal volume of Ca^{2+}–Mg^{2+} free Hanks balanced salt solution (HBSS), previously adjusted to 1000 mOsmol L^{-1} (22 g L^{-1} NaCl) to match the osmolality of invertebrate haemolymph (Coughlan *et al*., 2002). After haemolymph collection, the posterior and anterior adductor muscles were cut with a mounted razor and the shell halves opened. The gill tissue was cut away from the mantle along its ventral length with fine scissors, carefully removed with tweezers, and placed in an Eppendorf tube of adjusted HBSS making sure that the whole gill was submerged. Both gill and haemolymph samples were stored at 4 °C until further use. Specimens were dissected in pairs consisting of one randomly selected mussel from the noise treatment, and one randomly selected mussel from the control treatment, until all specimens were dissected. Sample storage was consistent across individuals and treatments. Any specimen that proved particularly difficult to dissect was noted and discarded when the five mussels were chosen for comet assay analysis.

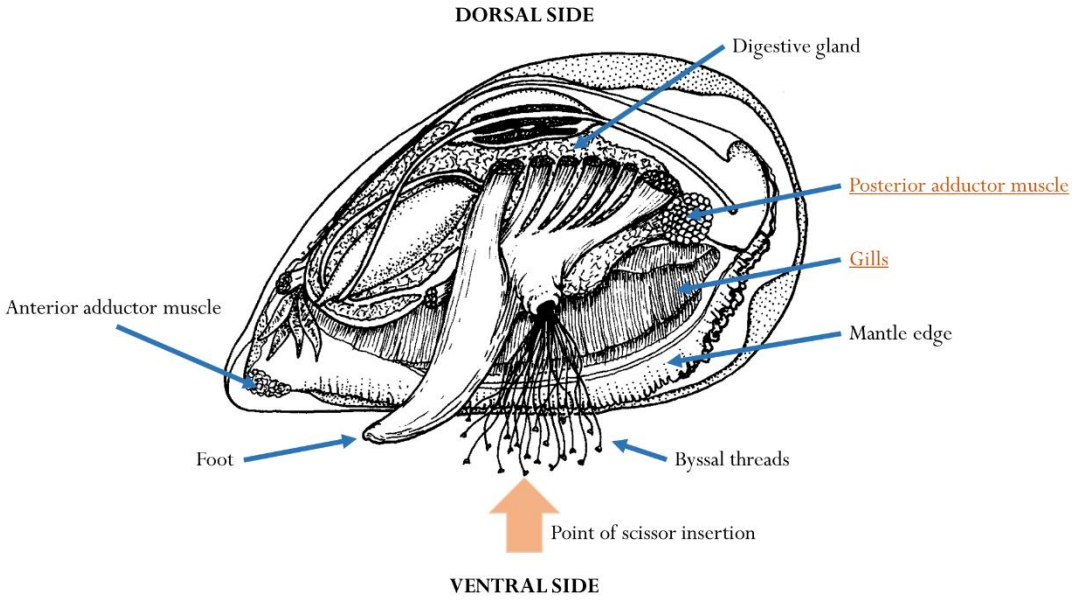

Figure 4.2.3| *M. edulis* morphology. Morphology of *Mytilus edulis* adapted after Livingstone (1999), highlighting the areas of dissection and sample collection.

To remove any potential bias, all samples were given six-digit code prior to laboratory work, these codes were not revealed to those conducting the assays until all results were generated. Gill epithelial cell suspensions were created following the procedure of Coughlan *et al*. (2002). Tissue samples were chopped 10 times with two scalpel blades in 0.2 ml of HBSS and transferred into a 15 ml centrifuge tube containing 2.5 ml HBSS and 0.3 ml trypsin (Final concentration 0.05%). The centrifuge tubes underwent 10 min of gentle rocking at room temperature on a platform rocker, followed by the addition of a further 10 ml of HBSS. The suspension was then passed through a 40 µm sieve to remove any remaining tissue fragments, and centrifuged for 5 min at 800 x *g*. The supernatant was discarded and the pellet carefully resuspended in 0.5 ml of fresh HBSS.

Five animals from each replicate tank were chosen for analysis, giving 10 animals for each treatment. Out of the six originally collected specimens, priority for removal was given to those that proved difficult to dissect (never more than a single animal fit this criteria per replicate). The decision to reduce these numbers was due to equipment constraints as the electrophoresis tank could only hold 40 slides at a time (10 slides for each cell type in each treatment). Comet Assay analysis was performed on the samples within 24 h of collection, following the procedure of Coughlan *et al*. (2002) modified by Al-Shaeri *et al*. (2013). 100 µl of 1% low-melting agarose (LMA) in PBS was added to pre-heated frosted microscope slides (150 µl normal-melting agarose in PBS), coved with a cover slip and solidified at 4 ^{o}C. Once solidified the cover slip was removed and a mixture of 70 µl LMA and 30 µl gill suspension or haemolymph was gently and quickly added on top of the first LMA layer. A cover slip was once more applied, and the slides left to solidify as before. A final layer of 100 µl LMA was added and solidified to protect the cell layer, and form an agarose "sandwich" with the cells held in the central layer. Once fully set the cover slips were removed and the slides lysed in a high salt buffer (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100, and 10% DMSO; pH 10.0) overnight (lysis completes after 1.5 h) at 4 ^{o}C, making sure no light reached the slides. This step removed the cell and nuclear membranes leaving the nuclear cage embedded in the agarose. Following lysis, the DNA was allowed to unwind in a horizontal electrophoresis tank (0.3 M NaOH, 1 mM EDTA; PH > 12) in the dark for 30 min at 4 ^{o}C, after which a 25 V 300 mA current was applied for 25 min to pull the fragmented DNA creating the comet shape. Once electrophoresis was complete, the slides were removed and neutralised with Tris buffer (0.4 M Tris-HCL; pH

7.4) three times at five minute intervals. The slides were then washed in distilled water and stained with GelRed (Biotum, catalog BT41003; solution 2 µl in 10 ml distilled water) three times at five minute intervals. A final washing was undertaken and cover slips placed over the slides. Slides were stored in the dark at 4 °C until use. Prepared slides were viewed under an epifluorescence microscope (Zeiss Axioplan, Carl Zeiss Microscopy, Oberkochen, Germany), using Comet Assay IV software (Perceptive Instruments, Bury Saint Edmunds, UK). DNA damage is expressed as % tail DNA (Olive *et al.*, 1990; Pavlica *et al.*, 2001) (Figure 4.2.4).

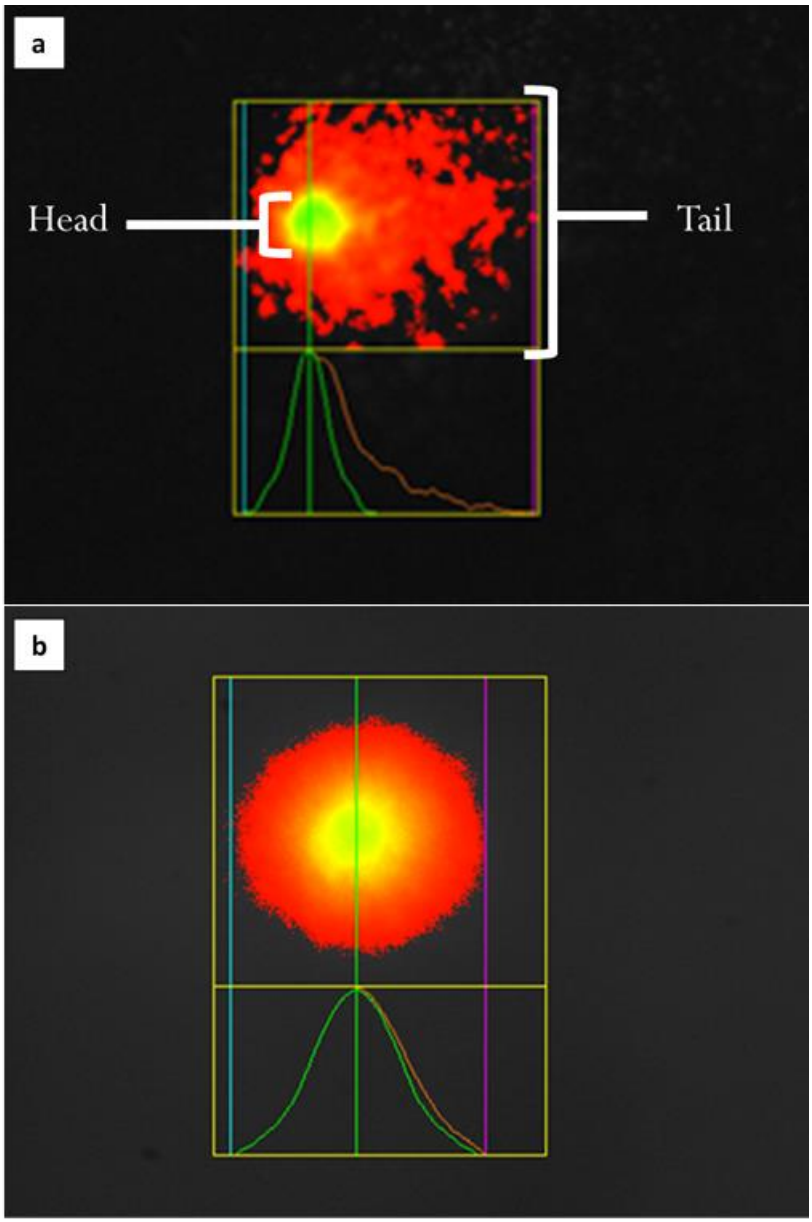

Figure 4.2.4|Comet assay output. Single cell after electrophoresis, viewed and measured in Comet Assay IV. The central mass of the cell (Head) and the fragmented DNA (Tail) are clearly visible. **(a)** a damaged gill cell with 43% tail DNA, **(b)** an undamaged gill cell with 6% tail DNA.

4.2.2.2 - Oxidative Stress

Gill samples for oxidative stress assays were collected at the same time and from the same animals as those for the Comet Assay and flash frozen in liquid nitrogen. Samples were stored at -80 °C until further analysis. In all assays the prepared microplates were read using a Spectramax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Due to sample restrictions the GSH and GPx assays were performed solely on gills collected during the November exposure.

Glutathione (GSH) and Thiobarbituric acid reactive substances (TBARS) assays were performed according to Smith *et al*. (2007). For TBARS, gill tissue samples were slowly defrosted on ice and homogenised in a glass homogeniser with 2.5 ml of ice-cold PBS (1 mmol L^{-1}EDTA; pH 7.4). 40 µl of homogenate was added to a 96-well microtiterplate in triplicate (Appendix B). To each well 10 µl BHT (1 mol L^{-1}butylated hydroxytoluene; 2,6-Di-O-tert-butyl-4-methylphenol), 140 µl PBS, 50 µl TCA (50% (w/v) trichloroacetic acid in distilled water), and 75 µl TBA (1.3% (w/v) thiobarbituric acid in 0.3% (w/v) NaOH) were added. A dilution series of 0.5 - 25 µMol TEP (0.5, 2.5, 5, 15, 25 nmol 1,1,3,3-tetraethoxypropane in ethanol; 1 mol=220.31 g L-1) was made, and added in triplicate to each 96 well plate (Appendix B) along with 10 µl BHT, 140 µl PBS, 50 µl TCA, and 75 µl TBA as above. Each plate was incubated at 60 °C for 60 min and cooled on ice. Absorbance (Abs) was recorded at 530nm and 630nm, with total absorbance calculated as:

$$Abs = A_{530nm} - A_{630nm} \quad \textbf{(I)}$$

Total protein content was determined following (Bradford, 1976). A dilution series of 1 – 0 mg ml^{-1} Bovine serum albumin (BSA) was prepared (1, 0.8, 0.6, 0.4, 0.2, 0 mg ml^{-1} dH_2O). 10 µl of each standard and each sample homogenate was pipetted into five separate wells of a 96 well microtiterplate (Appendix B). To each well 290 µl of Bradford reagent was added and the plates incubated at room temperature for 5 min. Absorbance was recorded at 595 nm. Total protein and TBARS of each sample was calculated against the standard curves, and final TBARS concentration calculated as:

$$TBARS\ \mu mol\ mg\ protein^{-1} = \frac{Abs}{Protein\ \mu mol\ mg} \quad \textbf{(II)}$$

GSH determination involved 20 µl of gill homogenate being added in triplicate to a 96 well plate (Appendix B) along with 20 µl of DTNB (10 mmol l^{-1}; 5,5-dithiobis-(2-nitrobenzoicacid)), 260 µl of assay buffer (100 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} EDTA; pH 7.5), and 20 µl of glutathione reductase (2 U ml^{-1}). The addition of 20 µl NADPH (3.63 mmol l^{-1}) started the reaction. After 5 min of incubation at room temperature absorbance at 4.12 nm was recorded and total GSH g^{-1} of wet tissue weight calculated against a standard curve (0, 5, 10, 15, 20 µM GSH in EDTA).

Glutathione Peroxidase (GPx) assays were conducted using the BioVision Glutathione Peroxidase Activity Colorimetric Assay Kit (Catalog #K762-100). Here 0.1 g of gill tissue was homogenised in 0.2 ml of ice-cold assay buffer and centrifuged at 10,000 x g for 15 min at 4 °C. 50 µl of supernatant was added in duplicate to a 96 well plate (Appendix B) containing 33 µl Assay Buffer, 3 µl NADPH (40 mM), 2 µl glutathione reductase, and 2 µl glutathione (lyophilized). A 0 to 100 nmol NADPH dilution series (25 µl 40 mM NADPH with 975 µl dH_2O, 100, 80, 60, 40, 20, 0 µl NADPH brought to 100 µl with Assay Buffer), a GPx positive control to test the absorbance of 100% GPx activity, and a reagent blank (ddH_2O) were added in the same way as above to the 96 well plate (Appendix B). The plate was incubated for 15 min at room temperature, followed by the addition of 10 µl Cumene Hydroperoxide solution to start the reaction. Absorbance at 340nm was measured immediately (A1) before 5 min of incubation in the dark at 25 °C. A second absorbance measurement was made after incubation (A2). NADPH concentration was then calculated against the NADPH standard curve and GPx activity in atomic mass unit (U) mg^{-1} was calculated using the following equations:

$$\Delta A_{340nm} = [(Sample_{A1} - Sample_{A2}) - (Blank_{A1} - Blank_{A2})] \quad \textbf{(III)}$$

$$GPx\ Activity\ U\ mg^{-1} = \frac{\Delta A_{340nm}}{Incubation\ Time\ min \cdot\ Sample\ Volume\ ml} \quad \textbf{(IV)}$$

For superoxide dismutase (SOD) assays the Sigma-Aldrich SOD determination Kit (19160) was used. Here, 20 µl of sample homogenate (created as per TBARS assay) was added in triplicate to a 96 well plate (Appendix B). To each well 200 µl of WST working

solution, and 20 µl of enzyme working solution were added. Three blank solutions were prepared in triplicate, Blank 1 replaced the sample solution with ddH_2O, Blank 2 replaced the enzyme working solution with dilution buffer, and Blank 3 replaced the sample solution with ddH_2O and the enzyme working solution with dilution buffer. The plate was incubated at 37 °C for 20 min followed by absorbance readings at 450 nm. SOD inhibition was measured using the following equation:

$$SOD\ inhibition\ \% = \frac{[(Blank\ 1 - Blank\ 3) - (Sample - Blank\ 2)]}{(Blank\ 1 - Blank\ 3)} \cdot 100 \quad \textbf{(V)}$$

The complete protocol for both the GPx and SOD kits can be found in the appendices (Appendix C).

4.2.2.3 - Statistical analysis

All statistics were performed in R version 3.3.1 (The R foundation for Statistical Computing). Data were tested for normality and heterogeneity of variance around the mean, normality was shown for all data without transformation. Therefore, appropriate parametric tests were used throughout. Two sample t-tests were used to compare the DNA damage between treatment tanks testing the % tail DNA (dependent variable) against treatment (independent variable). Two-way ANOVAs were employed to test the effects of both the run (independent variable) and treatment (independent variable) on the % tail DNA (dependent variable) for both gills and haemolymph. Additionally, two-way ANOVAs were performed to test the effects of run (independent variable) and treatment (independent variable) on both superoxide dismutase inhibition (dependent variable) and the presence of thiobarbituric acid reactive substances (dependent variable). Finally, two sample t-tests were used to test the effect of treatment (independent variable) on both glutathione concentrations (dependent variable) and glutathione peroxidase activity (dependent variable).

4.2.3 – Results

4.2.3.1 - DNA Integrity

No significant difference in % tail DNA was observed between the duplicate experimental tanks for either noise exposed (two sample t-test October: gill $t_{6.27} = 1.72$ $P = 0.13$, haemolymph $t_{4.64} = 0.67$ $P = 0.54$, November: gill $t_{7.27==52} = 1.98$ $P = 0.085$, haemolymph $t_{8.00} = 1.82$ $P = 0.11$) or control animals (two sample t-test October: gill $t_{7.27} = 1.95$ $P = 0.091$, haemolymph $t_{7.68} = 2.43$ $P = 0.10$, November: gill $t_{7.96} = 0.30$ $P = 0.77$, haemolymph $t_{6.52} = 1.58$ $P = 0.16$) allowing replicates to be combined for analysis. There was a significant difference between the amount of DNA damage observed in noise exposed animals between exposure dates (two-way ANOVA $F_{1,35} = 23.24$ $P < 0.001$ (Gill), $F_{1,35} = 60.80$ $P = 0.022$ (Haemo)). Animals exposed in October showed significantly more DNA damage in both the gills and haemolymph than those tested in November. This difference prevented the two trials from being combined for statistical analysis. DNA damage of the control animals did not differ significantly between the two months ($t_{17.36} = 0.009$ $P = 0.99$ (Gill), $t_{17.71} = 0.79$ $P = 0.42$ (Haemo)). However, to allow consistency and clarity in analysis, the October and November trials are presented separately (Fig. 4.2.5). In both the October and November exposures, noise-exposed mussels demonstrated significantly higher single strand breaks in the DNA of both haemocytes and gill epithelial cells than those of control animals (two-way ANOVA $F_{1,35} = 573.40$ $P < 0.001$ (Gill), $F_{1,35} = 346.82$ $P < 0.001$ (Haemo)). Approximately 25 - 33% tail DNA occurred in noise exposed cells, six times higher than control cells with only 5% damage.

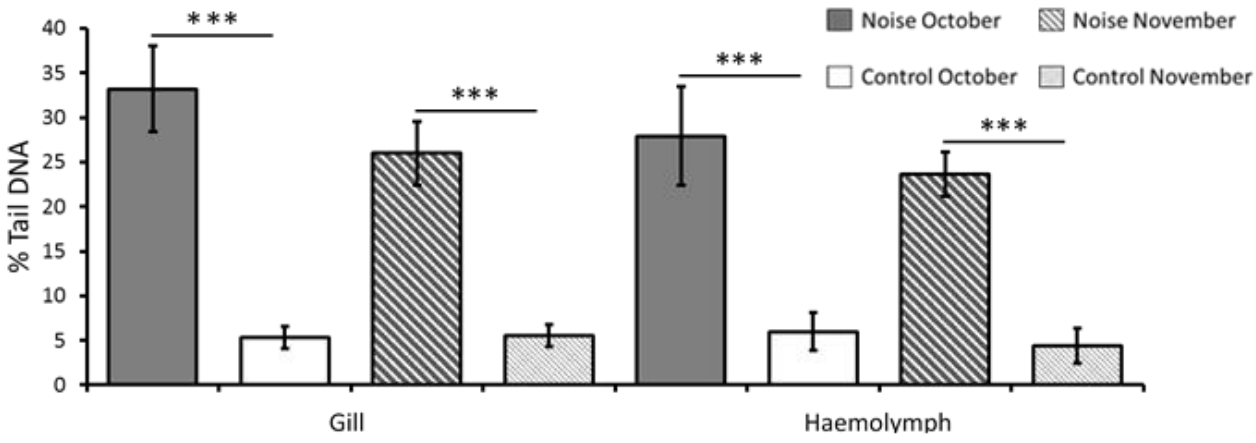

Figure 4.2.5| Noise induced DNA damage in *M. edulis*. Mean ± Stdev percentage tail DNA of gill and haemolymph*** (n = 9 for noise October, n = 10 for all other treatments and times).

4.2.3.2 - Oxidative Stress

The October and November exposures did not statistically differ for the SOD and TBARS assays (two-way ANOVA $F_{1,42} = 0.78$ $P = 0.38$ (SOD), $F_{1,37} = 0.25$ $P = 0.62$ (TBARS)). and were therefore combined for analysis. The SOD, GPx, and GSH assays did not identify significant oxidative stress (two-way ANOVA $F_{1,42} = 0.062$ $P = 0.80$ (SOD), Figure 4.2.6a, two sample t-test $t_{20.425} = 0.74$ $P = 0.47$ (GSH), Figure 4.2.6b, $t_{17.256} = 0.79$ $P = 0.44$ (GPx), Figure 4.2.6c). TBARS assays however revealed a significant 39% increase in malondialdehyde (two-way ANOVA $F_{51,37} = 4.93$ $P = 0.013$, Figure 4.2.6d), indicating lipid peroxidation in the gill epithelia of noise exposed specimens.

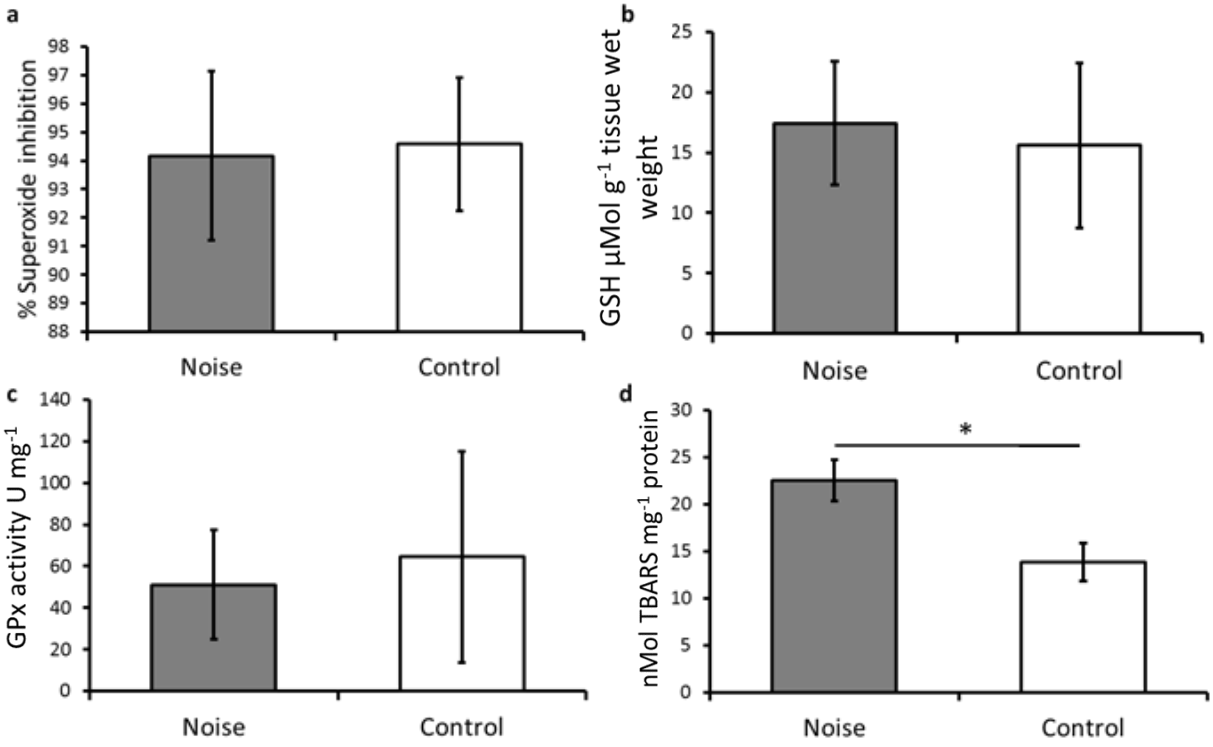

Figure 4.2.6| Noise induced oxidative stress in *M. edulis*. (a) Mean ± Stdev % SOD inhibition in gills (n = 21 for both treatments). (b) Mean ± Stdev GSH µMol g^{-1} tissue wet weight (n = 12 for both treatments). (c) Mean ± Stdev GPx activity U mg^{-1} (n = 12 control, n = 9 noise). (d) Mean ± Stdev nMol TBARS mg^{-1} protein in gills* (n = 21 for both treatments).

4.2.4 – Discussion

This study is the first that investigated DNA damage in response to noise in any marine species. It is also, to the best of my knowledge, the first to use oxidative stress endpoints as biomarkers of the effects of underwater noise in marine organisms. The results do not allow the rejection of the initial hypotheses, and indicate both elevated DNA damage and oxidative stress in response to acute exposure to ship noise playbacks. The cellular damage observed here may have occurred as a direct result of exposure to high intensity low frequency noise. Solé *et al*. (2013a, 2013b) showed that direct exposure to high intensity low frequency noise (SPL up to 175 dB re 1µPa) can cause severe damage to the structure of cephalopod statocysts, the hair cells of which were damaged to the point of extrusion from the statocysts body. Here however direct physical damage is unlikely due to the comparatively low exposure level (150-155 dB re 1 μPa^2Hz^{-1}). Instead, malondialdehyde (MDA), the end product of lipid peroxidation and the substance measured by the TBARS assays, has likely driven the DNA damage found in the gill tissue. MDA could then have flown from the gills into the haemolymph, due to the mussels open circulatory system (Yonge, 1976) and caused the observed damage in haemocytes. Links between oxidative stress and DNA damage are well known (Alves de Almeida *et al.*, 2007), and as a biochemical marker for stress the build-up of reactive oxygen species is the most likely cause of the observed DNA damage (Barzilai and Yamamoto, 2004).

No change was detected in SOD, GSH, or GPx activity. Superoxide (O_2^-), catalysed by SOD, is produced as a by-product of oxygen metabolism. Increased SOD activity would have indicated a build-up of superoxide and the associated oxidative stress. This response is understandable when considering the reduced oxygen consumption of noise exposed mussels (see sub-chapter 4.3.3.1, p120) and the corresponding reduction in oxygen metabolism. GSH concentrations and GPx activity are linked. GPx reduces lipid hydroperoxides and hydrogen peroxide by catalysing their reaction with GSH. It would therefore be expected that if there was no change in GSH, then there would be no change in GPx activity either (Bhabak and Mugesh, 2010). Within a cell there is a balance between SOD and GSH/GPx (Dunning *et al.*, 2013). Here, with noise having no effect on one of these substances it makes sense that the others are also unaffected.

Both DNA single strand breaks and oxidative stress are common responses of *M. edulis* to other anthropogenic pollutants including chemicals and heavy metals. DNA damage, if unrepaired, can lead to mutation and further degradation of the cell (Kurelec, 1993). This in turn causes increased energy expenditure in cell repair (Sancar and Sancar, 1988) and if this damage persists in multiple cells it can drastically effect the animal's fitness (De Flora *et al.*, 1991). Oxidative stress, if unregulated, will lead directly to DNA damage, mutation, and carcinogenesis (Cooke *et al.*, 2003), with high levels of ROS leading to cell necrosis (Evans and Cooke, 2004). Single strand breaks have been found in the DNA of *M. edulis* in response to styrene (Mamaca *et al.*, 2005), aromatic hydrocarbons (Large *et al.*, 2002), silver sulphide and cadmium sulphide (Munari *et al.*, 2014), while oxidative stress has been documented in response to gold nanoparticles (Tedesco *et al.*, 2008), copper, cadmium, and zinc (Géret *et al.*, 2002), and glass nanoparticles (Koehler *et al.*, 2008). It is therefore no surprise that noise, another anthropogenic pollutant, has similar effects. The effects of noise in fact appear greater than those of some chemical and heavy metal pollutants, with noise causing ≈ 30 % tail DNA, while only ≈ 19 % tail DNA was observed with styrene exposure (Mamaca *et al.*, 2005)), and ≈ 12 % with cadmium sulphide (Munari *et al.*, 2014). This difference highlights the large effect noise can have on sessile marine invertebrates, which have largely been unstudied in this regard.

As shown here, the application of established ecotoxicological techniques, such as the Comet Assay and oxidative stress assays, can greatly benefit the field of noise research. When investigating species that produce little visually obvious responses to anthropogenic noise, these assays can be used to investigate cryptic effects of noise, and enable a better understanding of how noise exposure affects the animal. In turn, this type of analysis will allow clarification as to whether an organism apparently unaffected by noise is truly unaffected or simply producing no visual indication of an effect. Given the negative effects of noise on *M. edulis* described in this study, it would be prudent for noise to be screened for, eliminated, or considered as a potentially confounding factor in any laboratory trials aiming to determine the effects of other stressors, such as chemical pollutants. Varying ambient noise levels between exposures may act to mask, or amplify the apparent effects of the target pollutant. Likewise, field monitoring programs for pollutants, e.g. the NOAA Mussel Watch Program (Kimbrough *et al.*, 2008), should regard noise as a potential (co)contaminant.

Uncertainties and Future Directions

Uncertainties of the experimental methods, and suggestions for improvements in future work are discussed in sub-chapter 4.5.

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Sub-chapter 4.3

Noise Induced Behavioural and Physiological Stress Responses in *Mytilus edulis*

The following sub-chapter forms part of the manuscript "Wale, M.A., Briers, R.A., Bryson, D., Hartl, M.G.J., Diele, K., From DNA to Ecological Performance: Effects of Man-Made Noise on a Reef-Building Mussel. *In Prep.* The manuscript contains a condensed version of the work in this sub-chapter.

K.D. and M.A.W. conceived the research; K.D. and M.A.W. designed the experiments with input from all co-authors; M.A.W. conducted the biochemical assays with M.G.J.H. and the behavioural and physiological assays with D.B.; M.A.W. analysed the acoustics and performed the statistical analysis with input from R.A.B. and K.D.; M.A.W. wrote the manuscript and K.D., M.G.J.H., and R.B. contributed revisions.

4.3.1 – Introduction

To create a rounded analysis of how noise is affecting the blue mussel *Mytilus edulis*, a series of experiments investigating the physiological and behavioural responses of these animals to ship noise playbacks were performed in addition to the biochemical responses presented in sub-chapter 4.2. Three metrics were chosen to cover common physiological/behavioural stress responses. Oxygen consumption was used as a representation of physiological stress, valve movement represented behavioural stress, and algal filtration was selected as an ecological response that bridged both physiological and behavioural processes.

Respiration rate/oxygen consumption is a metric commonly used to measure the response of an organism to environmental stressors such as temperature (Watson *et al.*, 2014; Srijaya *et al*, 2014), changes in salinity (Ern *et al.*, 2015; Yu *et al.*, 2013), and the addition of chemical (Haque and Kwon, 2016) and heavy metal (Capparelli *et al.*, 2016)

pollutants. Measuring variability in oxygen consumption provides useful insight into both individual and community level effects as a measure of metabolic demand, and how an animal adapts its physiology to environmental changes can provide insight into its resilience and fitness (Bridges *et al.*, 1980; Sommer and Pörtner, 2004). Changes in metabolic rate, through respiration, have been previously used in noise research. Regnault and Lagardere (1983) investigated the effects of ambient aquarium noise on the brown shrimp *Crangon crangon*. Here the authors decreased the noise of the aquarium rather than adding additional noise into the system. Animals in reduced noise conditions showed decreased oxygen consumption when compared with shrimp in ambient conditions, which along with a reduced ammonia excretion rate effectively showed a reduction in metabolic activity in lower noise conditions. These results can be interpreted as showing increased stress in conditions with higher noise levels. Wale *et al.* (2013) investigated the effect of acute and repeated ship noise playbacks on the shore crab *Carcinus maenas*. The authors identified an increased oxygen consumption in response to anthropogenic noise, and this response continued throughout repeated exposure. Metabolic changes in *M. edulis* have also been recorded during exposure to other aquatic pollutants. Manley (1983) showed a 58% reduction in oxygen consumption when *M. edulis* were exposed to copper, where the mussels would close their valves in response to the pollutant therefore lowering the oxygen uptake.

To feed, *M. edulis* filters biotic material such as bacteria, phytoplankton, and dissolved organic matter from the water column (Tyler, 2017). Algal filtration rate focuses on the phytoplankton component of *M. edulis* feeding by measuring the quantity of algal cells removed from the surrounding water over a set period of time. It is used not only as a measurement of individual food uptake (Thompson and Bayne, 1972), but also as a metric for the ecological performance of filter feeding animals (Garrido *et al.*, 2012). On the individual level, an extended period of reduced algal uptake will negatively affect the mussels' growth and survival (Clausen and Riisgsrd, 1996), and ultimately its fitness (Bayne and Widdows, 1978). Ecologically, a reduction in filtration rate can have deleterious knock on effects. *M. edulis* filter particulates from the surrounding water depositing them on the seafloor in the form of faeces and pseudofaeces (Garrido *et al.*, 2012). This process effectively transports energy from the water column into the benthic community (van

Broekhoven *et al.*, 2015), any reduction to which could shift the overall dynamics within the biogenic reef.

To allow oxygen consumption and algal filtration to occur successfully, the mussel's valves must open to reveal the gills and syphons held within. Changes in valve movements are therefore linked to changes in both oxygen consumption and algal filtration rate, and are one of the only visually detectable stress responses in *M. edulis*. They are a common response to anthropogenic pollutants in the environment, with valve gape and opening time reducing in response to environmental copper pollution (Curtis *et al.*, 2000), and valves completely closing in response to chemical stressors in the environment (Excis® sea lice treatment) (Gowland *et al.*, 2002). Previous studies of the responses of *M. edulis* to sediment vibration have identified valve movement as a behavioural representation of noise detection. Roberts *et al.* (2015) presented pure tones in a step up sequence, and recorded varying valve movement responses across frequencies. Both full and partial closure of the valves were repeatedly shown in response to sediment vibration. Here, the authors identified not only the ability of *M. edulis* to perceive this noise stimulus, but that valve movement is an appropriate metric to measure a noise induced behavioural change in these animals.

The aim of the experiments performed in this sub-chapter was to provide information on the physiological and behavioural responses of *M. edulis* to anthropogenic noise playbacks, and to identify any connections between such responses.

4.3.1.1 - Hypotheses

H_1: Acute exposure to ship noise playbacks will cause increased oxygen consumption in *M. edulis* when compared to those from a silent control.

H_2: Acute exposure to ship noise playbacks will cause a reduction in the algal filtration of *M. edulis* when compared to those from a silent control

H_3: *M. edulis* will display a reduced valve gape (partially closed valves) in response to acute exposure to ship noise playbacks.

H_4: *M. edulis* will display a reduced valve opening time in response to acute exposure to ship noise playbacks.

4.3.2 – Methods

Methods of animal collection and husbandry, along with the sound exposure conditions are covered in sub-chapter 4.1. Preliminary trials of the oxygen consumption and algal filtration experiment acting as a proof of concept were performed in March 2016 by MSc student David Bryson, the results of which are presented in his thesis (Bryson, 2016). The oxygen consumption results were not used in the following analysis, while the algal filtration data was reanalysed here. Method development for the valve movement experiment was performed in August 2016. Refined techniques were then employed for the here presented experiment. A single run of each of the below discussed experiments was performed and analysed in 2016: oxygen consumption November 10th – 15th, algal clearance – March 15th – 20th, and valve movement – October 25th – 30th.

4.3.2.1 - Oxygen Consumption

Following acclimation to the laboratory system, individual mussels were placed in a sealed section of a custom built transpartent acrylic respiration chamber (170 mm long and 85 mm diameter, Emitech Technische Komponenten, Figure 4.3.1b) held in the centre of a 120 L exposure tank (Figure 4.3.1a). Each chamber was set to hold 200 ml of natural seawater that was continuously refreshed by actively pumping water from the surrounding tank into the respiration chamber at 150 L h^{-1} throughout the acclimation period. During acclimation and exposure, the chamber was covered by a felt sleeve to eliminate any potential stimulus from the surrounding laboratory. After 23 h of acclimation to the respiration chamber, chosen so all exposures could start at the same time, the inflow and outflow valves were sealed to prevent any further water exchange, and noise exposure began. The mussels were exposed to ship noise playback (see sub-chapter 4.1.2.3, p77) or a silent control for one h.

During exposure, the changing oxygen saturation inside the sealed section of the respiration chambers was measured every s with a computer controlled setup using a fibox 3 trace v3 fibre-optic trace oxygen meter (Presens – Precision Sensing, Regensburg, Germany, Figure 4.3.1a, b) and a laptop (Acer E5-571 series, Acer inc., New Taipei City,

Taiwan). The oxygen sensor was attached to the top of the respiration chamber above a PSt3 oxygen sensor spot (Presens – Precision Sensing, detection limit: 0.03% oxygen, 15ppb) and connected to the Fibox with a fibre-optic cable. The sensor spot consists of a 3mm plastic circle covered in a red oxygen sensitive coating placed internally on the transparent outer wall of the respiration chamber. Non-destructive oxygen measurements are made by stimulating an immobilised luminophore with monochromatic light and measuring the luminescence decay time. The light signal emitted by the luminophore is delayed by the decay time allowing oxygen saturation to be measured.

Directly following the mussel exposure, a blank chamber containing water from the surrounding tank was exposed in the same way as the chamber containing the mussel to measure any bacterial respiration. If any bacterial respiration was present this could then be removed from the final respiration readings (see equation V). A total of four animals and four blank chambers were measured each day over a five day period (Figure 4.3.2). Mussels were measured individually and only used once. An alternating system of exposure (noise, control, noise, control) was employed and this order reversed each day.

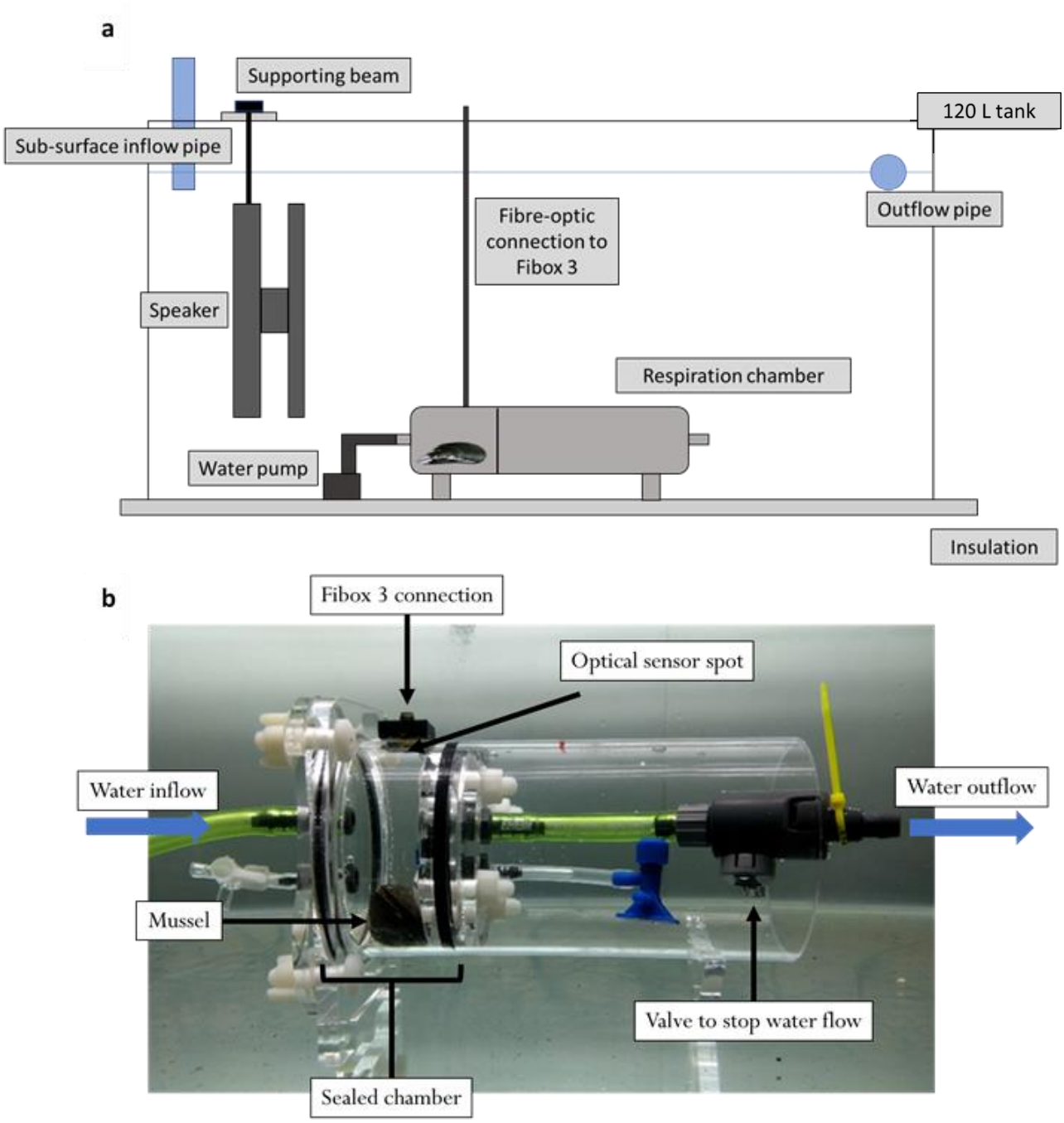

Figure 4.3.1 | Oxygen consumption set-up. (a) Aquarium set-up for the oxygen consumption experiment. (b) Custom built respiration chamber, sealed area calibrated to hold 200ml of water when empty. Tank dimensions: 788 x 528 x 306 mm, speaker placed 120 mm from the tank wall and 70 mm from the tank floor. Respiration chamber placed 250 mm from the speaker. Noise levels measured directly behind the sealed chamber holding the mussel without the end acrylic attached.

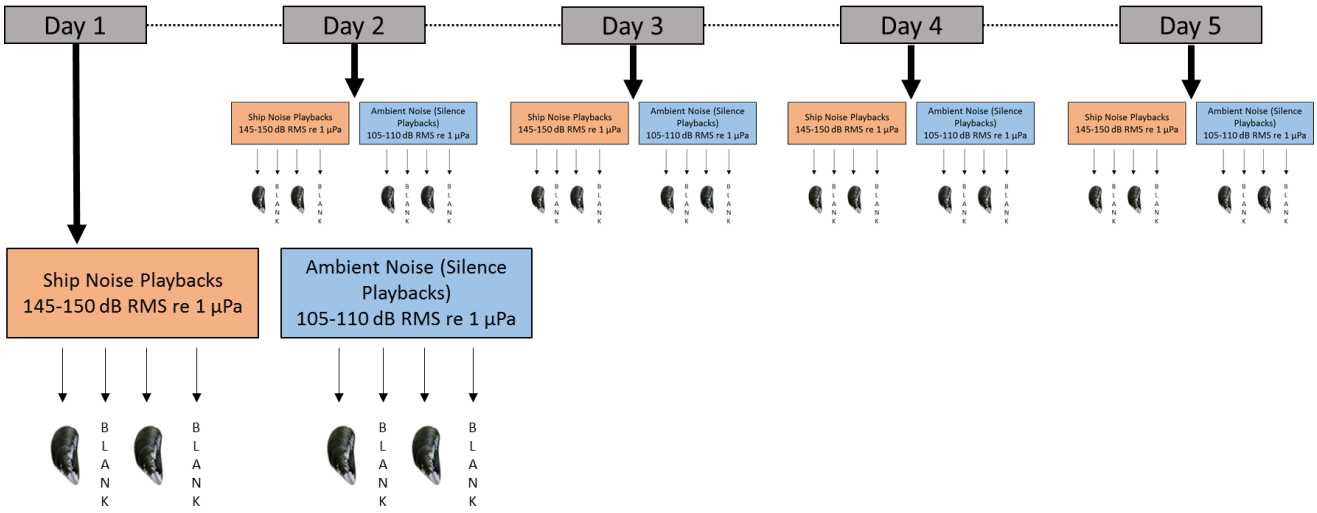

Figure 4.3.2 | Oxygen consumption experimental groups. Four individual mussels, two for each treatment, and their corresponding blanks tested each day for five days. Mussels were tested individually and only once. The process shown under day 1 above in magnification was repeated each day but with different animals.

Following noise exposure, the mussels were removed from the respiration chambers, dried of water by gentle blotting on tissue paper, and their wet weight recorded to the nearest centigram (Sartorius L420P, Sartorius AG, Göttingen, Germany). Each mussel was then submerged in a known volume of fresh water within a calibrated measuring cylinder to record the water displacement. Fresh water was used to prevent variable displacement caused by differing salinities, and to reflect the fresh water used when calibrating the respiration chamber volume. The length of the mussel from posterior to anterior shell tip, and width across the widest area of the shell were also recorded to maintain a comparable size range between treatments. The mean length of tested mussels was 51.4 mm for noise exposure, and 50.4 mm for control animals, and did not significantly differ (two-sample *t*-test: $t_{16.96} = 0.4275$, $P = 0.67$). Mussels were measured after exposure to prevent additional stress when being placed in the respiration chambers. Finally, the mussels were returned to a separate holding tank in the laboratory system, separated from future test animals. The temperature (°C, recorded via the Fibox 3 trace) and ambient air pressure (hPa, recorded using Met office data; Met Office, 2017) were recorded for each exposure period, for use in the below oxygen consumption calculations.

Oxygen consumption was then calculated using the below equations adapted from Presens (2006).

Oxygen saturation readings (%O_2) were converted to mg L^{-1} using the following equations:

$$C_{O_2}[mg\ L^{-1}] = \frac{P_{atm} - P_w(T)}{P_N} \cdot \frac{\%O_2}{100} \cdot 0.2095 \cdot \alpha(T) \cdot 1000 \cdot \frac{M(O_2)}{V_m} \quad \textbf{(I)}$$

P_{atm}: Atmospheric pressure at time of measurement

T = Temperature in Kelvin of water at time of measurement

$P_W(T)$: vapor pressure of water at T

P_N: Standard pressure (1013 hPa)

0.2095: Volume content of oxygen in air

$\alpha(T)$: Bunsen absorption coefficient at T; given in $cm^2(0_2)\ cm^{-3}$

$M(O_2)$: Molecular mass of oxygen (32 g mol^{-1})

V_M: Molar volume of oxygen (22.414 L mol^{-1})

$$P_w(T) = exp\left[A - \frac{B}{T} - C \cdot \ln T\right] \quad \textbf{(II)}$$

$A = 52.57$

$B = 6690.9$

$C = 4.681$

$$\alpha(T) = a + b \cdot \theta + c \cdot \theta^2 + d \cdot \theta^3 + e \cdot \theta^4 \quad \textbf{(III)}$$

a = 48.998

b = -1.335

c = $2.755*10^{-2}$

Θ = Temperature in °C of water at time of measurement

d = $-3.220*10^{-4}$

e = $1.598*10^{-6}$

Oxygen consumption was then calculated per g of mussel tissue using the following equations:

$$O_2 Consumption[mg\ L^{-1}] = \frac{Initial_{O_2} - Final_{O_2}}{Water\ Volume} \quad \textbf{(IV)}$$

$$O_2 Consumption[mg\ L^{-1}\ g^{-1}] = \frac{Mussel_{O_2} - Blank_{O_2}}{Mussel\ weight} \quad \textbf{(V)}$$

4.3.2.2 - Algal Filtration Rate

After a two week acclimation period, a group of 25 similarly sized mussels (see sub-chapter 4.1.1.2, p74) were placed in a 10 L tank, which itself stood inside a 120 L exposure tank (Figure 4.3.3) containing the noise source. The 10 L tank was raised off the floor of the 120 L exposure tank using an inverted plastic tray drilled through to allow the escape of air bubbles when submerging, and acoustically isolated from any transmitted vibrations using neoprene matting. Both tanks contained natural filtered seawater from the AquaLab aquaria system, although remained separate from each other with no water transfer occurring (Figure 4.3.3). Inside the 10 L tank the mussels were held on a raised mesh platform, allowing algal filtration but preventing the build-up of pseudofaeces around the mussels which may have been resuspended during sampling skewing the overall results.

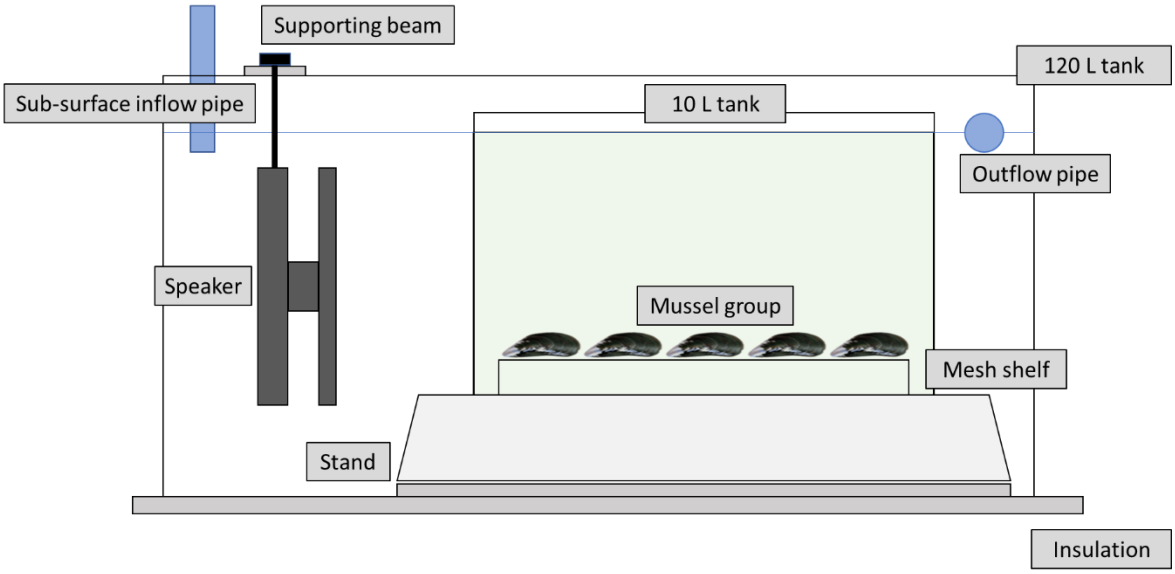

Figure 4.3.3| Algal filtration tanks. Aquarium set-up for the algal filtration experiment. Tank dimensions: 788 x 528 x 306 mm, speaker placed 120 mm from the tank wall and 70 mm from the tank floor. 10 L tank 280 x 180 x 190 mm held on stand 60 mm above the tank floor. Mussels held on mesh stand 40 mm from the floor of the 10 L tank.

Animals were starved for 48 h prior to noise exposure to remove any algae currently being digested and to create a level feeding state across all animals. After starvation, the 10 L tank was inoculated with ≈ 3,000 cells ml^{-1} dried *Tetraselmis suecica* (ZMSystems, Hampshire, UK), a large green alga often used in filtration studies (see Nielsen and Strømgren, 1991; Riisgård *et al.*, 1981; Vasconcelos, 1995; Wong and Levinton, 2004). Algal cells were purchased in a concentrated suspension at 1.5 billion cells ml^{-1}. A dilution of 300,000 cells ml^{-1} was made by mixing 1ml of suspension in 50 ml of seawater, which was then further diluted when to 3,000 cells ml^{-1} when 1ml of the diluted suspension was added to the 10 L exposure tank. Dried *T. suecica* were used to prevent changes in algal concentration during the exposure brought about by reproduction. The cells were mixed thoroughly into the 10 L tank to ensure an even distribution. Mussels were exposed to ship noise playback (see sub-chapter 4.1.2.3, p77) or a silent control for three h. Five replicate 1 ml water samples were taken from the centre of the tank midwater after 0, 90, and 180 min of exposure. The tank water was vigorously stirred (a glass rod was moved across the width and length of the tank) for 10 s and any created turbulence allowed to disperse prior to sample collection ensuring an even cell distribution. After exposure, the mussels were

removed, their outer shell dried, and bodies measured (weight, and water displacement) as with the oxygen consumption experiment above. A total of five tanks were used for both the noise and control treatments, with one noise and one control exposure taking place each day for five days (Figure 4.3.4). Each animal was used only once, and a staggered acclimation schedule allowed exposure on consecutive days.

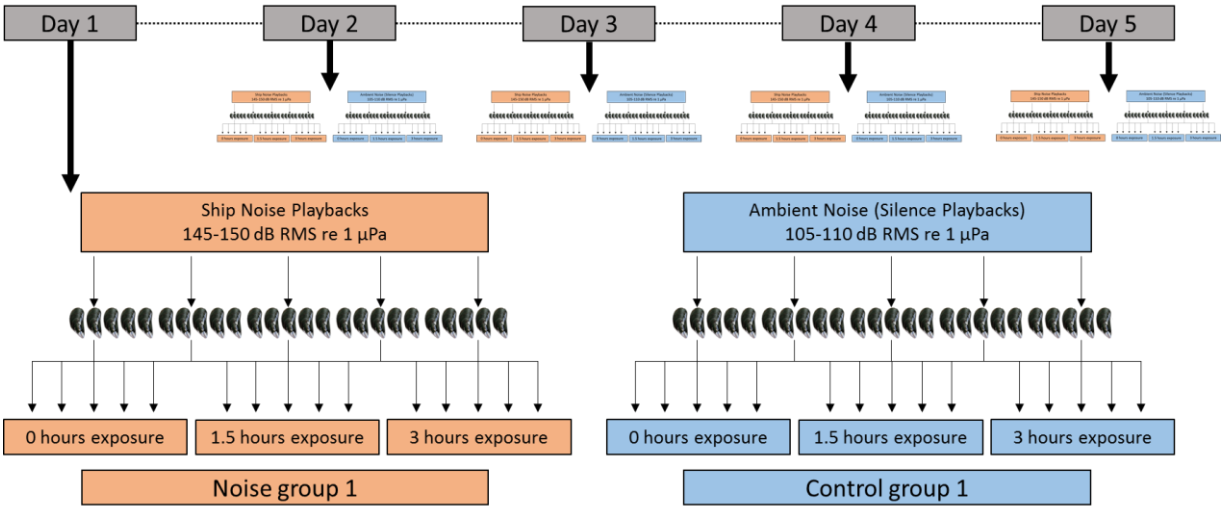

Figure 4.3.4| Algal filtration experimental groups. A group of 25 mussels used for each treatment, each day, for 5 consecutive days. The process magnified under day 1 above was repeated each day of the trial but with different animals.

Algal cells were counted using a Sedgewick-Rafter counting cell. Each 1 mm x 1 mm square was converted into an xy coordinate containing 1 µl of sample. 5 random squares per ml sample were imaged in cellSens (Olympus, Southend on Sea, UK) and coded to remove bias when the number of individual algal cells were manually counted. These readings were further converted to filtration rate per g of mussel and together with those for biomass per m^2 of mussel reef, extrapolated to produce a metric for estimating filtration rate reduction in the field. Reef biomass was calculated through photographic analysis of 250 cm^2 quadrats. Five quadrats where blindly placed within 5 m radius of a marker pole (yacht turning pole) in the area that the mussels were collected. These quadrats were then photographed. From these quadrats 10 individual mussels were blindly selected and removed from the quadrat. Their length was then measured from posterior to anterior tips of the shell. The mean mussel length was then converted to mean weight using the equation

generated from all mussel size data collected throughout sub-chapters 4.2-4.4 (Figure 4.3.5). Total biomass was then calculated by manually counting the number of mussels in each quadrat and multiplying this by the mean weight to gain biomass per m^2 of reef in the collection area (Appendix D).

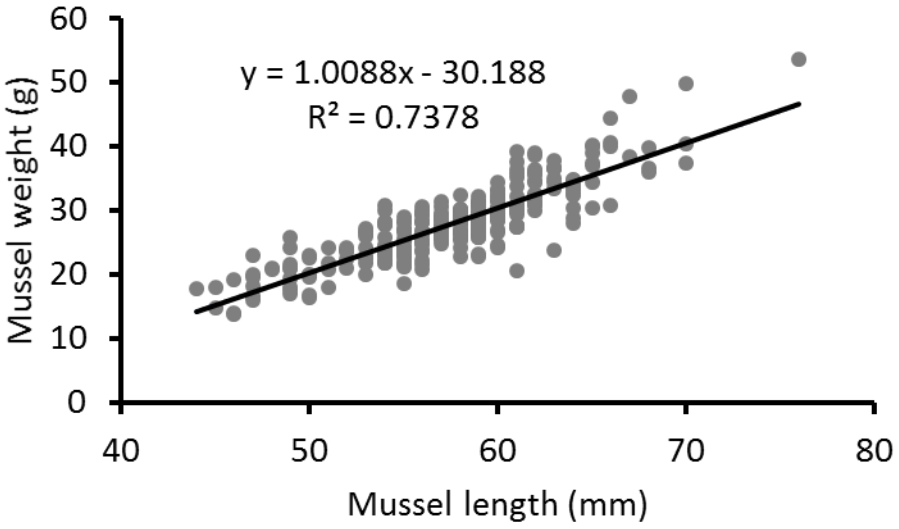

Figure 4.3.5| Mussel size calculation. Regression on scatter plot of average *M. edulis* size generated from the size metrics collected throughout all experiments in sub-chapter 4.2-4.4.

4.3.2.3 Valve Movement

After acclimation to the aquarium systems, individual mussels were placed on a custom built stand created from a GoPro camera mount facing a PVC pipe topped with a plastic pipe retainer (Figure 4.3.6a). The stand ensured that mussels remained in place throughout exposure and their valve opening faced towards a GoPro Hero 4 Silver camera (GoPro Inc, San Mateo, CA, USA). The stand was placed centrally inside the same 120 L tank used for the algal filtration rate and oxygen consumption experiments (Figure 4.3.6b). The mussels were acclimated to the exposure set-up for 24 h, after which they were exposed to either ship noise playbacks or a silent control (see sub-chapter 4.1.2.3, p77) for one h, with valve movements filmed throughout the exposure. To remove bias, video files were coded until fully analysed, and observed without sound. The resulting footage was manually analysed for valve gape in mm between valves (mean generated from readings at five min intervals, 13 total readings over the one h of exposure), and valve opening time to the

nearest s (presented as cumulative opening time). Any animal that remained closed from the start of the exposure for the entire exposure length was removed from the analysis (four animals in noise treatment, two animals in control treatment), to prevent skewing the results by zero inflation. A total of 10 mussels were filmed for each treatment, with two mussels filmed for each treatment each day, for five consecutive days (Figure 4.3.7). Each animal was used only once, and a staggered acclimation schedule allowed exposure on consecutive days.

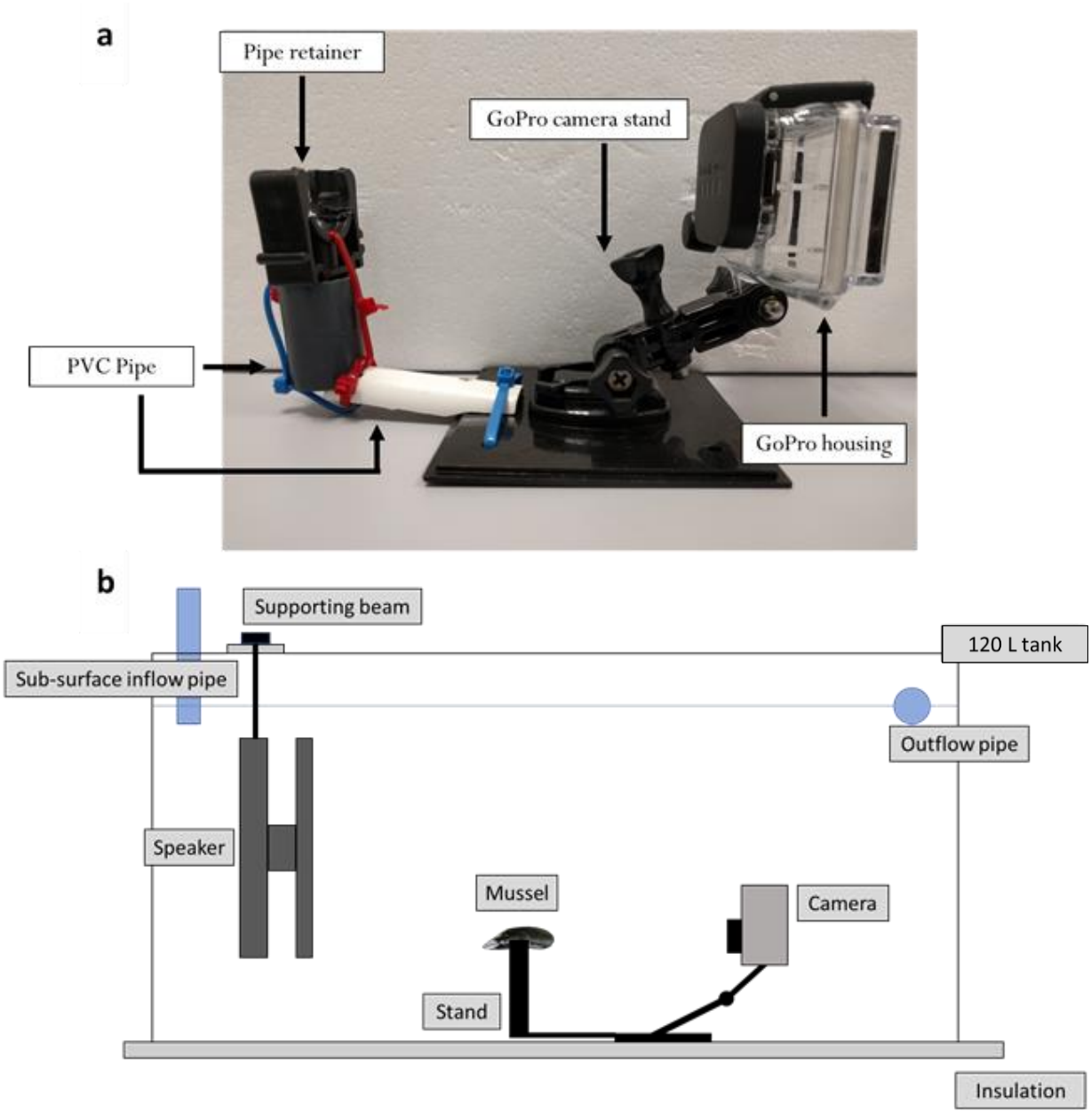

Figure 4.3.6| Valve movement set-up. (a) Custom stand to hold mussels for filming of valve movement. (b) Aquarium set-up for the valve movement experiment. Tank dimensions: 788 x 528

x 306 mm, speaker placed 120 mm from the tank wall and 70 mm from the tank floor. Stand placed with the mussel 300 mm from the speaker.

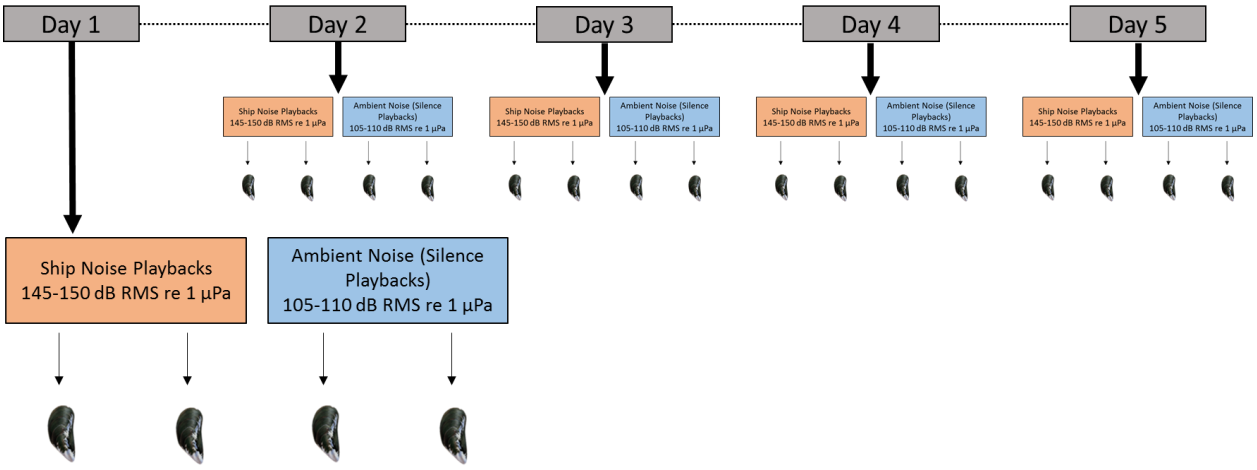

Figure 4.3.7| Valve movement experimental groups. Four individual mussels, two for each treatment, each day for five days. The process magnified under day 1 above was repeated each day of the trial but with different animals.

4.3.2.4 - Statistical analysis

Statistics were performed in R version 3.3.1 (The R foundation for Statistical Computing). Data were tested for normality and heterogeneity of variance around the mean, normality was shown for oxygen consumption without transformation, and algal filtration data with log transformation. Non-normality was identified for valve gape and opening time, and normality was shown for valve gape over time. A mixed-model ANOVA was used to assess the effect of the interaction between treatment (independent variable) and time (independent variable) on oxygen consumption in the form of O_2 saturation (dependent variable), the individual mussel was considered as a random effect in the model. A similar mixed-model ANOVA was used to assess the effect of the interaction between treatment (independent variable) and time (independent variable) on algal clearance in the form of log transformed algal cell count (dependent variable), the exposure tank was considered a random effect in the model. Wilcoxon rank sum tests were used to assess the effect of treatment (independent variable) on the valve gape (dependent variable) and valve opening time (dependent variable). A mixed-model ANOVA was used to assess the effect of the interaction between treatment (independent variable) and time (independent

variable) on valve gape (dependent variable), time was considered a random effect within this model.

4.3.3 – Results

4.3.3.1 - Oxygen Consumption

Changes in the oxygen consumption and thus the metabolic rate of mussels, in response to a one h exposure of ship noise playback are indicated by a highly significant interaction between treatment and time (mixed-model ANOVA, $F_{1, 218} = 4.90$, $P = 0.028$ Figure 4.3.8). Noise-exposed mussels consumed significantly less oxygen over time (b = 0.00017, SE = 0.00001) than the control animals (b = 0.00021, SE = 0.00002) with an overall reduction in oxygen consumption of 19%.

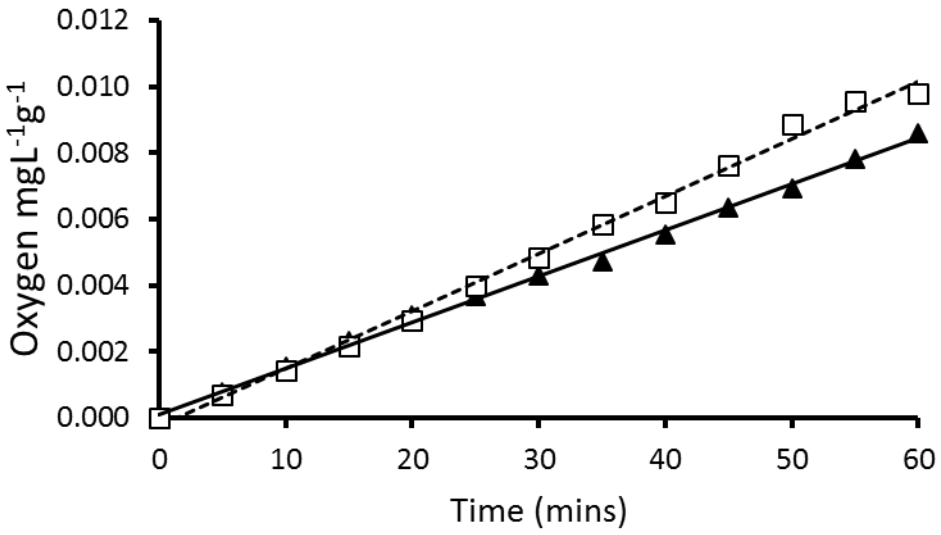

Figure 4.3.8| Noise induced changes in the oxygen consumption of *M. edulis*. Oxygen consumed (mg L^{-1}) per g of *M. edulis* tissue* (n = 10 for both treatments).

4.3.3.2 - Algal Filtration Rate

Noise exposed mussels consumed significantly less algal cells over a three h period than those in control conditions. The interaction between treatment and time was highly significant (mixed-model ANOVA, $F_{1, 138} = 41.96$, $P < 0.0001$, Figure 4.3.9). Mean cell count decreased significantly over time in the control treatment (b = -0.48, SE = 0.047), whereas there was no significant decline in the noise treatment (b = -0.077, SE = 0.060). This difference corresponds to an 84% reduction in algal filtration rate in response to noise. Extrapolating the observed reduced rate to the density of mussels from the reef where the experimental animals were sourced yields a reduction of 303.2 ± 15.4 million algal cells per L of surrounding water removed every h for each square meter of established mussel reef.

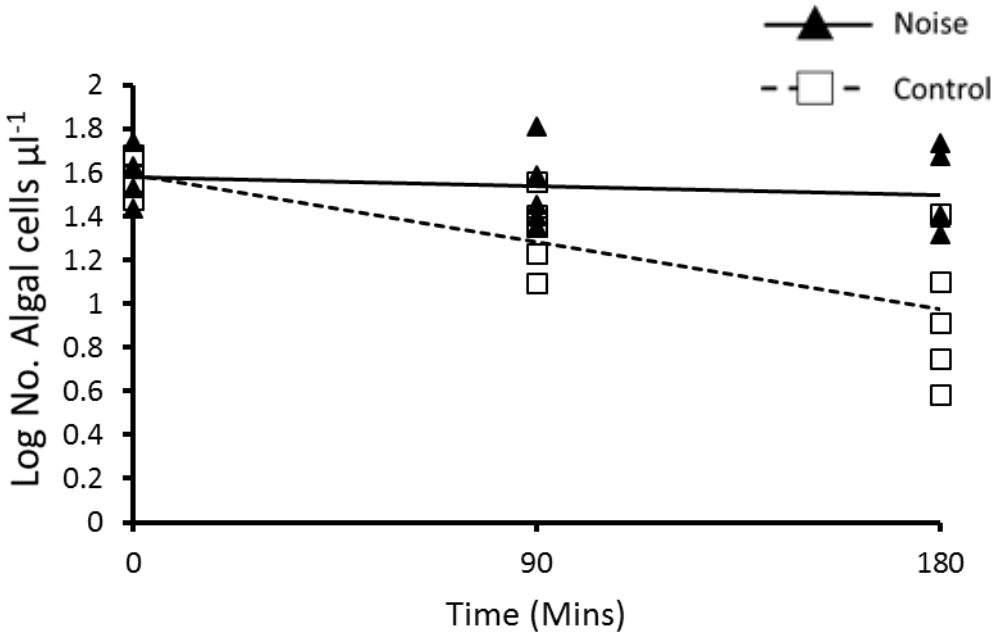

Figure 4.3.9| Noise induced changes in the algal filtration rate of *M. edulis*. Consumed algal cells μl^{-1} seawater*** (n = 5 for both treatments).

4.3.3.3 - Valve Movement

Valve gape was significantly increased 144% in noise exposed animals (Wilcoxon rank sum test, W = 7, *P* = 0.033. Figure 4.3.10a), while cumulative valve opening time did not differ between the two treatments (Wilcoxon rank sum test, W = 60, *P* = 0.46, Figure 4.3.10b).

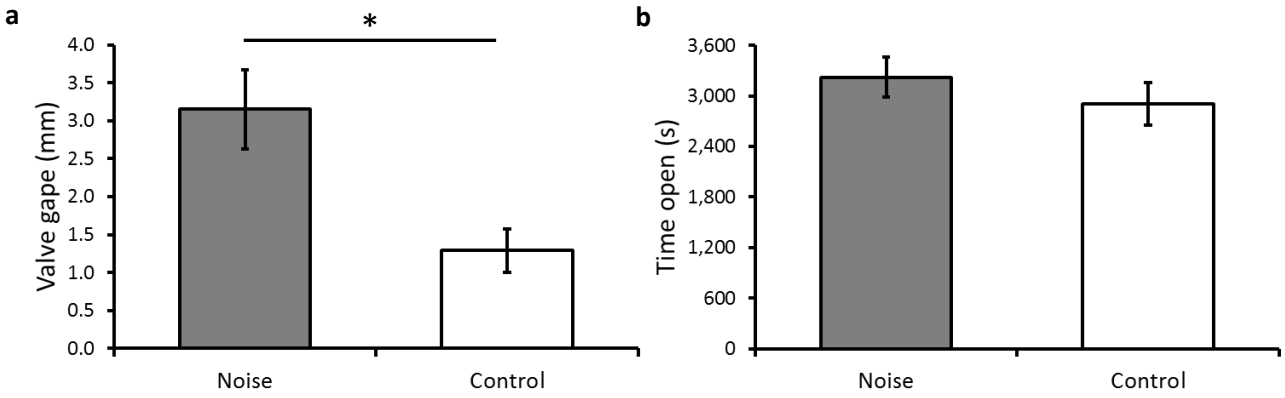

Figure 4.3.10|Noise induced changes in the valve movement of *M. edulis*. (a) Mean ± Stdev valve gape* (n = 6 for noise, n = 8 for control). (b) Mean ± Stdev s with valve open (n = 6 for noise, n = 8 for control), 3,600 s would mean the mussel was open throughout the experiment.

The valve gape of noise exposed mussels increased over the one h exposure, with the interaction between treatment and time highly significant (mixed-model ANOVA, $F_{1, 154} = 14.69$, *P* 0.0002, Figure 4.3.11), and mean valve gape increasing significantly (b = 0.044, SE = 0.013). This increase was not seen in the control treatment where no significant change in valve gape was observed during the exposure (b = -0.0037, SE = 0.0081).

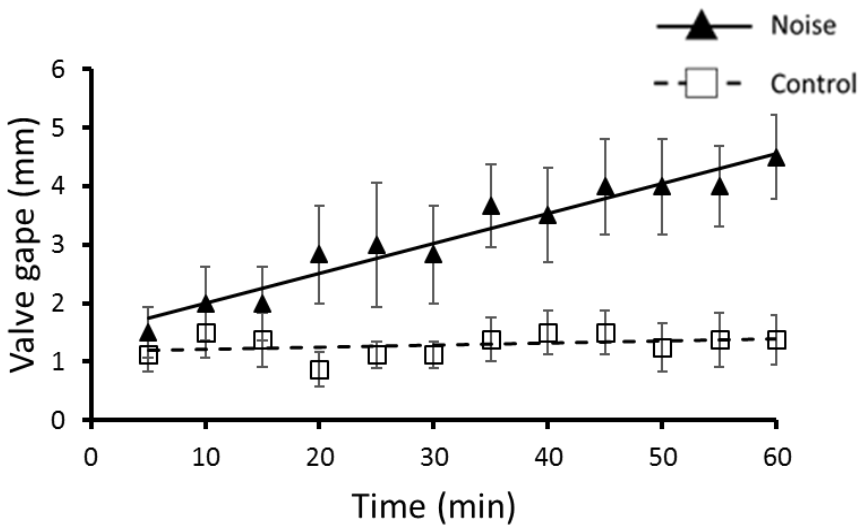

Figure 4.3.11 | Noise induced changes in the valve gape of *M. edulis* over time. Mean ± SE valve gape (n = 6 for noise, n = 8 for control).

4.3.4 – Discussion

The aim of the here presented experiments was to provide information on the physiological and behavioural responses of *M. edulis* to anthropogenic noise playbacks. The noise exposed mussels responded in the form of reduced oxygen consumption, reduced algal clearance, and increased valve gape, with these reactions contrasting several of the initial hypotheses. For example, it had been assumed that the mussels would increase respiration, rather than decreasing it. An elevated oxygen consumption is often associated with elevated stress. For example, this type of response was identified by Wale *et al*. (2013) in *Carcinus maenas*, with crabs exposed to ship noise playbacks consuming more oxygen than those in ambient conditions. Regnault and Lagardere (1983) similarly showed how noisier conditions could increase the oxygen consumption of crustaceans, with *C. crangon* consuming more oxygen in louder ambient noise conditions. Reduced oxygen consumption in bivalves is more common when exposed to chemical stimuli. Manley (1983) identified reduced mantle oxygen tensions in *M. edulis* exposed to copper. Wenguang and Maoxian (2012) showed reduced respiration in the noble scallop *Chlamys nobilis* with lowering water pH.

It was also hypothesised that the valve gape and opening time would be significantly reduced when exposed to ship noise playbacks, rather than being increased as observed here. This was expected since *M. edulis* close its valves in response to predation (Freeman, 2007) and noise (specifically the particle motion component) might evoke a similar response. Roberts *et al*. (2015) showed full and partial valve closure in response to sediment vibration, with a number of responses occurring during a single vibration period. However, conversely to hypotheses H_1, H_3, and H_4 (see section 4.5.1, p148) mussels exposed to ship noise playbacks had a significantly reduced oxygen consumption, and an increased valve gape. As such, none of the associated null-hypotheses can be rejected.

A reduction in oxygen consumption and an increased valve gape are seemingly converse reactions. One would have expected that a greater valve gape would permit more gill surface to be in contact the surrounding water, allowing a greater exchange and consumption of oxygen. Similarly, if oxygen consumption was to be lowered, it would be expected that the valves would fully close. This reaction is seen when mussels are exposed to a chemical pollutant (Gowland *et al.*, 2002; Manley, 1983). Closing their valves lowers contact between the contaminated water and soft tissue. This in turn assists in preventing the build-up of contaminants within the animal (Gowland *et al.*, 2002; Jing *et al.*, 2006; Kádár *et al.*, 2001). The responses identified here however are more akin to a shock response than that of a general stress response. A freeze response is a common initial reaction to a stressor in prey animals (Bracha, 2004), where the animal remains vigilant of its surroundings, conserving energy for successive reactions. Here, with the onset of noise, the exposed mussels likely startled and attempted to reduce energy, and therewith reduce oxygen demand. Relaxation of the posterior and anterior adductor muscles, resulting in valve opening, lowers the energy demand associated with keeping the valves actively closed (Livingstone, 2013). The acute onset of noise presented in this experiment would have led to an unfamiliar environment for the mussels, and would explain the wider valve gape when exposed to anthropogenic noise (to conserve energy). With repeated or chronic exposure to noise, this response may no longer occur. There was no evidence of habituation or tolerance build up, which would have resulted in a decreasing valve gape over the exposure. The increasing valve gape over the one hour of noise exposure likely occurring as the adductor mussels relaxed fully (Livingstone, 2013).

It was further hypothesised that noise exposure would cause a reduction in the algal filtration of *M. edulis*. The results of this study corroborate this hypothesis, with noise exposed mussels filtering half the algae over a three-h period than those in control conditions. A similar reduction in algal filtration occurs in the presence of chemical pollutants, as shown by Manley (1983) where a 30 ppb concentration of copper resulted in a 50% reduction in the filtration of *M. edulis*. Likewise, the mussel *Perna perna* filters up to 80% less with increased environmental mercury (Anandraj *et al*. 2002). A reduction in filtration is likely to reduce the energy uptake of the mussels, and if continued over an extended period would have knock on effects to growth. Riisgård (1991) presented data that showed a direct correlation between algal filtration rate and growth in *M. edulis*, with the energy gained through algal uptake having to exceed that of the mussel's energy expenditure.

In addition to the negative effects identified here at the organismal level, which may influence the mussels' growth, survival and reproductive success, the observed decline in algal clearance rate indicates that noise can also reduce their ecological performance. A similar 84% reduction in algal clearance rate in the field, such as observed here in the laboratory, would have a detrimental effect on the surrounding reef community. *M. edulis* clears particulates from the surrounding water and deposits them on the seafloor in the form of faeces and pseudofaeces (Garrido *et al.*, 2012). This process effectively transports nutrients from the surrounding water into the benthic community (Kautsky and Evans, 1987). Deposited particulates also aid in the sedimentation of coastal habitats, although to a much lower extent than physical process (Callier *et al.*, 2006; Hatcher *et al.*, 1994). A reduction in the overall filtration rate caused by noise would therefore have important carry-over effects to the surrounding ecosystem by modifying the mussels' performance in benthic-pelagic coupling. It is difficult to accurately extrapolate from laboratory experiments to a field environment due to the varying conditions, and then additional stimuli present in the field. However, this extrapolation can serve as a base for a field experiment studying algal filtration or biodeposition of *M. edulis* in response to noise in situ.

Uncertainties and Future Directions

Uncertainties of the experimental methods, and suggestions for improvements in future work are discussed in sub-chapter 4.5.

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Sub-chapter 4.4

Noise Induced Molecular Stress Responses in *Mytilus edulis*

The following sub-chapter is a collaborative work between M.A. Wale, R.A. Briers, and K. Diele of Edinburgh Napier University, and T. Henry of Heriot-Watt University.

K.D. and M.A.W. conceived the research; M.A.W. designed the experiments with input from all authors; M.A.W. conducted the genetic assays and qPCR with instruction from T.H. M.A.W. analysed the acoustics and performed the statistical analysis with input from R.A.B. and K.D.

4.4.1 – Introduction

The assessment of genetic responses to noise is understudied (see chapter 2.3.1, Fig. 2.5, p30), with only a single previous investigation of changes in invertebrate gene expression during underwater noise exposure. Peng *et al*. (2016) exposed Chinese razor clams *Sinonovacula constricta* housed in buckets, to 1 kHz sine waves at 80 and 100 dB re 1 µPa, and investigated changes in the expression of 10 genes (6-phosphofructokinase-1, Pyruvate kinase, Acetyl-CoA carboxylase, Arylformamidase, Citrate synthase, Isocitrate dehydrogenase(NAD^+), Isocitrate dehydrogenase($NADP^+$), Oxoglutarate dehydrogenase, Dihydrolipoamide succinyltransferase, and Dihydrolipoamide dehydrogenase). There was an increased expression of all genes of the razor clams exposed to the 80 dB sine wave, however this response was not produced with the 100 dB signal, suggesting a lowering of metabolic rate as the clams retreated into the mud during the higher noise intensity. Despite the current lack of research in this area, it is important to understand the underlying molecular mechanisms that drive the more frequently studied physiological and behavioural responses to man-made noise. The role of increased gene expression depends on the specific genes that are being expressed, the end product of which, often a protein, will most likely have an explicit function within the organism (Bork *et al*, 1998). The time at which a change in the expression of a specific gene occurs depends on the stimulus. It can occur quickly,

after a prolonged period, or at multiple points during exposure to said stimulus (Auch and Church, 2001). Following the work described in sub-chapter 4.2 and 4.3 on noise induced biochemical, physiological, and behavioural responses of the *Mytilus edulis*, here it is assessed whether anthropogenic noise playbacks alter the expression of heat shock protein 70 (Hsp70) using quantitative polymerase chain reaction (qPCR).

The term heat shock protein refers to a group of proteins produced when cells are exposed to adverse, stressful conditions. Although first described as a response to temperature shock (Ritossa, 1962), the experienced stress can come from many different sources including heavy metals (Dondero *et al.*, 2006; Radlowska and Pempkowiak, 2002), and ocean acidification (Moya *et al.*, 2015; Wang *et al.*, 2016). An increase in Hsp70 has also been observed at the biochemical (protein) level in response to anthropogenic noise exposure. Filiciotto *et al.* (2016, 2014) and Vazzana *et al.* (2016) studied the spiny lobster *Palinurus elephas*, the common prawn *Palaemon serratus* and the Mediterranean mussel *Mytilus galloprovincialis* respectively, all of which displayed an increased quantity of Hsp70 in their cells (*P. elephas* – haemocytes; *P. serratus* – brain; *M. galloprovincialis* – mantle and gill). Hsp70 is widespread in animals, plant, and bacteria (Depledge, 1994), and its expression represents a general response to an environmental stressor. It functions as a chaperone, stabilising the production of proteins within the cell and assisting in the repair of proteins that were damaged during cell stress (De Maio, 1999). This in turn increases overall protein integrity, and limits the potential for mutation and cell degradation (Muller *et al.*, 2013; Wegele *et al.*, 2004). This stress protein is prevalent in *M. edulis* experiencing stress (Dondero *et al.*, 2006; Luedeking and Koehler, 2004; Radlowska and Pempkowiak, 2002; Rola *et al.*, 2012), and along with the evidence of its upregulation in noise exposed *M. galloprovincialis*, led to it being chosen as a potential indicator of noise induced genetic responses in *M. edulis* investigated here.

Quantitative polymerase chain reaction (qPCR) is a commonly used genetic technique to monitor and quantify the amplification of targeted DNA molecules, in real time, during thermocycling and enzyme driven DNA replication (Franzen, 2001). The generated C_T values (the number of thermocycles needed to amplify a gene above a set threshold) indicate the abundance of the targeted gene, which can then be compared against the abundance of a reference/housekeeping (constantly exhibited) gene to give a quantified

measure of the expression of the target gene. Changes in the expression of the target gene can then be identified across different treatments or times. Identifying the time at which gene expression peaks will allow future studies to utilise this information when planning their exposure lengths.

The aim of this experiment was to identify potential changes in heat shock protein expression in *M. edulis* exposed to anthropogenic noise playbacks, and to establish an ideal time period for sample collection to assess this expression.

4.4.1.1 - Hypotheses

H_1: Acute exposure to ship noise playbacks will cause increased expression of Hsp70 in *M. edulis* gills when compared to those from a silent control.

H_2: Noise induced Hsp70 expression will vary depending on the time since the onset of exposure.

4.4.2 – Methods

Methods of animal collection and husbandry, along with the creation and presentations of the ship noise playbacks used here are covered in sub-chapter 4.1. A single trial was performed on November 14th 2016 and all samples were collected during this time. All animals were exposed under the same conditions as those for the biochemical experiments in sub-chapter 4.2 (Figure 4.2.1, p90). After a 24 h period of acclimation a total of 36 mussels were subjected to either ship noise playback or a silent control (see sub-chapter 4.1.2.3, p77) for five h. Six mussels were randomly removed from the exposure tank at 0, 30, 60, 120, 180, and 300 min of exposure (Figure 4.4.1) to produce an expression curve. By collecting samples after varying periods in the noise playbacks, changes in Hsp70 expression can be plotted, and the produced expression curve used to identify the ideal exposure length when assessing gene expression changes in *M. edulis*. Once removed from the experimental tanks, the mussels were opened as in sub-chapter 4.2 and samples of gill, mantle, and digestive gland taken from each animal. Samples were stored in pairs

(Figure 4.4.1) where the tissue from 2 animals was pooled to reduce the impact of individual genetic variability on the subsequent analysis (Foster *et al.*, 2015). This led to a total of three replicate samples for each treatment at each time point. All samples were flash frozen in liquid nitrogen following collection and stored at -80 °C until use.

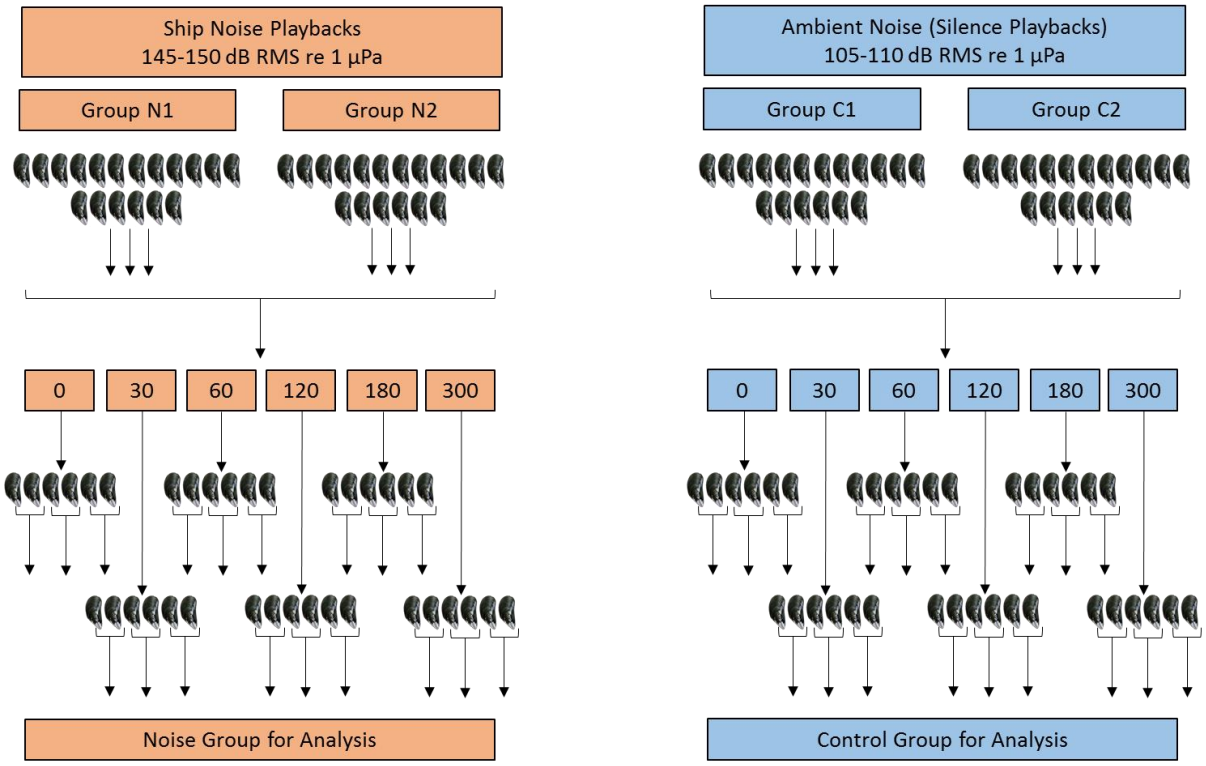

Figure 4.4.1| Experimental set-up. Mussels exposed in four groups of 18 mussels (two noise, two control). At each time point, three mussels were removed from each group and combined into a group of six mussels for each treatment. Samples from two mussels in each group were stored together to give a total three samples for analysis at each time point.

4.4.2.1 - RNA Extraction

For RNA extraction and all subsequent procedures, only gill tissue was used, since it has previously been identified as tissue the most susceptible to Hsp70 expression changes in *M. edulis* (Rola *et al.*, 2012). It was therefore decided that initial analysis would be performed on these samples as a proof of concept, after which other samples could be tested depending on the success of the gill tissue analysis. RNA extraction was conducted as directed by the Ecotoxicology and Pathophysiology of Aquatic Organisms Research

Laboratory SOP:238 (Appendix C), based on the Qiagen RNA easy mini kit (cat#74106, Qiagen, Hilden, Germany).

A small quantity of the target tissue, approximately 10% of the pooled sample, was taken from the total sample and placed in an Eppendorf tube to thaw on ice. Thawed samples remained on ice throughout the procedure. To each Eppendorf 350 µl RLT lysis buffer was added and the sample was homogenised with a sterile sonicator until 90% of the tissue had dissolved and the liquid had visibly yellowed. Following homogenisation, the sample was centrifuged for three min at max power, and the resulting supernatant (350 µl) transferred to a new Eppendorf tube containing an equal volume of 70% ethanol, and mixed well by pipetting. The resulting 700 µl of liquid, including any precipitate, was transferred onto an RNeasy minicolumn and centrifuged for 15 s at max speed. The flow through was discarded following centrifugation. This step was repeated with 350 µl of RW1 buffer pipetted onto the minicolumn.

70 µl of RDD buffer and 10 µl DNase stock (550 µl of molecular water added to a lyophilized vial of DNase) was pipetted directly onto the minicolumn gel membrane and left to incubate on ice for 15 min. 350 µl of RW1 buffer was further added to the minicolumn and centrifuged for 15 s on max speed, with the resulting flow through discarded. Centrifugation was twice repeated with 500 µl of RPE buffer replacing the RW1. The RNeasy minicolumn was then dried in a centrifuge for 2 min on max speed. Finally, the minicolumn was transferred to a new Eppendorf tube, and 30 µl of RNase-free water pipetted directly onto the gel membrane for 5 min of incubation. The minicolum was then discarded after one minute in the centrifuge at max speed, which resulted in the final RNA solution.

Extracted RNA was checked for quantity (> 100 ng μl^{-1} (in mussel tissue usually above 500 ng μl^{-1})) and quality (optimum ratios: 260/280 at 1.8-2.1 and 260/230 above 1.5) using NanoDrop software and the accompanying spectrophotometer (Thermo Scientific NanoDrop 2000, Thermo Fisher, Waltham, MA, USA). Samples were tested in triplicate and averages used for assessment (Appendix D). If samples did not reach the quantity or quality desired they were discarded and RNA extraction for that sample repeated.

4.4.2.2 Reverse Transcription

Reverse transcription, a process by which complementary DNA (cDNA) is generated from an RNA template, was performed as directed by the Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory SOP:240 (Appendix C), based on the Primer Design Precision nanoScript™ 2 Reverse Transcription kit, Instruction for cDNA synthesis using up to 2 µg of RNA (Handbook HB05.09.02). Extracted RNA was added in a 9:1 ratio with RT primer to a 0.5ml Eppendorf tube. The resulting mix was then annealed in a thermocycler (Applied Biosciences Vertical 96 Well Thermocycler, Applied Biosystems, Foster City, CA, USA) at 65 °C for 5 min, followed by immediate cooling in an ice bath. A reverse transcriptase stock mix was prepared by mixing in a 5:1:3:1 ratio nanoScript2 4x Buffer, dNTP mix 10nM, RNase/Dnase free water, and nanoScript2 enzyme. 10 µl of both the annealed RNA and the reverse transcriptase stock were mixed on ice and placed in the thermocycler for 15 min at 65 °C. The resulting cDNA was stored at -20 °C until use. It is assumed that the produced cDNA is in a 1:1 ratio of quantity and quality as the initial RNA.

4.4.2.3 - Primer Optimisation

Choosing the correct primers is essential if the qPCR process is to succeed. A primer is a short strand of RNA (or DNA) that binds to a specific sequence within a target gene. A pair of primers, forward and reverse, bind at each end of a longer RNA fragment allowing the enzymic amplification of this known sequence. Forward and reverse primers were selected from the GenBank database nucleotide collection, *Mytilus edulis* heat shock protein 70 mRNA, partial cds (AF172607.1, www.ncbi.nlm.nih.gov/nuccore/AF172607.1). Primers were selected with no self-complementarity, hairpins, or self-annealing (Table 4.4.1), that would cause them to bind to themselves rather than the target sequence. To assess the viability of the primers and to optimise the temperature used in the qPCR process, agarose gel electrophoresis was run on the PCR products of the produced cDNA. PCR was conducted according to the Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory SOP:131 (Appendix C), and developed from the Sigma: ReadyMixTM Taq PCR Reaction Mix with $MgCl_2$ (cat#P4600). Forward and reverse primers were

reconstituted according to the manufactures instructions and prepared as 1 µmole L^{-1} working solutions by diluting 5 µl of stock solution with 500 µl of nuclease-free water.

Table 4.4.1| Primers. Primers selected to detect Hsp&0 expression, taken from GenBank nucleotide sequence AF172607.1, showing length, start position, end position, annealing temperature (Tm), guanine-cytosine content (GC%), and self-complementarity (Self. Comp).

Nucleotide Sequence
GCTATTGCTTATGGTTTGGATAAGAAAGTAGGTGGAGAAAGAAATGTACTCATCTTTGACTTGGGTGGTGGAACTTTTGATGTGTC AATCCTTACAATTGAGGATGGTATTTTTGAAGTGAAATCAACCTCTGGTGATACCCACTTGGGTGGTGAAGACTTTGACAACAGAA TGGTCAATCATTTCATTCAAGAATTCAAACGCAAGCACAAAAAAGACATTAGTGAAAACAAGCGTGCTGTCCGACGACTTAGAACT GCTTGTGAAAGGGCAAAGAGAACCCTTTCTTCAAGCACACAAGCAAGTGTTGAGATTGACTCTTTGTTTGAAGGAGTTGACTTTTA TACAAGCATCACAAGAGCCAGGTTTGAGGAATTGAATGCAGATCTTTTCAGAGGAACCATGGAACCAGTTGAAAAAGCTCTACGTG ATGCCAAACTAGACAAGGCTGCTGTCCATAAAATTGTCTTGGTAGGTGGATCAACCAGAATTCCAAAAATCCAGAAGTTACTTCAG GACTTTTTCAACGGCAAAGAATTGAACAAATCCATTAACCCTGATGAAGCTGTA

Primer Pair								
	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self. Comp
Fwd	GTGCTGTCCGACGACTTAGA	Plus	20	236	255	59.48	55	5.00
Rev	ACCTGGCTCTTGTGATGCTT	Minus	20	367	348	59.60	50	3.00

Product length	132

Forward Primer	no self complementarity; no hairpin found; no self annealing
Reverse Primer	no self complementarity; no hairpin found; no self annealing

To prepare the PCR plates, a 0.5 ml nuclease-free tube was filled with S x 25 µl of ReadyMix (1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCL, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, stabilizers) S x 10 µl of forward primer, and S x 10 µl of reverse primer were added, where S is the number of samples to be tested. 45 µl of the created PCR Master Mix was added to a 0.2 ml PCR tube along with 5 µl of cDNA (Diluted to 10%), or 5 µl of water for use as a "no template control" (NTC). PCR was run in a thermocycler under the following parameters: denaturation of the template at 98 °C for 2 min, 30 cycles of amplification at 98 °C for 15 s, annellation of primers for 30 s, extension at 72 °C for 30 s, hold the samples at 72 °C for 10 min. Annealing took place from 56 to 61 °C at 1 °C intervals, to optimise the annealing temperature for qPCR. The PCR products were then run on an electrophorese gel (Figure 4.4.2).

To separate the genetic components amplified in the above PCR, a northern blot was performed. Here gel electrophoresis is employed to separate RNA fragments of

different sizes. Smaller fragments are transported further along the gel by the supplied current, and their size is compared against a ladder containing many fragments of known sizes. Gel electrophoresis was conducted according to the Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory SOP:130 (Appendix C). An electrophoresis gel was created out of 2% agarose in 50 ml of 1 x TBE (10 x = 109 g Tris base, 55 g Boric acid, and 4.8 g EDTA, dissolved in 900 ml deionised water, adjusted to pH 8.3 with NaOH, and made up to 1 L with deionised water), heated until all agarose was dissolved. The molten solution was stained with 5 µl GelRed (Biotium, Freemont, CA, USA) and poured into a mini gel mould ensuring no bubbles were present. A gel comb was added and the gel left to set for a minimum of 30 min. Once set, the gel comb was removed, the gel was removed from the mould, placed into the electrophoresis tank, and the tank was filled with 1 x TBE as a running buffer.

The gel was prepared by adding to each well 10 µl of PCR product mixed with 2 µl of gel loading buffer (40% sucrose, 0.25% bromophenol blue, dissolved in 5 ml of deionised water), ensuring the gel was not pierced by the pipette tip. To the first well 2 µl of DNA ladder (peqGOLD Ultra Low range DNA-Ladder I (PEQL25-3110 VWR), 10 to 300 base pairs, VWR International Ltd, Leicestershire, UK) was added and mixed with 10 µl 1 x TBE. Once all wells were loaded, a lid was placed on the tank and the electrodes were connected to the power supply. Electrophoresis took place for 25 min at 250 V, 350 mA, 25 W. The subsequent gel was read on Image Lab™ (Bio-Rad Laboritories Ltd, Watford, UK) and the connected gel reader (Gel Doc XR+ Gel Documentation System, Bio-Rad, Hertfordshire, UK) (Figure 4.4.2). The resulting image shows the amount of the selected gene annealed to the primers at each temperature, a brighter line shows higher annealing and indicates the optimum temperature for qPCR. The position of the line on the gel indicates the length of the product and highlights whether the primers are annealing to the desired gene, here 132 base pairs (Figure 4.4.2).

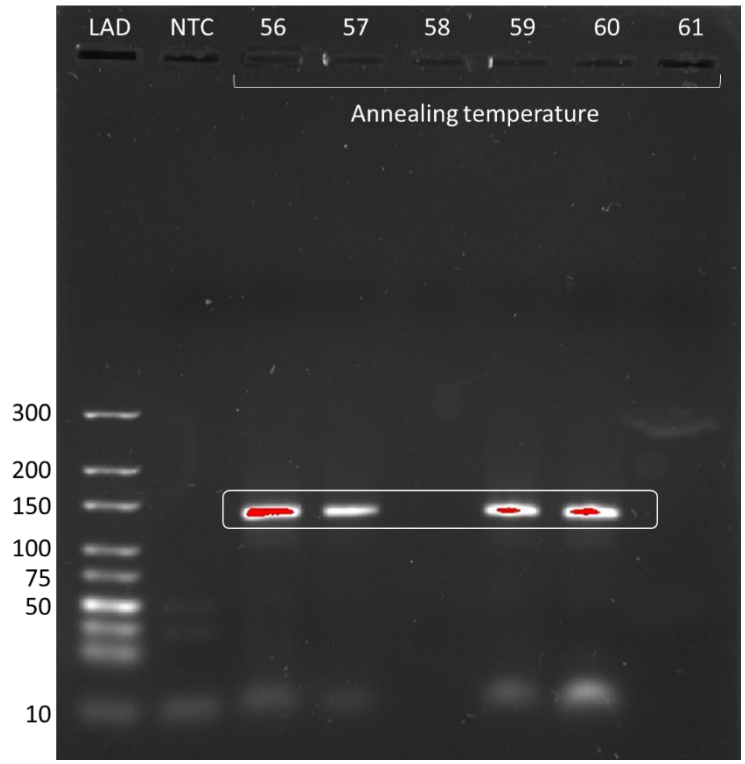

Figure 4.4.2| Electrophoresis gel of PCR products. PCR products at different annealing temperatures, produce from a sample of *M. edulis* gill cDNA. The detected product length matches the 132 base pair sequence formed between the primer binding sites.

To test the primer efficiency, a dilution series of template cDNA (1:10, 1:50, 1:100, 1:1000) was created and run as per the qPCR steps below. Efficiency of the PCR reaction (Figure 4.4.3, Efficiency = 97.86%) was determined by comparing the change in C_T value for the gene transcript relative to the concentration of the standard, using the following equation:

$$Efficiency\ (\%) = [10^{\left(-\frac{-1}{slope}\right)} - 1] \times 100 \quad \textbf{(I)}$$

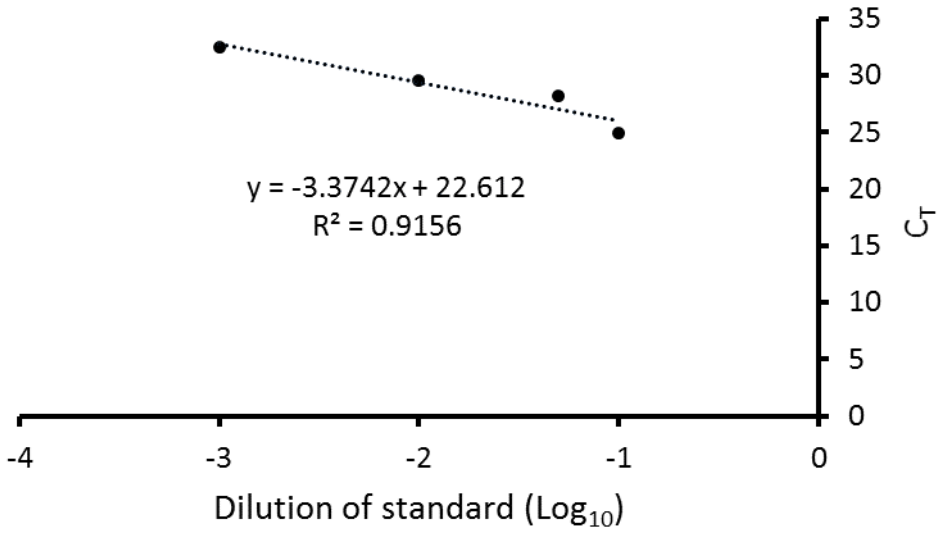

Figure 4.4.3| Primer efficiency. Efficiency of designed primers for detection of Hsp70 expression. Efficiency = 97.86%.

4.4.2.4 - qPCR

In quantitative (q)PCR, targeted DNA molecules are amplified, in real time, during thermocycling and enzyme driven DNA replication (Franzen, 2001). The generated C_T values (the number of thermocycles needed to amplify a gene above a set threshold) indicate the abundance of the targeted gene. Methods for qPCR analysis were performed as directed by the Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory SOP:243 (Appendix C), based on the procedure outlined in the Primer Design hand book "Instructions for use of Primer Design PrecisionPlus Mastermix and PrecisionFAST Mastermix". An initial qPCR Mastermix was created combining 10 µl PrecisionPlus, 1 µl Forward Primer, 1 µl Reverse Primer, and 3 µl RNase/DNase free water for each planned reaction. The Mastermix was stored on ice and covered in tin foil to alleviate photodegradation. All samples were then diluted to 10% (2 µl cDNA template, 18 µl RNase/DNase free water), and 5 µl cDNA was combined with 15 µl Mastermix in triplicate, in each well of a 48 well qPCR reading plate (Appendix C). Two wells on each plate were used as a non-template control (NTC) combining 15 µl Mastermix with 5 µl RNase/DNase free water to test the C_T change when no cDNA is present. A third well

contained only RNase/DNase free water (20 µl) to record evaporation over the qPCR procedure (Figure 4.4.4). Once all wells were filled, a plate cover was securely attached and qPCR (48 wells, standard curve, SYBR green, ~2 h run) (Figure 4.4.4) was run in the "Step1 software" and attached plate reader (Applied Biosystems StepOne real-time PCR system, Applied Biosystems, Foster City, CA, USA).

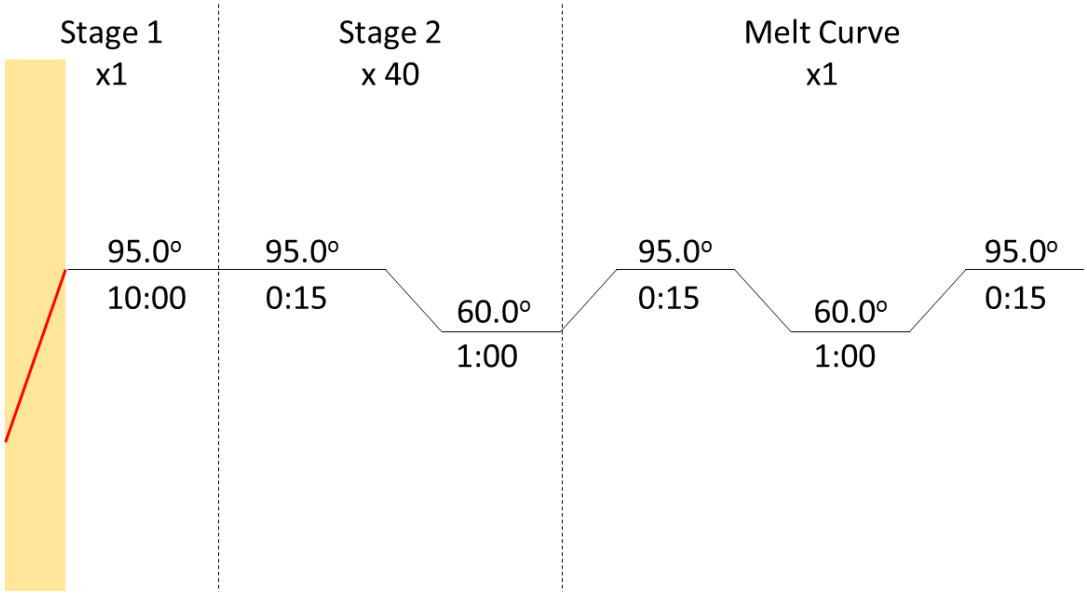

Figure 4.4.4| qPCR set-up. Set up for Hsp70 expression qPCR run – Annealing temperature 60 °C, run length ~2 h. Temperature presented above the line (°C), and time presented below the line (min:s).

A second qPCR was run for each sample, testing the expression of 18S ribosomal RNA for use as a housekeeping gene (a gene expressed at a constant level irrespective of stressors). Primer efficiency and annealing temperature for 18S were previously assessed by researchers in the Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory, Heriot-Watt University, and these parameters were followed here. Change in expression of Hsp70 was then assessed using the $\Delta\Delta C_T$ (delta-delta C_T) method. Here the fold change in expression of the target gene (Hsp70) is determined relative to the reference gene (18S) using the following equation:

$$\Delta\Delta C_T = 2^{-(C_T 18S_{control} - C_T HSP70_{control}) - (C_T 18S_{treatment} - C_T HSP70_{treatment})} \quad \textbf{(I)}$$

A $\Delta\Delta C_T$ of 1 would indicate no change in expression, a number above 1 would indicate a change in expression relative to that number e.g. a $\Delta\Delta C_T$ of 10 would equate to a relative fold change of 10 times the expression. At each time point a random 18s CT value was combined with a random Hsp70 CT Value to give a ΔCT value, from this a random noise ΔCT value was combined with a random control ΔCT value to give a ΔΔCT value. This created three ΔΔCT values for each time point along the expression curve.

4.4.2.5 - Statistical analysis

All statistics were performed in R version 3.3.1 (The R foundation for Statistical Computing). Linear regression analysis was performed assessing the fold change ($\Delta\Delta C_T$) in Hsp70 expression (independent variable) against time after noise onset (dependent variable).

4.4.3 – Results

Hsp70 expression did not change in the gill tissue of mussels exposed to ship noise playbacks at any time interval up to 5 h of exposure. No significant difference in the fold change was observed between exposure time ($R^2 = 0.0407$, $P = 0.996$, Figure 4.4.5).

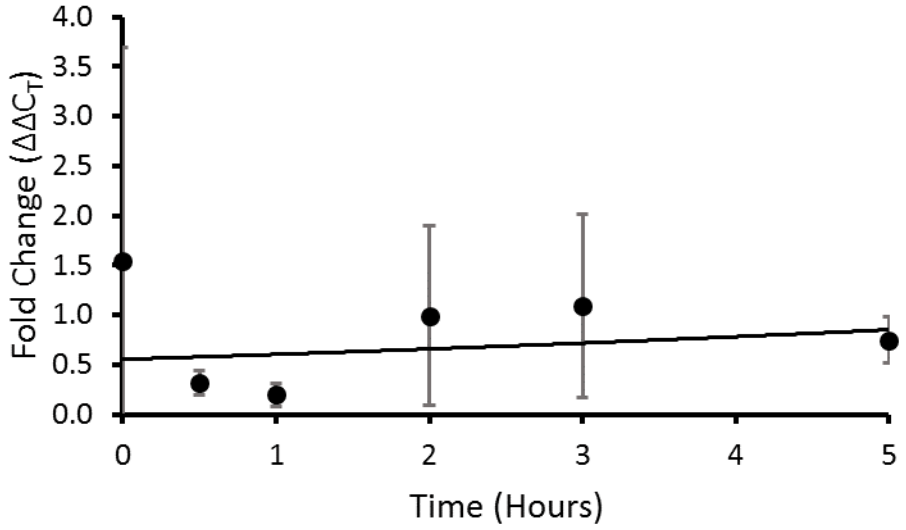

Figure 4.4.5| Noise induced changes in Hsp70 expression of *M. edulis*. Mean ± Stdev Hsp70 fold change $\Delta\Delta C_T$ of *M. edulis* (n=3 for each time point). Regression line plotted through data.

4.4.4 – Discussion

This study is the first to investigate noise induced changes in the gene expression of *Mytilus edulis*. It is also the first study to utilise qPCR to investigate the effects of anthropogenic noise playbacks in any marine species, and the first to use heat shock proteins at a genetic level as a metric for noise induced stress. Previously Hsp70 concentrations were only investigated at the protein level (Filiciotto *et al.*, 2016, 2014; Vazzana *et al.*, 2016). In this study, it was hypothesised that noise exposure would cause an increased, time varying, expression of Hsp70. This hypothesis was based upon the prolific nature of Hsp70 as a stress protein expressed during exposure to many different environmental stressors (Dondero *et al.*, 2006; Moya *et al.*, 2015; Radlowska and Pempkowiak, 2002; Ritossa, 1962; Wang *et al.*, 2016). Evidence of increased Hsp70 (at the protein level) in response to noise is available for several other marine invertebrates (Filiciotto *et al.*, 2016, 2014; Vazzana *et al.*, 2016), one of which, *M. galloprovincialis* (Vazzana *et al.*, 2016), is a member of the same genus as the species investigated in this study, *M. edulis*. However, the analysis here identified no changes in Hsp70 expression over the course of noise exposure. As no change in Hsp70 expression was identified here at the genetic level, analysis at the protein level would also reveal no response. These two methods of analysis essentially measure the same response, however genetic (qPCR) analysis allows the intensity of the expression to be quantified.

The lack of Hsp70 expression could have resulted for a number of reasons, first among them is a lack of discernible genetic response to noise exposure in *M. edulis*. However, given the strong responses previously identified in biochemistry, physiology, and behaviour of *M. edulis* (see sub-chapters 4.2, p86 and 4.3, p106) it is likely that the lack of response observed here is not representative of all possible genetic responses. Only a single stress protein (Hsp70) was tested, and although Hsp70 is readily expressed in stressed *M. edulis* (Rola *et al.*, 2012), there are many other stress proteins (e.g. Hsp27, Hsp40) not analysed here that could be expressed in the presence of noise. Hsp70 expression has been shown to vary between populations. Sørensen *et al.* (2001), using natural populations of the fruit fly *Drosophila buzzatii*, showed that expression of Hsp70 varies between populations depending on their environmental contexts. As all *M. edulis* in this study were taken from a

single population, the lack of expression identified may be typical for this, but not other populations of the same species. However, *M. edulis* for many populations have been shown to express Hsp70 when exposed to a number of stressors, and it seems highly unlikely that the Musselburgh population is the exception to this.

Brennecke *et al*. (1998) identified species specific Hsp70 expression in two species of Hydra, with *Hydra oligactis* expressing significantly less Hsp70 than *Hydra magnipapillata* under the same conditions. This variability between species in the same genus could explain the fact that *M. galloprovincialis* showed noise induced Hsp70 expression (Vazzana *et al*., 2016), while *M. edulis* did not. In this scenario, *M. edulis* Hsp70 expression would be unaffected by noise exposure, although it may be affected by other stimuli. Alternatively, the sound intensity used in this exposure may have been too loud to produce a response. Peng *et al*. (2016) showed in the razor clam *Sinonovacula constricta* that although gene expression can be altered by noise exposure (~80 dB re 1 µPa), when the noise intensity is too great (~100 dB re 1 µPa) these changes no longer occur. These result however were produced using 1 kHz pure tones, a lower frequency than the peak pressure used in this exposure. The exposure conditions presented by Peng *et al*. (2016) would additionally create sediment vibration around the buried bivalves, which may have had a greater influence on the Hsp70 expression than the sound pressure. The exposures presented in the present study, although high, were reminiscent of levels the mussels would experience in the field and are unlikely "too high" for elicit a response. The most likely of the discussed explanations for the lack of Hsp70 expression is a species specific reaction to noise, with *M. edulis* not expressing Hsp70 in response to noise to the same extent as other *Mytilus* species.

Uncertainties and Future Directions

Uncertainties of the experimental methods, and suggestions for improvements in future work are discussed in sub-chapter 4.5.

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Sub-chapter 4.5

From DNA to Ecological Performance: Effects of Anthropogenic Noise on a Reef-Building Mussel – A Synthesis

Sections of the following sub-chapter are included in the manuscript "Wale, M.A., Briers, R.A., Bryson, D., Hartl, M.G.J., Diele, K., From DNA to Ecological Performance: Effects of Man-Made Noise on a Reef-Building Mussel. *In Prep.*

M.A.W. wrote the manuscript and K.D., M.G.J.H., and R.B. contributed revisions.

All experiments conducted in sub-chapters 4.2-4.4 are here discussed in a holistic manner, considering the noise induced responses identified at the different levels of biological organisation. A range of noise induced negative effects on the animals' biology, from biochemistry, through physiology and behaviour were uncovered in these sub-chapters. DNA damage and oxidative stress endpoints as a response to anthropogenic noise are shown for the first time in any marine species. Decreased oxygen consumption, decreased algal filtration, and changes to the valve movement of bivalve molluscs have additionally been identified in response to this noise. Finally, the expression of heat shock proteins was investigated at the genetic level as a biomarker for noise induced stress.

4.5.1 - Response Links and their Implications

Using a mechanistic multi-method approach for investigating the effects of noise on *M. edulis* allowed the characterisation of individual and sometimes subtle effects. By conducting research in this way, following the research strategy proposed in chapter 2, responses could be mapped and their underlying drivers and interactions seen (Figure 4.5.1). Areas which require further investigation can also be identified in this way.

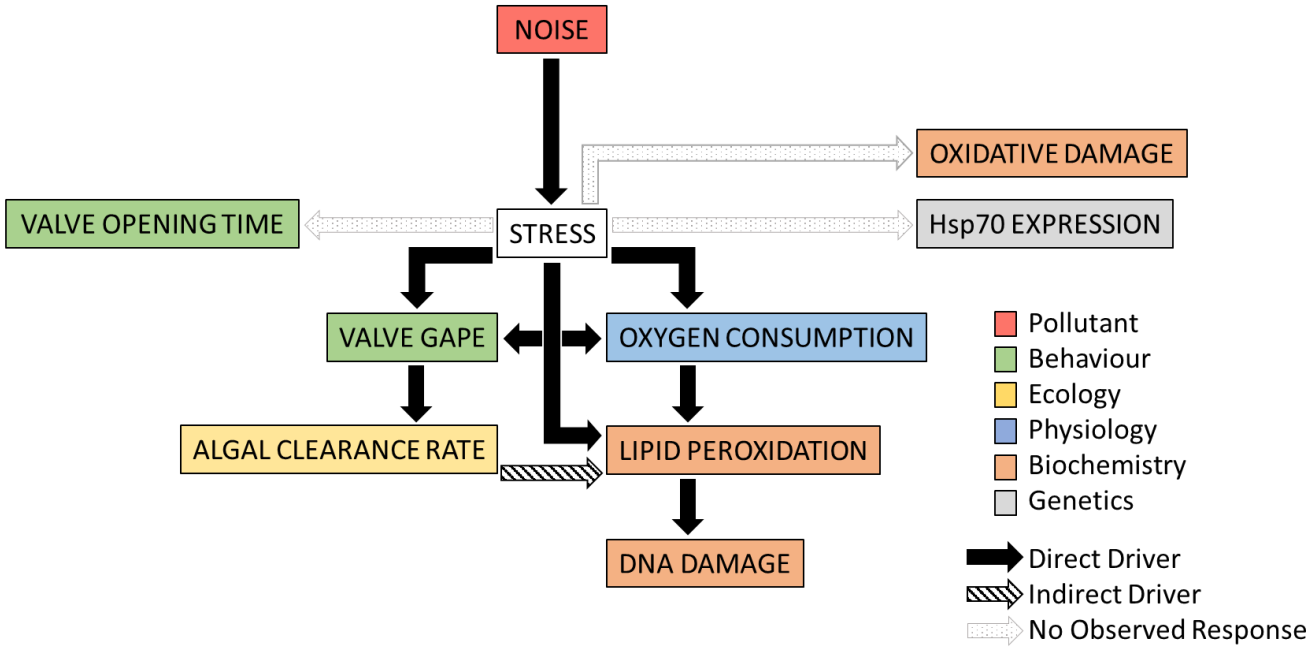

Figure 4.5.1 | Response map. Responses of *M. edulis* to anthropogenic noise playbacks, and the prospective links between these responses.

As shown by the response map (Figure 4.5.1), noise induced stress can have a number of negative effects on *M. edulis*. When stressed by acute noise, the mussel appears to exhibit a shock response. The mussel freezes and attempts to exert as little energy as possible. A freeze response is a common initial reaction to a stressor in prey animals (Bracha, 2004), where the animal remains vigilant of their surrounds, conserving energy for successive reactions. The intense vibration of the particle motion component of noise present in this exposure may have been a stressor previously unencountered by the mussels. This freeze response therefore may have been initiated whilst the mussel determined the source and extremity of the stimulus. In this reaction, relaxation of the adductor muscles caused the valves to fully open, resulting in an increased valve gape. For the mussel to remain in a closed or semi open state, energy is required by the adductor mussels (Livingstone, 2013). A lower energy demand, created when relaxing the adductor mussels, requires lower respiration which likely explains the lower oxygen consumption in mussels exposed to noise. Similarly, if mussels are conserving energy by freezing their body movements, they will be unable to filter algae from the surrounding water, evidenced here by the reduced algal filtration rates.

The observed lipid peroxidation in the gill epithelial cells of noise exposed mussels was then likely driven by the lowered oxygen consumption. Hypoxic conditions in the cells created by extended periods of low oxygen consumption can cause increased levels of lipid peroxidation (Woo et al., 2013), such as the increased level of malondialdehyde seen here. Lipid peroxidation could also be linked to the reduced algal filtration rates. Reduced algal consumption will over many weeks lead to a reduction in Vitamin E and iron (Iron -Knutson et al., 2000; Vitamin E -Tappel, 1972), which act as antioxidants regulating lipid peroxidation. This is unlikely to have occurred over the short length of the here performed exposures, but it is potentially a concern if the animal receives large levels of noise exposure over an extended period. Finally, the observed DNA damage has likely been driven by increased lipid peroxidation, the end point of which, malondialdehyde, is known to cause single strand breaks (Vöhringer et al., 1998).

Although no genetic response was observed in this study when using Hsp70 as a biomarker, this likely does not reflect all possible genetic responses to noise. The lack of Hsp70 expression could have occurred for a number of reasons including expression variability between populations, species, and noise intensities (see section 4.4.4, p143). There are a number of other heat shock proteins (e.g. Hsp27, Hsp40), amongst other stress proteins, that are expressed readily when an animal is under stress, and could potentially be expressed when exposed to anthropogenic noise. Therefore, to fully understand the genetic responses to noise a range of additional genes would need to be tested.

There are a number of implications of the observed responses, both on the individual, and ecosystem levels. Individual consequences can come in the way of increased predation risk when valves are fully open, and reduced growth through lower algal filtration rates and the associated reduction in nutrient uptake. Increased energy expenditure is likely to occur in an attempt to repair the DNA single-strand breaks. An increase in energy expenditure can additionally reduce growth in *M. edulis* as shown by Widdows and Johnson (1988). Lipid peroxidation, if uncontrolled can cause mutation (reviewed by Cooke et al., 2003), cellular damage (discussed as biomarkers for tissue damage by Gutteridge, 1995), DNA adducts (disscussed by Franco et al., 2008), and potentially mortality (reviewed by Evans and Cooke, 2004), with all of the above reactions being observed in many vertebrate, invertebrate, and bacteria species. On the individual level, each separate response can

compound upon the others, leading to a potentiational response where the animal is affected to a much greater extent than would first appear if only a single response was measured. These negative effects can then impair growth, survival, and reproductive success. This situation is seen readily in birds (Halfwerk et al., 2011 [*Paris majopr*]; Slabbekoorn and Ripmeester, 2008 [*P. major*, *Turdus merula*]), and invertebrates (de Soto *et al.*, 2013 [*Pecten novaezelandiae*]; Nedelec *et al.*, 2014 [*Stylocheilus striatus*]) where increased anthropogenic noise leads to a reduction in survival and reproductive success.

At the ecosystem level, a reduction in algal filtration can be directly linked to a reduction in the ecological performance of the mussels. A reduction in the particulates sequestered by the mussels would drastically reduce their ability to perform their role in benthic-pelagic coupling (Broekhoven *et al.*, 205; Garrido *et al.*, 2012; Tyler, 2017). A more indirect ecological consequence would result from the individual level response of reduced growth, and potential reproductive success. In this scenario, the number of animals in the ecosystem would eventually decrease, lowering the overall energy available within said system, and having knock on effects to higher trophic levels that rely on mussels as prey items.

4.5.2 – Uncertainties

All experiments conducted in this study were run in aquarium tanks. Most tanks used were large enough to avoid exposing the animals to unrealistically high particle motion intensities (see chapter 2.1.2, p22), however some were unavoidably small (oxygen consumption, valve movement, and algal clearance) and as such would have higher particle motion than if the animals were exposed in large tanks or in the field. However, given the paucity of information on many of the topics explored in the here conducted laboratory experiments, indications of potential effects are still valuable. Since tank playbacks do not perfectly replicate natural sound fields (Parvulescu, 1967, 1964), follow up field studies would be useful, especially regarding the findings of the experiments run in small tanks if at all possible (see above), giving accurate in situ readings of responses to noise. Field experiments on the other hand make it hard to control for a single stressor, and are therefore easily confounded. A combined lab-field approach is therefore desirable (see below).

Despite efforts to refine the procedures used in this study, there were a number of uncertainties. For the biochemical experiments, the Comet Assay employed to detect structural DNA damage is limited by its ability to only detect damaged DNA, and not to quantify any DNA repair that may have taken place throughout the exposure time, or would take place thereafter. To improve upon this, a number of samples could be collected at a period post exposure, and the proportion of damaged DNA to intact DNA compared across time periods. However, more serious DNA damage in the form of double strand breaks are not quantifiable by the Comet Assay. This limitation is however unavoidable, as methods currently available to detect double strand breaks rely on very "clean" DNA samples, such as those from cell cultures, and are unreliable on samples collected from wild specimens (M. Hartl, personal communication, 2016). The assessment of single strand breaks is a commonly used method in ecotoxicology to assess the genotoxic nature of pollutants within the environment (Everaarts and Sarkar, 1996) and, due to their easy quantification, allow comparisons to be drawn between the effects of different pollutants, as well as the monitoring of contaminants over large time scales.

In the oxygen consumption experiment, the blank chamber used to quantify any bacterial respiration in the water of the aquarium system was measured after the mussel exposure, rather than alongside. Due to equipment restrictions, only a single chamber could be measured at any one time, leading to these staggered measurements. The water measured in the blank, due to the flow through nature of the aquarium system could therefore potentially have a slightly different microbiota to the water in the mussel exposure. However, as the bacterial respiration was low throughout the trial, it can be assumed that the blank is representative of the water conditions during exposure of the mussels and the same across treatments.

For the valve movement experiments the filming presented some uncertainties, due to the difficulty to always achieve full focus. This varying ability to focus across videos, prevented a more detailed image analysis of the mussels' behaviour. For example, it would have been beneficial if the mussel's syphon, and its associated movement, could have been filmed along with the valve movement. This would have provided information on the water movement through the mussel and allowed better links to be drawn between the valve movement and oxygen consumption experiments. For this to be possible higher definition

cameras need to be utilised for filming, and they should be positioned in a custom position for each mussel. This however would extend the experimental time, and require the animal to remain in experimental stand for a longer period before the exposure can take place, which is likely to increase unwanted stress. To optimise this procedure, custom stand positions could be recorded for each individual mussel at the start of the two weeks acclimation to the holding system. The stand could then be adjusted to the recorded positions prior to the start of the experimental run.

In the genetic analysis of noise effects, the expression of only a single gene was studied. Hsp70 was chosen due to its high susceptibility to environmental changes, however to fully assess whether anthropogenic noise affects *M. edulis* at a genetic level it would be beneficial to study a range of additional genes in future studies. This can begin with RNAseq (Wang *et al.*, 2009), a technique based on whole transcriptome analysis, which assesses the total expression change within a specified tissue allowing any change to be quantified. This technique does not specify which genes are being expressed more readily, but would highlight whether noise could induce a genetic change in these animals. Specific genes could then be selected to try and identify the exact influence of anthropogenic noise exposure.

Acute noise exposure was chosen as the stimulus in all performed experiments to establish initial responses to anthropogenic noise in the environment. Longer noise exposures, either chronic or repeated (see chapter 2.1.1, p21), may produce different responses. The shock response identified by the physiological and behavioural changes reported in sub-chapter 4.3 may not have occurred in repeated or chronic exposure to noise. Similarly, the response may have changed over time in a longer noise exposure. Testing a range of exposure lengths would allow the identification of any habituation or tolerance. However, chronic exposure is less likely to occur in the natural environment than acute noise exposure, especially for stressors such as ship noise. Therefore, the results generated here will guide future studies focusing on the effects of varying exposure lengths, intensities, and noise sources.

4.5.3 – Conclusions and Future Directions

Conducting research following the mechanistic integrative approach set forth in this chapter allows a more accurate assessment of the way noise is affecting the individual animal. When only a single aspect of the animal's biology is measured, the effects of noise can go unnoticed, i.e. conducting only the genetic analysis (sub-chapter 4.4), or appear less severe, i.e. conducting the valve movement experiment without the accompanying algal clearance and oxygen consumption measurements (sub-chapter 4.3). By measuring a large range of responses in a single study a better understanding of the way anthropogenic noise is affecting the animals can be garnered. The integrated approach to noise research employed here, initially suggested by Kight and Swaddle (2011) for terrestrial noise research, can also be used as a model for other invertebrate species and faunal groups (Kunc et al., 2016; Sabet et al., 2012) and inform the development of effective methods for assessing and monitoring noise impacts.

The identified effects of noise on *M. edulis*, a filter feeding sessile species, highlight the need to assess animals from all trophic levels before the ecosystemic effects of noise can be understood. They also exemplify the importance of considering species with different biological and ecological traits, such as both sessile and mobile animals, before any potential negative effects of noise on an ecosystem level can be dismissed.

Following the work conducted here there are a number directions future work could take. Firstly, the genetic analysis started in sub-chapter 4.4 could be continued, examining a large range of genes and creating a more complete assessment of how noise affects the genetics of *M. edulis*. In addition to this, work should be expanded to include field studies to show how noise affects *M. edulis* in situ. Finally, taking the methods used here, *M. edulis* should be exposed to varying noise lengths and intensities to explore how responses vary with repeated and chronic exposures, and to pinpoint the levels at which noise induced changes occur. Such work would also allow information on the habituation to, and recovery from noise exposure to be generated. Chronic and repeated exposures would additionally allow the assessment of noise induced changes in the growth and reproductive success of *M. edulis* if employed in a long tern study. This work could be expanded to cover mussels from areas that receive high levels of anthropogenic or natural sound and comparing their

responses to those of mussels from low noise areas, potentially identifying a tolerance build up over long term noise exposure to sound. The here generated results act as a base for many future studies, having effectively identified the potential for noise to affect *M. edulis* on multiple levels of biological organisation.

In this study, a number of noise induced negative responses are shown in *M. edulis*. Due to the use of *M. edulis* as a model in ecotoxicology studies, future research in this area should consider noise as a potential (co)contaminant that could affect any results generated. As such researchers working in these fields should screen for noise exposure prior to sampling to eliminate any noise induce effects. *M. edulis* is a highly commercial species and the results identified in this study raise concern over the noise levels experienced in both aquaculture and near commercial fishing grounds. The results highlight both the sensitivity of *M. edulis* to anthropogenic noise and that established ecotoxicological techniques are suitable for use in noise research.

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Chapter 5

Noise Level Preference in the European Lobster *Homarus gammarus*

Chapter 5

Behavioural Responses of the European Lobster *Homarus gammarus* to Anthropogenic Noise Playbacks

The following chapter forms part of the manuscript "Wale, M.A., Briers, R.A., Diele, K. Seasonal Changes in the Noise Induced Behaviour of the European lobster *Homarus gammarus*. *In Prep*." The manuscript contains a condensed version of the work in this chapter.

M.A.W. and K.D conceived the research; M.A.W., K.D., and R.B., designed the experiments; M.A.W. conducted all exposures; M.A.W. analysed the acoustics and video footage, and performed the statistical analysis with input from R.A.B. and K.D.; M.A.W. wrote the manuscript and K.D and R.B. contributed revisions.

5.1 – Introduction

The European, or common, lobster *Homarus gammarus* is abundant throughout the eastern Atlantic. Its geographic range extends from the Lofoten Islands of Norway and the southeast coast of Denmark and Sweden in the north (Dyntaxa, 2013; Hayward and Ryland, 2017), along the coasts of western Europe, the UK, and Ireland, to the coast of Morocco in the south (Hayward and Ryland, 2017; Lloris and Rucabado, 1998). The species is additionally found throughout the Mediterranean and western Black Sea, albeit in smaller numbers (Holthuis, 1991). Mature *H. gammarus* live predominately subtidally on the continental shelf at depths of 0-50 m, although can occur up to 150 m depth (Holthuis, 1991), and on occasion intertidally in rockpools (Personal observations). *H. gammarus* is nocturnal and territorial living a solitary lifestyle on hard substrates in holes and crevices, and emerges at night to feed on benthic invertebrates.

Female lobsters become sexually mature at five to seven years of age, or at a carapace length of 75 – 80 mm. Mating occurs shortly after the female has moulted and is soft, with the males' sperm retained in spermathecae until spawning occurs, usually in summer. The eggs are gestated on the abdomen of the female for 9-12 months after which the larvae

emerge and begin their planktonic phase. The planktonic phase lasts for 15-35 days and encompasses three distinct stages, a final fourth metamorphosis will change the larvae into a form more closely resembling that of an adult, and settlement will occur (Beard and McGregor, 2004). Juveniles are rarely seen since they spend most of their time in crevices or extensive burrow networks (Beard and McGregor, 2004) until reaching a carapace length of 15 mm and beginning to live an adult lifestyle (National Lobster Hatchery, 2017).

Lobsters have always been highly regarded as luxury food stock, and as such hold a high commercial value. In Scotland 1,000 tonnes of *Homarus gammarus* were fished, mainly through creels, in 2013 (Barreto and Bailey, 2015) with a value of £10.6 million. Additionally, a number of facilities specialising in lobster aquaculture are beginning to appear (Holthuis, 1991). However, these facilities focus more on conserving native populations and increasing wild fish-stocks, than on farming the lobsters directly for market (Hatchery, 2017; National Lobster Hatchery, 2017). As with many invertebrates, the commercial importance of lobsters is expected to rise as fishery targets switch from historically exploited fish species to animals from lower trophic levels (Pauly *et al.*, 2002). In addition to its commercial importance, *H. gammarus* is a keystone species essential for biodiversity maintenance of hard bottom communities (Schmalenbach and Franke, 2010). Acting as both a predator of lower trophic levels, and a food source for higher trophic levels, these animals are important components in the food webs of subtidal coastal ecosystems.

Tazaki (1977) evidenced mechanosensory hairs on the antennal flagellum of *H. gammarus* which are highly sensitive to water vibrations, and likely able to detect sound in addition to the detection provided by the statocysts (internal structures used in taxis). As yet, no evidence of sound production has been identified in *H. gammarus*, however the similar American lobster *Homarus americanus* has been shown to produce acoustic signals through vibration of the second antenna (Henninger and Watson, 2005), a process potentially mirrored by *H. gammarus*. With such a high degree of both ecological and commercial importance, evidence of sound detection and the potential for sound production, *H. gammarus* is ideally suited to act as a model species when assessing the effects of anthropogenic noise on marine crustaceans, sharing many behavioural, physiological, and biochemical characteristics with other crustaceans.

Due to the large size of these crustaceans, behavioural changes can be recorded far easier than those of smaller species, especially when the noise stimulus is presented in large aquaria and cages where the sound field more closely resembles that of natural environments. In this study, mature *H. gammarus* were exposed to an intensity gradient of anthropogenic noise, and their movement/preference relative to noise intensity measured. Noise induced behavioural changes have previously been documented in a number of crustacean species. Filiciotto *et al*. (2014) identified numerous changes in the movement behaviour of the spiny lobster *Palinurus elephas* when exposed to boat noise playbacks in aquaria. Here, lobsters moved more readily and covered a longer distance when in noisy conditions. Wale *et al*. (2013) showed negative changes to the foraging and antipredator behaviour of the shore crab *Carcinus maenas*. The feeding behaviour of the crabs was disrupted by ship noise playbacks, as well as time to return to shelter after a predator interaction was extended. Nousek-mcgregor *et al*. (2016) exposed hermit crabs *Pagurus bernhardus*, to ship noise playbacks and identified increased distraction and delayed reaction to a predator stimulus whilst in noise compared to control conditions. These laboratory studies highlight the viability of behavioural responses as an indicator of noise detection in crustaceans.

One of the most common behavioural responses to unwanted anthropogenic sound for many marine species is to move away from the source. This behaviour has been documented for both fish (cod *Gadus morhua* and haddock *Melanogrammus aeglefinos*, Engäs *et al*., 1996; bluefin tuna *Thunnus thynnus*, Sára *et al*., 2007) and mammals (small fast moving odontocetes, Stone and Tasker, 2006; fin whale *Balaenoptera physalos*, Castellote *et al*., 2012). Such a response has also been observed in mobile invertebrates such as the southern reef squid *Sepioteuthis australis*, where caged individuals moved away from air gun noise, and spent larger proportions of time in the cages' sound shadow, the area with the lowest noise within the sound field (McCauley *et al*., 2000; Fewtrell and McCauley, 2012). These behaviours highlight, that when possible, animals will travel away from unwanted noise towards quieter environments.

The aim of the present study was to provide information on a behavioural response of *H. gammarus* to anthropogenic noise playbacks, testing if the animals moved away from,

or towards intense noise exposure, or showed no behavioural change in response to this noise.

5.1.1 - Hypotheses

H_1: *Homarus gammarus* will actively move away from high intensity anthropogenic noise playbacks.

H_2: *Homarus gammarus* will spend a higher proportion of time in the lowest noise zone of the experimental tank during noise exposure.

5.2 – Methods

5.2.1 - Permits and Ethical Approval

The work conducted required no specific permits but was conducted following the ethical guidelines of Edinburgh Napier University.

5.2.2 – Animal Sourcing and Husbandry

A total of 20 *H. gammarus*, three females and 17 males, of varying size (250 to 580 g weight and 51 to 94 mm carapace length) were provided by the creel fishers working out of St Abbs, Berwickshire, UK (Figure 5.1), all lobsters were provided free of charge and their condition (size, missing limbs etc.) assessed prior to their inclusion in the experiments, with poor condition lobsters discarded. Lobsters were collected between May and November 2016 by the fishers, and by manually deployed creels set in shallow water off the coast of St Abbs Marine Station by marine station staff during the same period. This area is fished by a small number of fishers, specialising in pot caught shellfish including *H. gammarus*. During the summer the surrounding waters experience anthropogenic noise regularly from the fishing vessels and small pleasure craft, used for recreational diving. Larger ships pass regularly off shore at cruising speeds in turn adding to the anthropogenic component of the marine soundscape.

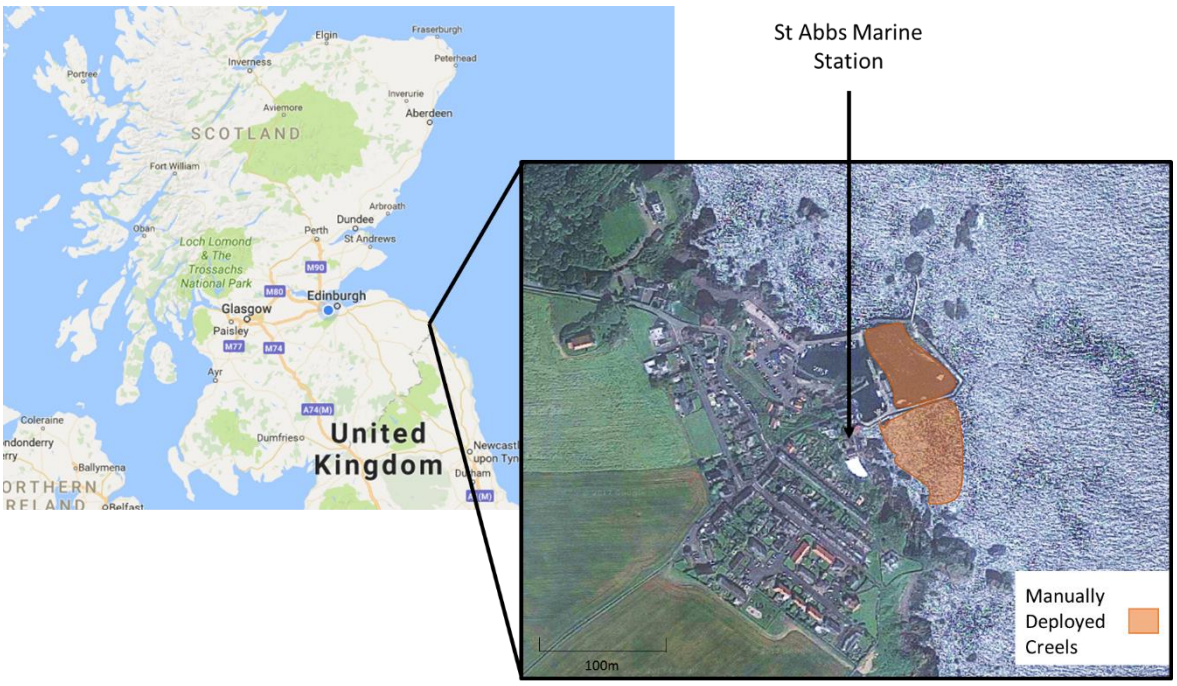

Figure 5.1 | St Abbs. Location of marine station and lobster collection at St Abbs, Berwickshire, UK. Adapted from Google Maps, 2016.

Following collection, the lobsters were transported the short distance back to St Abbs harbour and passed to St Abbs Marine Station where the animals were housed for the duration of acclimation and exposure. The lobsters were subsequently kept in small groups of similarly sized animals inside a 675 L holding tank with flow-through natural seawater. All lobsters had their claws banded to prevent fighting, and were provided optional shelters, plastic piping weighed down with large rocks, to allow them to separate if desired. The lobsters were fed daily ad libitum on cooked mussel meat and any uneaten food was removed from the tank at the end of the day. The holding tank was fitted with a subsurface inflow pipe to prevent noise from falling water or collision with the tank floor, reducing the ambient sound levels. The flow rate of this system varied over the course of acclimation depending on tides, due to the raw seawater draw design of the Marine Station. Salinity and temperature in the tanks matched the surrounding coastal waters (10 - 14 °C, salinity 32 - 35 ppt; NO_2: < 0.3 mg L^{-1}; NO_3^-: 0 mg L^{-1}; NH_3^+: ≤ 0.25 mg L^{-1}; pH: 7.8 - 7.9). All animals were acclimated in holding tanks for a minimum of two weeks prior to exposure, and allowed an additional 12 h acclimation prior to any experimental exposure. All possible

effort was made to keep the sound levels in the holding tanks as low as possible and matching those of the control treatments (Figure 5.2).

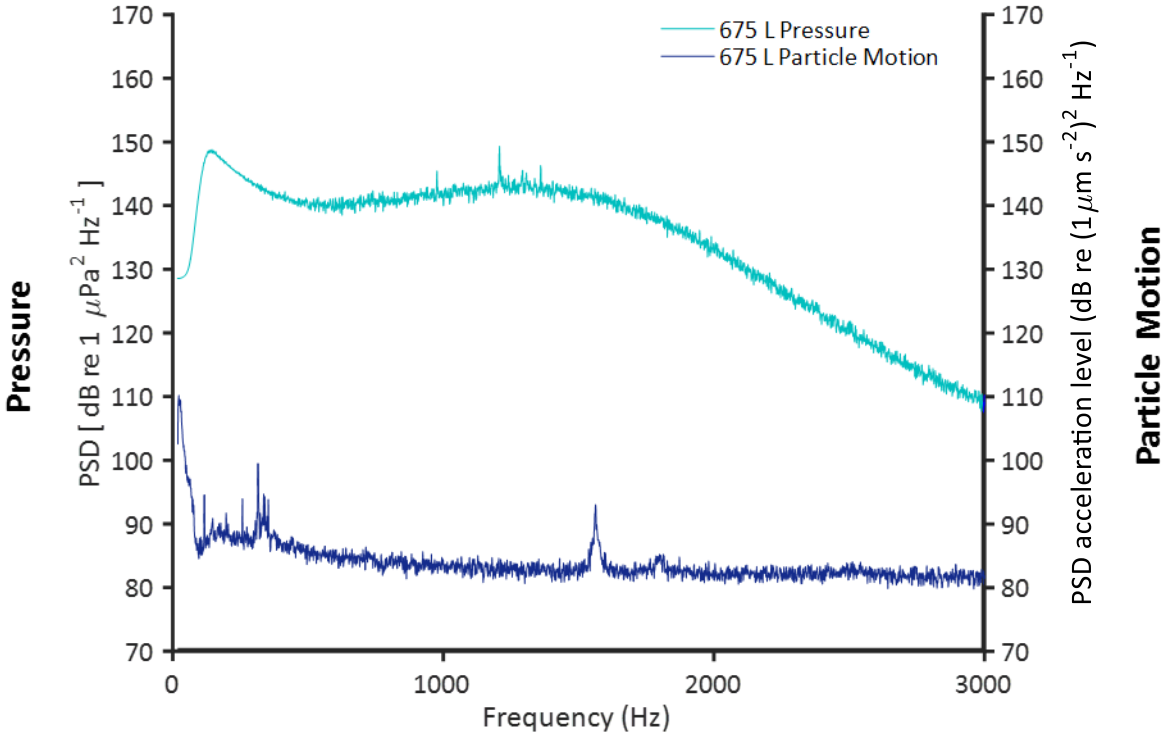

Figure 5.2| Acoustic analysis of holding tanks. Mean power spectral density of 30 s of each sound condition of acoustic pressure and particle acceleration for holding conditions.

5.2.3 - Sound Recordings and Playback

Ship noise playbacks produced by Wale *et al.* (2013) were used in all experiments, the details of which can be found in Chapter 4.1. Briefly, recordings of large vessels travelling at constant speeds (<10 knots) at approximately 200 m distance were taken at three major UK ports. All recordings were made with a calibrated omnidirectional hydrophone (HiTech HTI-96-MIN with inbuilt preamplifier, High Tech Inc., Gulfport, MS, U.S.A.) and an Edirol R09- HR 24-Bit recorder (44.1 kHz sampling rate, Roland Systems Group, Bellingham, WA, U.S.A. Sound samples of 60-140 s, incorporating the highest amplitude of the ship passes were repeated to create noise tracks in Audacity 1.3.13 (http://audacity.sourceforge.net/).

Experimental tracks were compiled in Audacity 2.0.5 and included a 30 s fade in, 6.5 min of ship noise and a 30 s fade out for each of the recorded vessels. A random selection of these tracks was compiled to create a two h playback track of continuous ship passes (Figure 5.3a), preceded by eight h of silence playback (to allow nocturnal deployment). Experimental tracks were played back as WAV files. The set-up consisted of a laptop (Acer E5-571 series, Acer Inc., Xizhi, New Taipei, Taiwan); amplifier (Samson Servo 300; 150W; frequency response: 20-20,000 Hz, Samson Technologies, Hauppauge, New York, USA); and Clark Synthesis AQ339 underwater speaker (effective frequency range 20-17,000 Hz, Clark Synthesis Inc., Littleton, CO, U.S.A). To map the sound field of the experimental tank (Figure 5.3), tracks were re-recorded at marked points (HiTech HTI-94-SSQ with inbuilt preamplifier, Roland R-26 24-bit recorder – calibrated as of chapter 4.1 in PAMGuide; Merchant *et al.*, 2015) and modified (uniform amplification or attenuation, 1 kHz low pass filter, 48 dB Rolloff, filter applied 5 times, Figure 5.3b) to give the desired exposure pressures and attenuation over the 7m length of the exposure tank chosen for this experiment (Figure 5.3). Particle acceleration was measured using a calibrated sensor (Wale *et al.*, *In Prep*; see chapter 3), consisting of a STMicroelectronics LIS344ALH triaxial accelerometer (STMicroelectronics, Geneva, Switzerland) potted in clear epoxy resin and suspended via 1.0mm diameter elastic cord to two interlocking 3D printed nylon rings. The accelerometer was linked to a Roland R-26 24-bit recorder for recording, and chosen due to its mobility. Particle acceleration was recorded separately for all three axes and combined internally within paPAM during the analysis process.

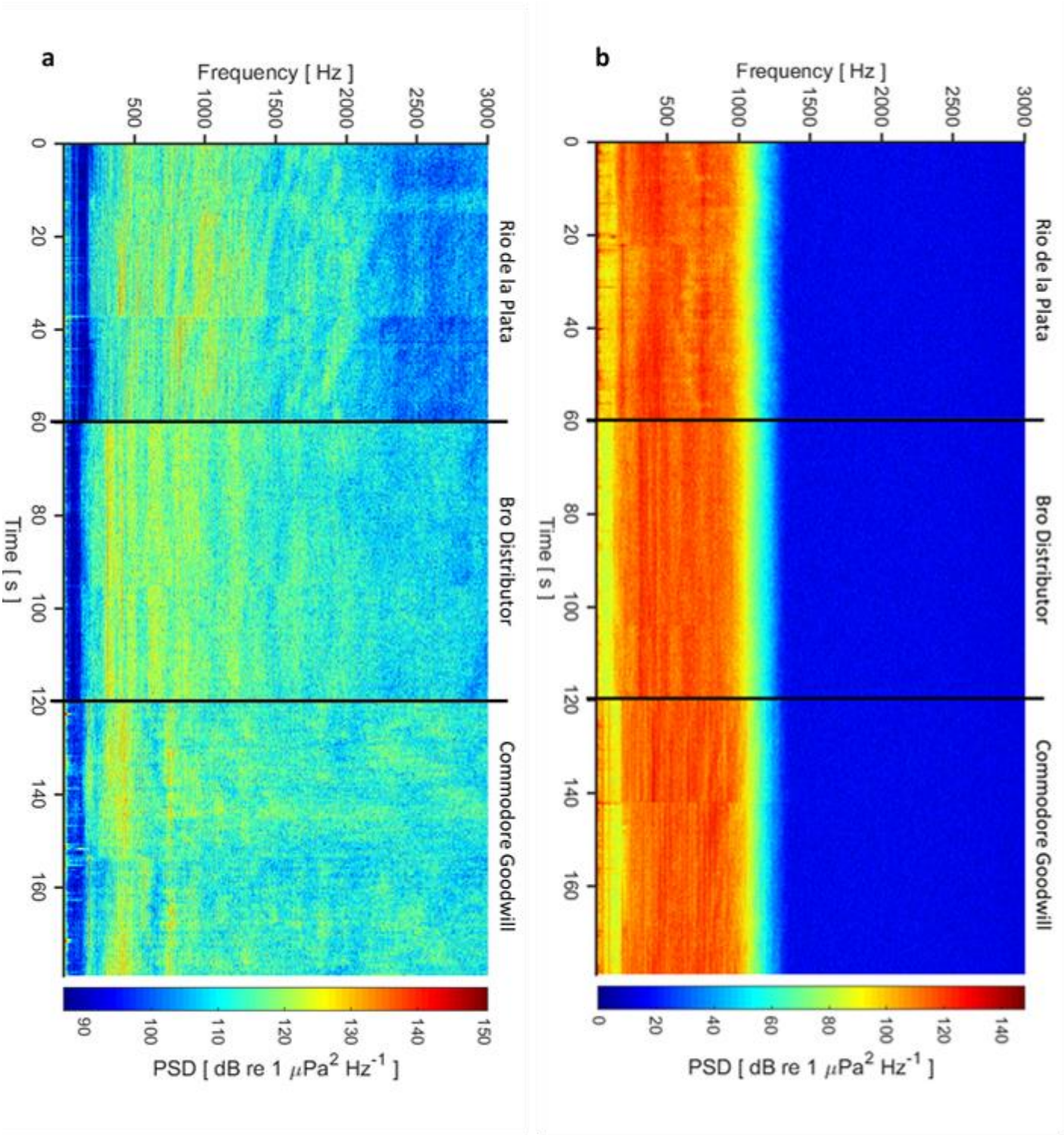

Figure 5.3| Spectral analysis of acoustic stimuli. Power spectral density of 1 min of each (a) ship passes used in the generation of the playback tracks (b) the same ship passes after low pass filters have been applied. Analysis performed in MATLAB R2015b, Hann window with 1 s length and 50 % overlap, fft length = 48 kHz resulting in 1 Hz bands.

Reference sound pressure and particle motion readings were taken at 18 points throughout the seven m tank to represent the sound source, the tank corners, and the middle of each exposure zone (Figure 5.4). Attenuation curves were then modelled in excel to generate sound levels for the rest of the tank. To do this the tank was split into excel cells (15 by 20) each given an arbitrary value to represent its position in the tank. Values ranged from 0.6 to 3.4 in 0.2 increments along the tank width, and 0.6 to 4.4 in 0.2 increments along the tank length. Integers represented the position of the initial measurements taken along the centre line of each noise zone. A 2nd order polynomial trend line was fitted to the reference readings along the width of the tank, the equation of which was used to generate sound levels for the empty cells. The position value for each empty cell was used as x in the produced equation. This process was repeated along the length of the tank using the reference readings along with the newly generated sound levels. A 4th order polynomial trend line was fitted to these numbers and the equation was used to generate the remaining sound levels (Appendix D).

Received sound pressure levels peaked at 138-141 dB re 1 μPa^2Hz^{-1} for zone 1 and 127-130 dB re 1 μPa^2Hz^{-1} for zone 4, with the lowest pressure level in the tanks present in zone 4 at 105-106 dB re 1 μPa^2Hz^{-1} (Figure 5.4a, Figure 5.5a), as measured in PAMGuide (Merchant *et al.*, 2015). Particle acceleration peaked at 190-195 dB re 1 $(\mu m\ s^{-2})^2Hz^{-1}$ for zone 1 and 160-166 dB re 1 $(\mu m\ s^{-2})^2Hz^{-1}$for zone 4, the lowest particle motion was found in zone 1 at 140-156 dB re 1 $(\mu m\ s^{-2})^2Hz^{-1}$ and zone 4 at dB re 1 $(\mu m\ s^{-2})^2Hz^{-1}$ 154-158 (Figure 5.4b, Figure 5.5b), as measured in paPAM (Nedelec *et al.*, 2016). Although tank mapping was conducted as fine scale as feasible, the exact sound levels will vary throughout the tanks. However, the aim of this study was to determine the potential impact of additional anthropogenic noise in the environment, rather than establishing the precise links between a given sound levels and responses.

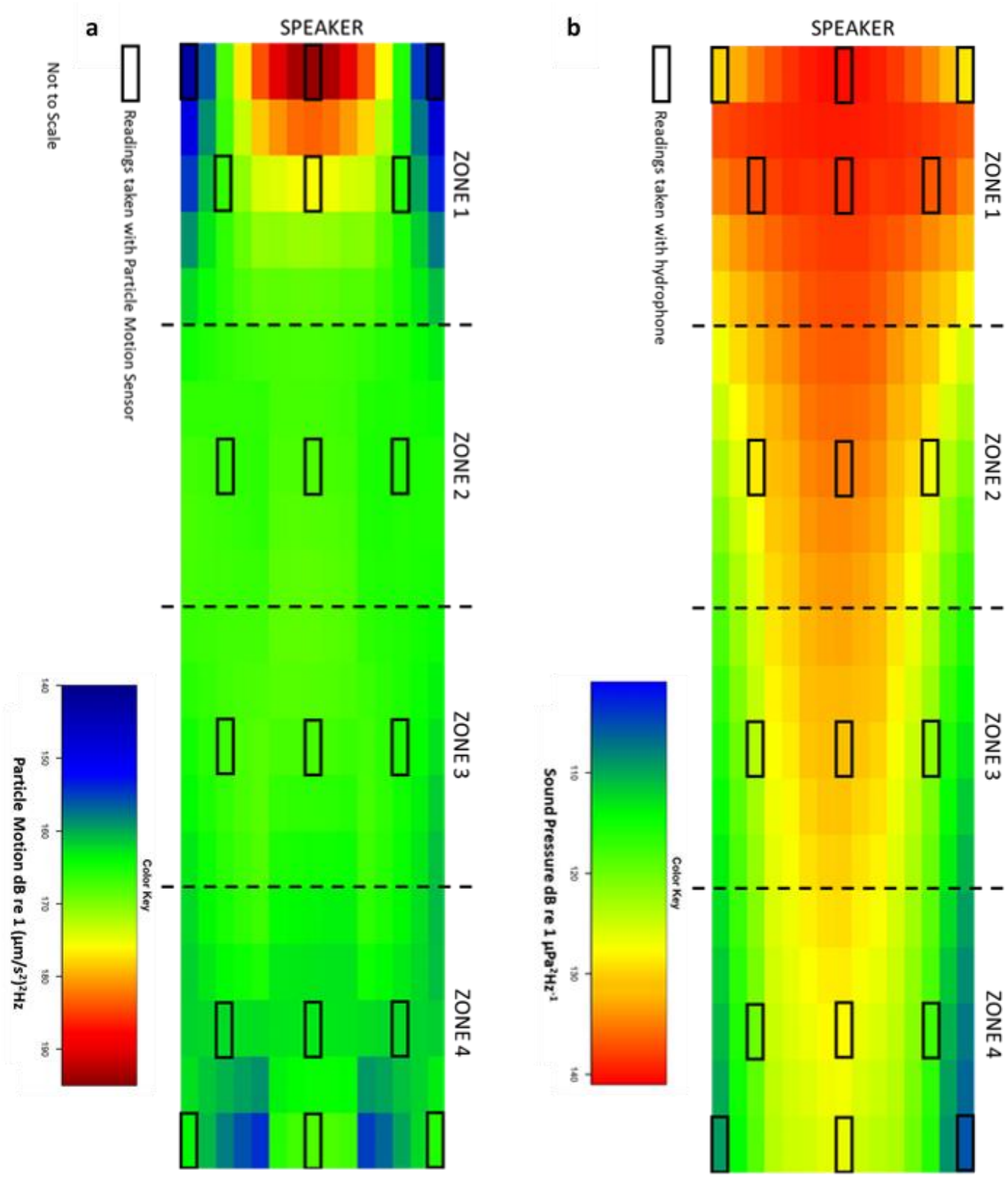

Figure 5.4 | Sound exposure map. Mean power spectral density of 30 s of each sound condition of acoustic pressure and particle acceleration for experimental tank conditions. Measurements taken at 18 locations in the seven m exposure tank and sound maps modelled from these readings. Particle motion averaged across axes in paPAM (Nedelec *et al.*, 2016). Colour representative of measurement central to each rectangle. Tank 7 m long and 2 m wide.

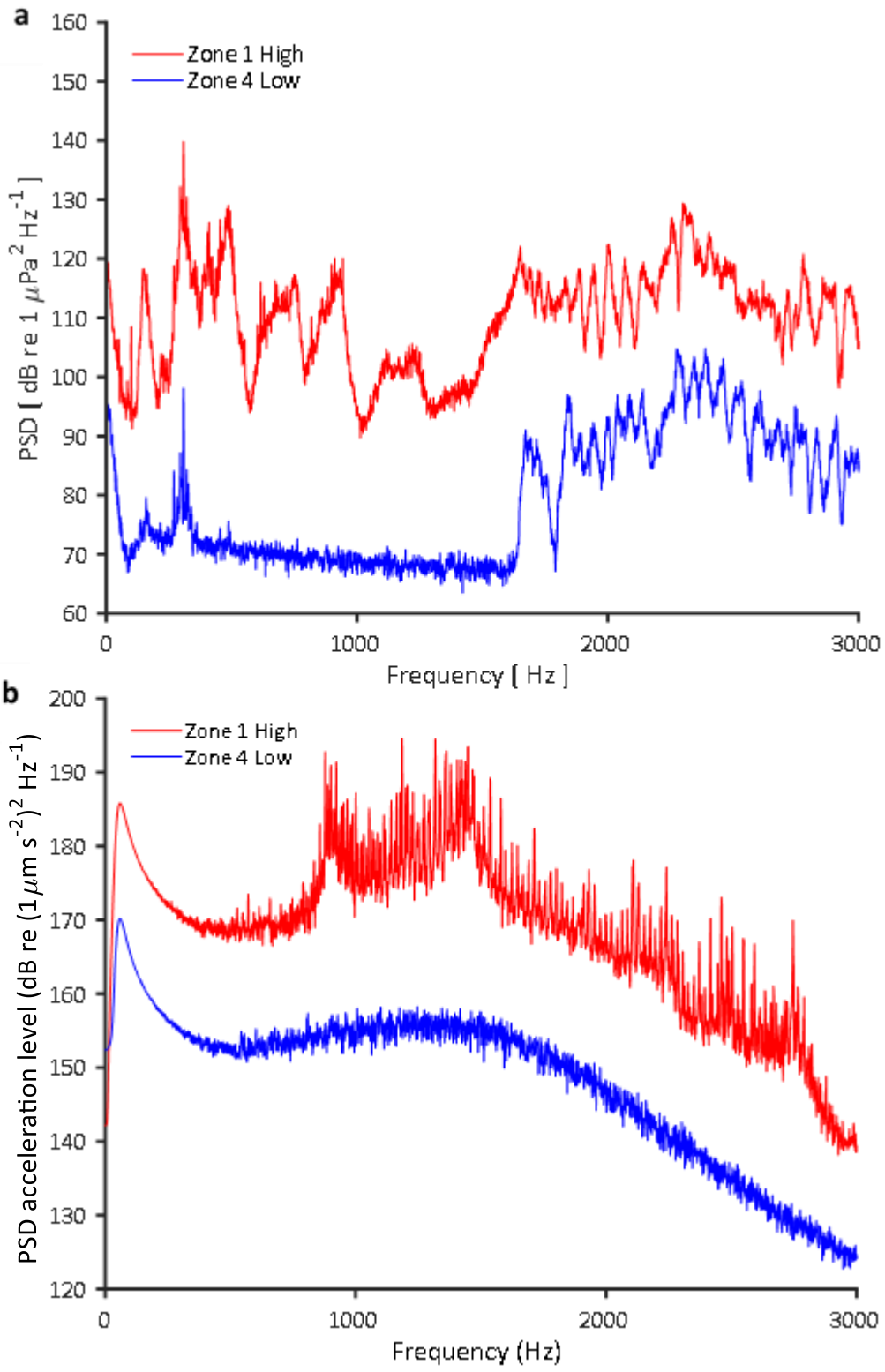

Figure 5.5| Analysis of acoustic stimuli and sound playback conditions. Mean power spectral density of 30 s of each sound condition of (a) acoustic pressure and (b) particle acceleration for centre of zone 1 (red) and centre of zone 4 (blue).

5.2.4 - Exposure

A total of three exposures were performed. An initial exposure was conducted throughout May 2016 acting as a preliminary trial to establish robust methods and appropriate filming conditions. No biological data were collected or analysed from this exposure. The second (summer) exposure took place from 12th - 27th of August 2016, and the third (winter) exposure from the 10th - 21st of January 2017. Data from both runs are analysed below.

Individual lobsters were placed in a seven m long, two m wide exposure tank, with a water level sitting at 600 mm (water volume 8400 L) 12 h prior to exposure onset. No shelter was provided in the experimental tank to remove the potential for lobsters to remain in shelter despite the noise intensity, preventing any changes to their movement being measured. One animal at a time was placed in the centre of the tank, but allowed to freely roam during acclimation. The tank was set up with two speakers, one at either end (Figure 5.6). One speaker played back ship noise and the other was powered on to control for potential electromagnetic effects, but did not produce sound. Eight h prior to noise exposure the playback tracks where started (4pm) allowing eight h of silence playback (4pm-12am) before two h of ship noise playback (12am-2am) at night when lobsters are most active (Smith *et al.*, 1999). Throughout exposure the lobsters where filmed by four adjacent night vision CCTV cameras (Sannce 4CH DVR CCTV system, Sannce, Mongkok, Hong Kong) each covering an exposure zone encompassing a quarter of the tank area (Figure 5.6). The tank area was additionally lit with six infrared spotlights (Fuloon IR 140 LED illuminator, wavelength 850nm, Fuloon, Shenzhen City, China) to penetrate the water and provide suitable conditions for filming during the night, without disrupting the lobster's behaviour. Filming took place over the two h of noise exposure (summer and winter), one h before the start of the noise exposure (winter), and one h after the end of the noise exposure (winter). A malfunction with the filming equipment prevented the periods before and after noise exposure form being filmed during the summer exposure. A total of ten lobsters of varying size (carapace length [mm] and mass [g] summer: 51.4 – 93.9, 82.3; 306.9 – 479.9, 403.89; winter: 72.0 – 100.9, 84.3; 231.1 – 581.5, 380.3) and sex (see animals and husbandry above) were exposed individually over a 10 day period (Figure 5.7), although all effort was taken to expose all animal consecutively there were a number of

unavoidable sequence breaks (Figure 5.7). Each lobster was exposed to noise only a single time, and a different lobster was used in each run of the experiment, with the tank water flushed to remove any potential chemical cues.

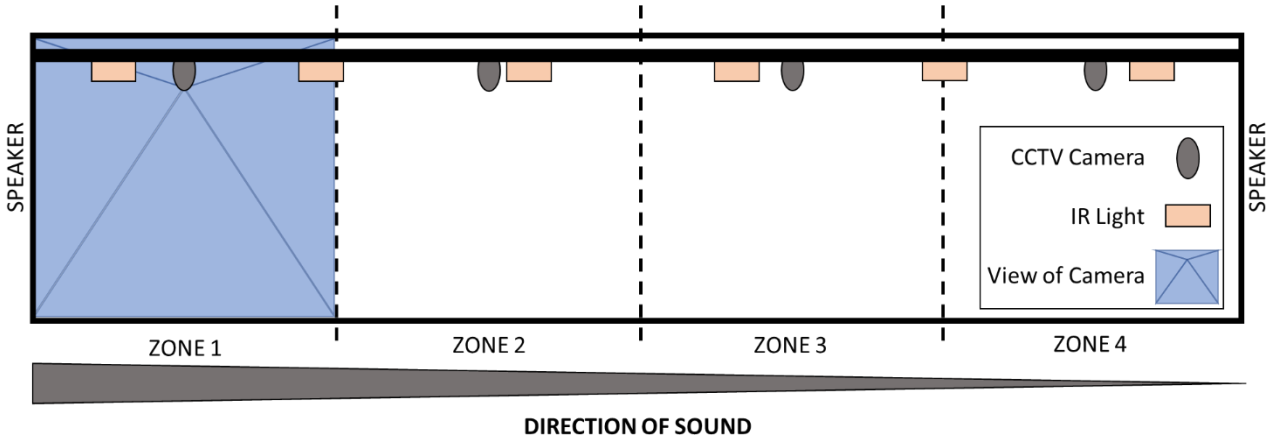

Figure 5.6| Lobster choice chamber set-up. Experimental tank for lobster exposure. Four night vision CCTV cameras and six infrared spotlights cover the entire exposure area for filming. One speaker played back ship noise, whilst the other was powered on but produced no noise. Tank 7 m long, 2 m wide and water depth 600 mm. Speakers held 200 mm from the tank wall.

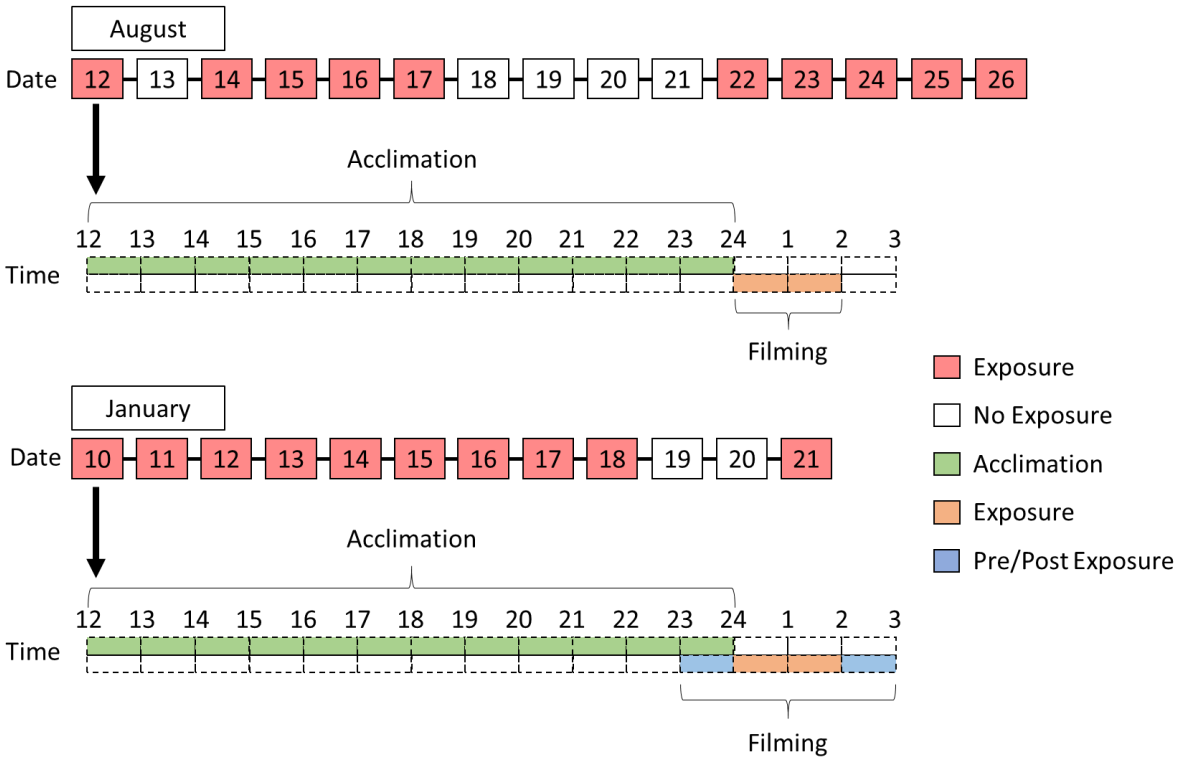

Figure 5.7| Experimental set-up: Ten lobsters exposed individually in two replicate runs, one in August 2016 and one in January 2017. Each lobster was used only once, and in only one run of the experiment. Lobsters were exposed in a seven m long, two m wide tank, with a water level sitting at 60 cm (water volume 8400 L). The magnified timeline was followed on all exposure dates.

Once all exposures were completed, all video files were extracted from the CCTV system as .avi files. Each file consisted of one h of footage for a single exposure zone (e.g. four h of exposure for zone one would be split into four .avi files). All videos were manually analysed, and the time spent in frame for each video recorded. Time in frame was measured as the time from when 50% of the lobsters' length was present on entering the frame until 50% of the lobsters' length remained on leaving the frame, movements in and out of frame were not recorded to account for the varying number of walls in each exposure zone. Cameras were positioned so that there were no overlapping sections, therefore the aggregate time spent in each frame was taken as the total time in that exposure zone. Time was recorded in s and converted to a proportional of total exposure time.

5.2.5 - Statistical analysis

All statistics were performed in R version 3.3.1 (The R foundation for Statistical Computing). Data were tested for normality and heterogeneity of variance around the mean, normality was shown for all data with logit transformation. A two-way ANOVA was used to assess differences between logit transformed time spent in each zone (dependent variable) as a factor of the zone (independent variable) and the exposure (independent variable). One-way ANOVAs were used to assess the logit transformed time spent in each zone (dependent variable) as a factor of the zone (independent variable), this was repeated separately for each exposure. Finally, a mixed-model ANOVA was used to assess the time spent in each zone (dependent variable) as a factor of the zone (independent variable) and the period of exposure, before, during, and after exposure (independent variable), period of exposure was treated as a random effect in this model.

5.3 – Results

One lobster from the winter exposure did not move from the corner of the tank (next to the speaker) for the entire period of filming (before, during, and after exposure). As such it was considered an outlier and removed from the analysis (see Appendix D). The time spent in each exposure zone differed significantly between the two exposures (two-way ANOVA, $F_{7,68} = 56.56$, $P < 0.0001$), and the two seasons were subsequently analysed separately. During the summer exposure, the relationship between exposure zone and time spent in that zone was highly significant (one-way ANOVA, $F_{3, 36} = 184.90$, $P < 0.0001$, Figure 5.8). Lobsters spent significantly more time in zone 4 than any other zone (Figure 5.7), with this zone corresponding to the lowest noise level of the tank.

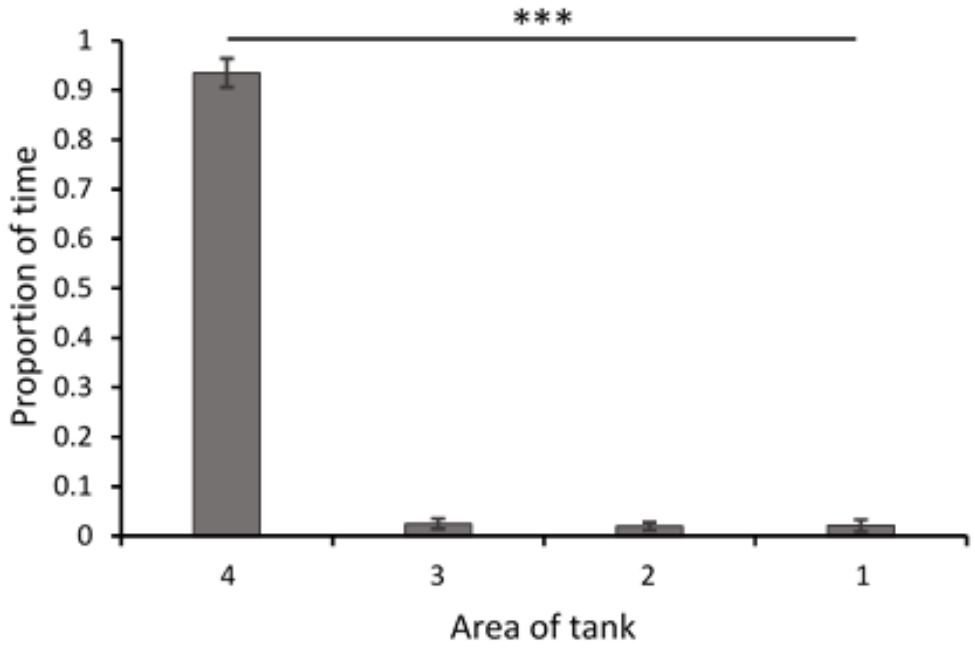

Figure 5.8| Apparent noise intensity preference in *H. gammarus* (summer). Mean ± SE time spent in each exposure zone during the summer exposure*** (n=10).

The interaction between exposure zone and the time the lobster spent in that zone was also significant in the winter exposure although to a lesser degree (one-way ANOVA, $F_{3, 32} = 4.13$, $P = 0.014$, Figure 5.9a). There was also a significant difference in the relationship between exposure zone and the time spent in that zone, across time period (Mixed Model ANOVA, $F_{1,3.78} = 6.49$, $P = 0.012$, Figure 5.9b). Here lobsters spent significantly less time in zone 1, and significantly more time in zone 4 once noise exposure began.

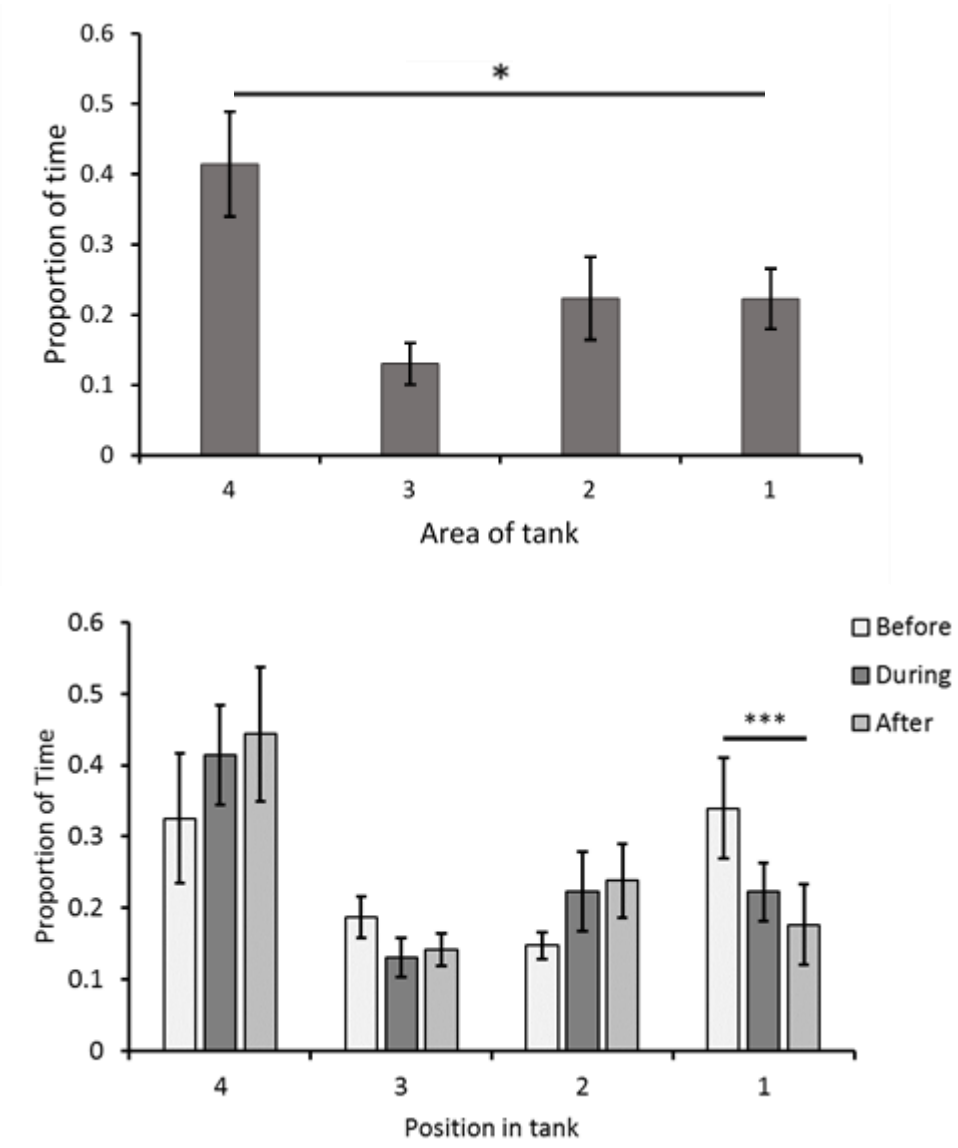

Figure 5.9| Apparent noise intensity preference in *H. gammarus* (winter). (a) Mean ± SE time spent in each exposure zone during the winter exposure* (n=9). (b) Mean ± SE changes in time spent in each exposure zone before and after noise exposure during the winter exposure*** (n=9).

5.4 – Discussion

This study is the first to investigate the effects of anthropogenic noise on the *H. gammarus*. Here, using movement as a metric, effects on the behaviour of these lobsters were shown, for the first time in anthropogenic noise research. Noise altered the behaviour of these animals and caused them to move away from the noise source.

It was hypothesised that lobsters would move away from the noise source and that they would spend the majority of their time in the zone with the lowest noise. These two hypotheses cannot be rejected, as in the winter experimental run there was a tendency for lobsters to move away from high intensity anthropogenic noise, and towards the lowest noise zones of the exposure tanks. In both the summer and winter exposure the lobsters spent the majority of their time away from the noise source, and during winter there was strong evidence that the lobsters actively moved away from areas of high noise.

The apparent seasonal nature of the observed response, with lobsters spending far more time in the high noise zone in winter than in summer (22% in winter, 2% in summer), is likely due to the natural seasonally changing behaviour of *H. gammarus*. During winter months, adult lobsters will spend the majority of their time in shelter (McLeese and Wilder, 1958; Smith *et al.*, 1999). The seasonal variation in behaviour was shown by Smith *et al.* (1999) who identified a drastic decrease in the activity levels of *H. gammarus* in the low temperatures of late winter. As no shelter was provided during the exposure, the lobsters may have moved around the exposure tank, a behaviour seen during video analysis, in an attempt to locate an appropriate shelter. The urge to find shelter may have had a stronger influence over the animals' movement behaviour than a desire to avoid high noise levels. Remaining in high noise levels for prolonged periods can increase the lobsters risk of noise induced physiological or biochemical changes, and further increase the potential negative effects that noise is having on these animals. In the summer, lobsters have a lower drive for sheltering (Smith *et al.*, 1999) and move between areas of their environment for foraging and social interactions. Here, the effects of noise, such as moving away from the noise source as observed here, become more apparent, with lobsters readily moving away from areas of high noise. In the field, this could lead to actively displacing lobster populations in high noise

environments, effectively changing the food web dynamics and species interactions of that area.

If lobsters are displaced from their territory by the introduction of high noise, they potentially may come into conflict with another *H. gammarus* as they move away from the noise source and into the territory of a competitor. Long range movements of lobsters are rare (Jensen *et al.*, 1994) and their territories are small often staying within a single small reef system or rocky outcrop (Jensen *et al.*, 1994; Skog *et al.*, 2009). As such, the likelihood of displacement into another lobster's territory is greater. Any movement away would have negative connotations for fisheries. European lobsters are primarily caught with creels (Marine Scotland, 2016) which are placed in areas with a known lobster population. Displacement of lobsters from these areas, due to increased noise, would subsequently affect fisheries yields within these areas, and potentially move the lobsters to areas where fishing is restricted.

5.5 – Uncertainties

Although all attempts were taken to refine the procedures used in this experiment, there are some associated methodological limitations. The first of these limitations appear in the presentation of the noise exposure. Due to the width of the exposure tank, the lobsters could escape the high intensity noise by moving to the side walls of the tank, rather than along the tank towards its end wall. To avoid this, a corridor-like structure could be constructed running centrally along the length of the exposure tank, thus creating a more direct representation of the lobsters' movement away from the noise source. It should also be noted that examining the noise field of the experimental tank revealed that the one lobster excluded from the analysis spent the entire experiment in the area of the tank with the lowest particle motion, and as such may have followed that pattern seen in the other lobsters by avoiding the higher noise areas. The corridor-like structure mentioned above would prevent this from happing in subsequent experiments. The second obvious limitation concerns the filming process. To allow the whole tank floor to be covered by the CCTV cameras, a large distance was required between the individual cameras. This distance prevented more detailed behavioural observations from being produced, and therefore

reduced the quantity of responses recorded. In future work, it would be beneficial to create a larger camera network allowing more close-up filming of the lobsters. Finally, due to equipment constraints during the experimental trials, noise playbacks were only presented from a single end of the seven m tank. The end of the tank acting as the high noise zone was switched between the summer and winter experiments, however it remained constant between each individual exposure each season. The results seen here therefore could be attributed to a tank effect, and an additional run of the experiment where the noise is switched between exposures would allow this potentially confounding factor to be removed.

The lobsters were provided no shelter in this experiment, allowing more accurate recordings of their movement within the exposure thank. However, this is not truly representative of the natural environment of the animals. As such, the results generated here may not have occurred if the animals were able to shelter, potentially remaining in shelter despite high noise conditions. This study however is useful in showing that the behaviour *H. gammarus* can be affected by anthropogenic noise, allowing future studies to be developed and implemented to identify how these responses vary in different environments.

The noise exposure presented in these experiments only lasted for two h, between 12pm and 2am, and although occurring at a time where the lobster is most active, and potentially susceptible to noise induced effects, is a short exposure length. As such, the reactions seen in this study may change, becoming less pronounced or even stopping, if the lobster is exposed to noise for a longer period. A short exposure to ship noise however is not unrealistic, and the periods of no noise following exposure may allow the lobster to "forget" the noise producing the same effect of repetitive exposure. Therefore, as suggested below, experiments examining responses of *H. gammarus*, to repeated and chronic noise exposure should be performed in the same set-up to determine any changes in response.

5.6 – Future Directions

To further this work a number of follow on steps are proposed. Initially, to appropriately disentangle the seasonal behaviour, a second run of the summer trial incorporating video footage from before and after noise exposure, as per the winter trial, is planned for August 2017. From this, changes in behaviour caused by the onset of anthropogenic noise can be compared across seasons. This experiment will also utilise a design where the noise is presented from alternating ends of the tank between exposures in an attempt to remove any tank based effects. Following the mechanistic integrative approach to noise research proposed in chapter 2 (and realised in chapter 4), a number of physiological and biochemical experiments will aid in obtaining a more complete understanding of how anthropogenic noise affects these animals. Samples for these analyses can be taken following the exposures performed in the above discussed experiment. Variation in the lobsters' responses during repeated and chronic noise exposure, and across life history stages should be additionally explored. A further study should be conducted exposing lobsters to the same stimuli as presented here, but with the inclusion of shelter within their environment. Any potential trade-offs between noise and shelter can then be identified, and a more representative assessment of behavioural change can be made. Finally, field trials of this experiment should be undertaken, exposing lobsters in situ to anthropogenic noise, either through playbacks or through source noise (see chapter 2.1.2, p22), and any behavioural changes recorded.

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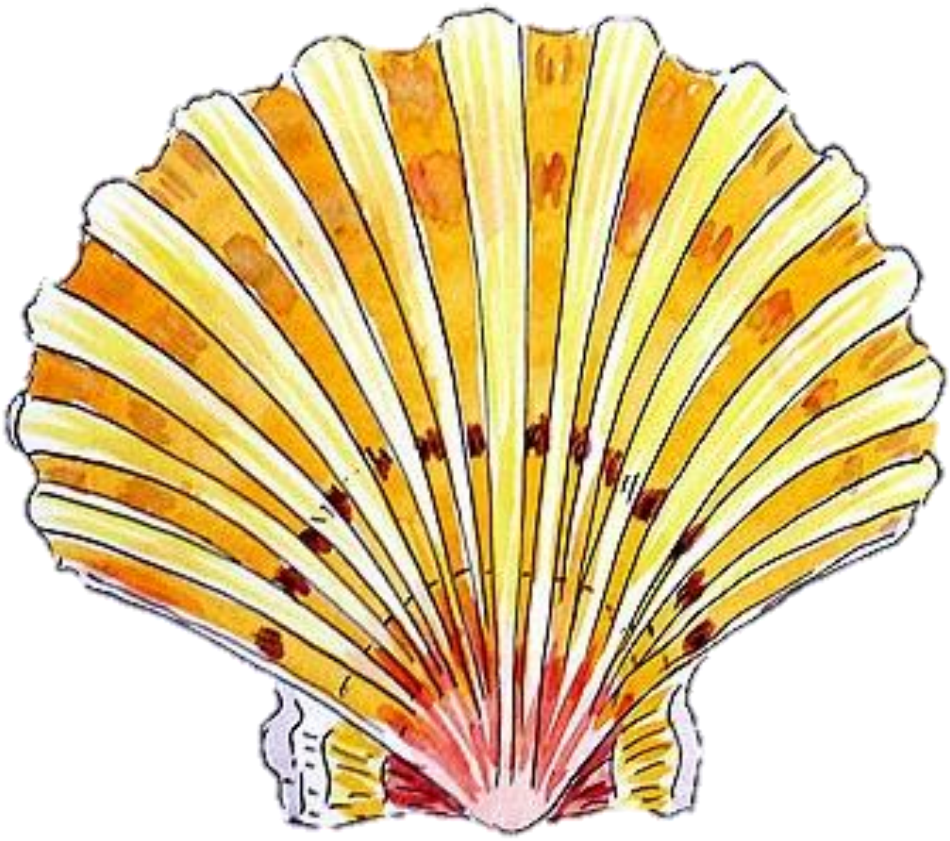
Chapter 6

Synthesis



Chapter 6

Synthesis

In this thesis, I have analysed the current state of the art of marine invertebrate anthropogenic noise literature, showing how methodological trends have developed as the topic evolved. From this analysis, I presented three research strategies that can be employed to better study the effects of noise on these animals. I then addressed two current knowledge gaps surrounding marine invertebrates and anthropogenic noise. The first of these gaps pertains to the current inclusion of particle motion data in underwater sound research. Here, by having developed two functional, easily accessible, low cost particle motion sensors, I have improved the access to particle motion readings in bioacoustic research, and enabled this noise metric to be easily considered in all future studies in aquatic bioacoustics. The second gap that I began addressing was the lack of information on the effects man-made noise is having on many marine invertebrate species. Here, focusing on two model species, the blue mussel *Mytilus edulis* and the European lobster *Homarus gammarus*, I investigated noise induced responses on a range of biological processes. The new data increases the available information on how anthropogenic noise affects ecologically and commercially important sessile and mobile marine invertebrate species.

6.1 - Main Findings

Through analysis of the invertebrate anthropogenic noise literature, a number of methodological trends were identified showing how, in the last 5 years, researchers have moved studies away from previously identified limitations/uncertainties. Through this analysis, three research strategies were formulated as general guidelines to inform the development of investigations into the effects of noise on marine invertebrates. The first two of these strategies mirror the methods suggested by Kight and Swaddle (2011) for terrestrial noise research, and propose that when possible the effects of noise should be assessed on multiple levels of biological organisation. The second of these strategies expands

on the first to suggest that when assessing ecological responses or fisheries yields, multiple species should be assessed in a single study. The final strategy focusses on multiple species experiments, and suggests that when investigating multiple species the study should focus on a single biological response, sufficiently comparable across all species used.

Two fully functional particle motion sensors were successfully developed utilising commercially available accelerometers, and lacking the previously identified limitations of other available particle motion sensors. The accelerometers these two new sensors are based on cost £1200 and £3.50 respectively, which is significantly cheaper than any commercially available particle motion sensors to date (£10,000 at the start of this project). Of the two workable sensors, the cheaper one, although less precise, is recommended as an appropriate accelerometer for adaptation into a particle motion sensor. This is due to its accuracy, ease of use; as it can easily be attached to hand held recorders providing high mobility, and low cost; with the total cost of the potted sensor ~£30. The higher cost sensor is more precise and accurate than the low cost sensor, and can also be employed in bioacoustic studies. However, the high cost, low mobility, and low ease of use, make it less ideal for field studies and experiments where space and power are limited.

The effects of anthropogenic noise on benthic marine invertebrates, were studied for two species, *M. edulis* and *H. gammarus*. *M. edulis* was chosen to represent a sessile benthic invertebrate unable to move away from any noise sources, and, due to its limited complexity, likely to display few visually obvious responses to environmental stressors. Experiments were therefore conducted following a mechanistic integrative approach (see chapter 2.4.1, p39, Chapter 1.1, p6, and Kight and Swaddle 2011) where responses to noise were investigated on multiple levels of biological organisation from behaviour and physiology, through to biochemistry and genetics.

For the first time in noise research, DNA damage was investigated as a response parameter. When exposed to ship noise playbacks *M. edulis* exhibited significantly higher levels of DNA single stand breaks than in control conditions. Oxidative stress endpoints were also used for the first time as a biomarker of the effects of underwater noise in marine organisms. Significantly higher thiobarbituric acid reactive substances (here, malondialdehyde) were identified in noise exposed mussels compared to those in a silent control, with the build-up of MDA the likely driver for the observed DNA damage.

M. edulis additionally displayed a number of physiological and behavioural stress responses when exposed to anthropogenic noise, in the form of decreased oxygen consumption, decreased algal filtration, and increased valve gape. The combination of all three of these responses indicates a shock response where, with the sudden onset of noise, mussels freeze and attempt to reduce energy, and therewith reduce their oxygen demand. Relaxation of the posterior and anterior adductor muscles lowers the energy demand associated with keeping the valves closed (Livingstone, 2013). Relaxing the adductor muscles (and as a result increasing the gape) of the valves requires less oxygen consumption and allows the mussel to conserve energy for successive responses to the experienced stressor. The reduced algal filtration, through cessation of the mussel-pump process used in active algal filtration (Riisgård et al., 2014) will further contribute to a reduction in energy expenditure.

Finally, noise induced genetic responses were explored in *M. edulis*, utilising the expression of heat shock protein 70 (Hsp70) as an indicator. This again is the first study in noise research to use a heat shock protein at the genetic level as a possible indicator of stress. However, no change in the expression level of Hsp70 was detected during noise exposure. The lack of Hsp70 expression could have resulted from a number of reasons including the lack of discernible genetic response to noise exposure in *M. edulis*. However, this is unlikely given the previously identified biochemical, physiological, and behavioural responses to noise. It is more likely that the lack of Hsp70 expression is produced by population (Sørensen et al., 2001) or species (Brennecke et al., 1998) variability, and is not representative of all genetic responses. In summary, the applied holistic approach has revealed a multitude of responses of this sessile benthic invertebrate to noise.

The second study species *H. gammarus* was chosen to represent mobile benthic invertebrates, with a more complex behaviour, and therefore potentially more directly observable responses. Here the focus was on the investigation of movement behaviour in response to noise playbacks. A noise intensity gradient was presented along the length of a seven m aquarium tank, and the time that lobsters spent in each intensity level recorded. The lobsters actively moved away from high intensity noise during the winter run of the experiment and spent the majority of their time in the lowest intensity areas of the exposure tanks during both summer and winter. This movement was more significant during the

summer exposure than the winter exposure, likely due to seasonally changing behaviours, and an increased desire to find shelter during winter. This seasonal variability in the responses of marine invertebrates exposed to noise in their environment has never before been described.

6.2 - Implications

The analytical framework demonstrated in chapter 2 can be used as a guide for the development and assessment of future research in the field of anthropogenic noise and marine invertebrates. The presented approach to selecting the optimal methods for noise research can be used across taxa as well as in other research areas with specific descriptors chosen for that field.

The work presented in chapter 3 on the development and construction of new low cost and easily accessible particle motion sensors can be followed by those working in the field of bioacoustics, and allow the construction of similar sensors for use in their own research. The low cost of these sensors will remove a current barrier present to researchers new to the topic trying to include particle motion data in bioacoustic studies, enabling these readings to be reported more often in the literature. This will increase the repeatability of studies, and allow more accurate comparisons between experiments.

The findings of the comprehensive experiments conducted on *M. edulis* (chapter 4) highlight the need to assess both visually obvious and cryptic responses to noise exposure in sessile marine invertebrates, and possibly other benthic species. By conducting research in such a way, identified responses can then be mapped together and any underlying drivers or interactions uncovered. Further to this, the biochemical effects of noise identified in this chapter highlight the need to consider noise as a potentially confounding factor in areas of research investigating the effects of other marine pollutants. As such, it is suggested that field monitoring programs for pollutants, e.g. the NOAA Mussel Watch Program, should regard noise as a potential (co)contaminant.

The information on the behavioural responses of *H. gammarus* presented in chapter 5 can potentially be used by those introducing noise into the marine environment, such as

renewable energy companies. Once the results if these experiments have been corroborated by follow-up studies in more realistic conditions, such companies, along with the fishers operating in the same areas, can then plan for potential species migration and reduction of lobster stocks. Any seasonal difference in the observed response (corroborated by further research) could be factored into the planning of activities such that the time period of minimal disturbance is used. The generated data on *H. gammarus*, along with that for *M. edulis*, can be used to inform government and industry of the effects that anthropogenic noise has on invertebrates. This in turn can help generate strategies to limit the effects of noise in the marine environment. The results obtained here for both sessile and mobile benthic marine invertebrates will also be useful when aiming to understand any ecosystemic effects of anthropogenic noise.

6.3 - Future Directions

To further the work conducted in this thesis, a number of avenues can be explored. Firstly, the established ecotoxicological methods effectively transferred to the discipline of underwater noise research can be used as a basis for further experiments. These methods were shown to provide reliable information on how noise affects the biochemistry of bivalve molluscs and can be expanded to cover other species within this taxon, as well as marine invertebrates as a whole. The methods for physiological and behavioural examination of noise induced responses can similarly be employed in other species in noise research to investigate and compare reactions. In addition to this, repeating the experiments conducted in this thesis utilising chronic or repeated noise exposure will allow comparisons to be drawn between exposure length and response. Similarly, exploration of context dependent exposures involving animals from environments naturally high or low in sound should be explored. Any evidence of habituation or tolerance to noise can then be detected, and any potential recovery from the initial response, e.g. the observed structural DNA damage, observed.

The work conducted on *H. gammarus* in chapter 5 should be repeated during summer to test if the "summer response" can be replicated, which would corroborate the current

conclusion that the lobsters' behaviour changes seasonally. This experiment can be further expanded to cover trade-offs between noise and other stimuli, such as the availability of shelter or food (e.g. Lima and Dill, 1990). Here the animals' response to noise may change. For example, it could be more beneficial for the lobster to remain in a high noise area in shelter than in a low noise area out of shelter. These trade-offs can also be explored in different species, and in multi species experiments exploring the interactions between predators and prey.

In addition to the development of laboratory based research, several methods of analysis trailed here can be taken into the field, or a field like environment, in experiments exploring the effects of noise in a more natural environment. Animals can be exposed in situ to playbacks of noise, or to the actual noise sources themselves. Although field based studies provide a number of challenges, once proof of concept is shown, such as the laboratory experiments conducted in this thesis, more effort can be placed on ensuring an accurate noise exposure. In this situation, it is also possible to expose multiple species present in the environment to noise at the same time, and build an ecosystem level analysis of the effects of anthropogenic noise.

Finally, further research can utilise the methods developed here to investigate the effects of noise, and add additional stimuli into experiments including other pollutants or stressors. Such multi-stressor experiments would allow a more realistic representation of the changing marine environment, and uncover and interactions between stressors. These stressors can combine additively, synergistically, potentiationally* or antagonistically, and it is therefore important to analyse their interactions to determine their ultimate effects.

* Dependent action in which a substance or physical agent at a concentration or dose that does not itself have an adverse effect enhances the harm done by another substance or physical agent (Wexler, 2000)

References

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Appendices

Pictures used

All drawings used for the chapter title pages were provided with permission by Richard Bramble (www.richardbramble.com).

Chapter 1 – "Langoustine"

Chapter 2 – "Native Oyster"

Chapter 3 – "Spiny Lobster and Crawfish"

Chapter 4 – "Mussels"

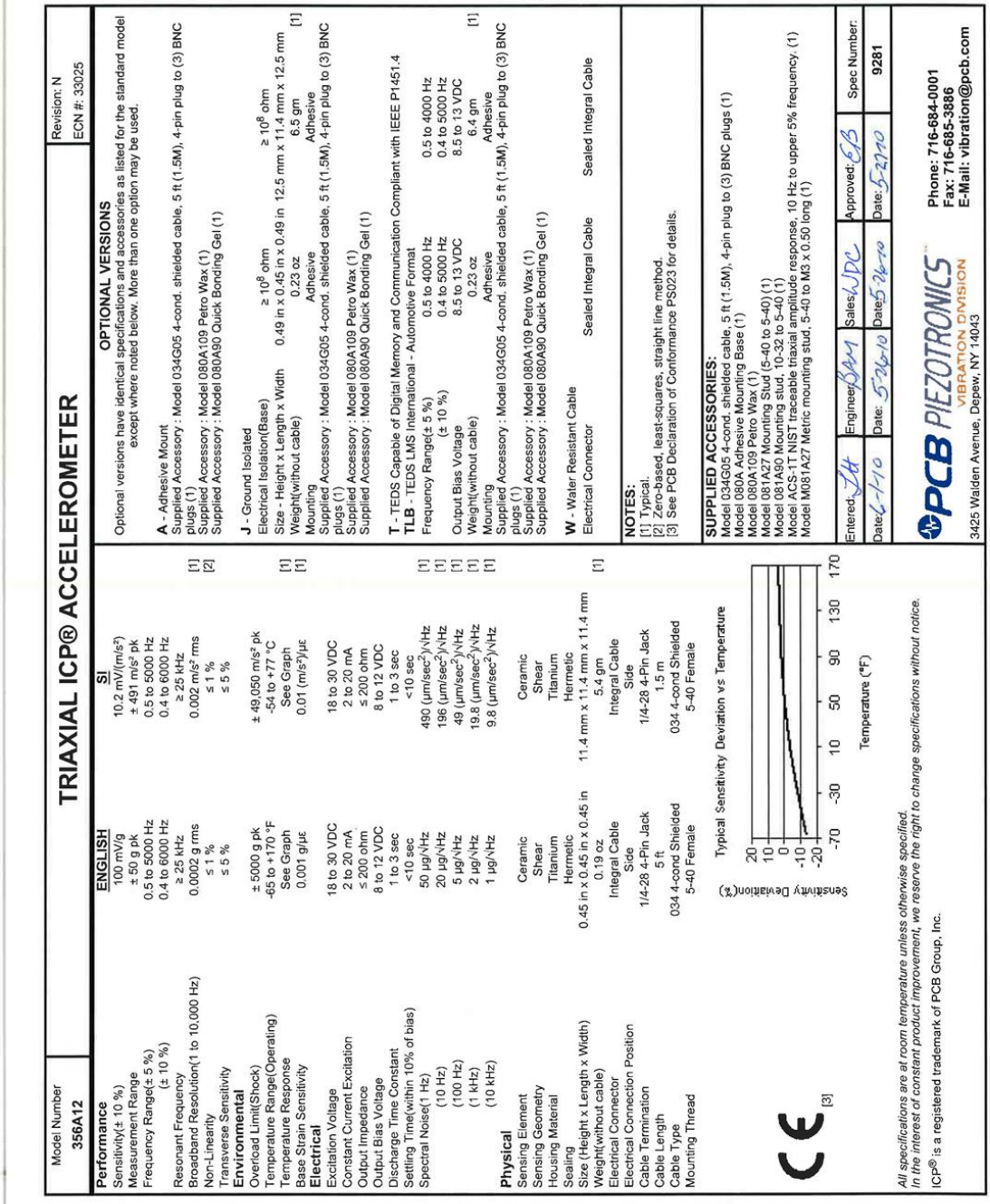
Chapter 5 – "Lobster"

Chapter 6 – "Scallop Study"

Appendices – "Squid"

Appendix A

Technical specifications and data sheets for the three commercially available accelerometers adapter for use as particle motion sensors in Chapter 3.



Model Number 356A12

TRIAXIAL ICP® ACCELEROMETER

Revision: N
ECN #: 33025

Performance	ENGLISH	SI	
Sensitivity(± 10 %)	100 mV/g	10.2 mV/(m/s²)	
Measurement Range	± 50 g pk	± 491 m/s² pk	
Frequency Range(± 5 %)	0.5 to 5000 Hz	0.5 to 5000 Hz	
(± 10 %)	0.4 to 6000 Hz	0.4 to 6000 Hz	
Resonant Frequency	≥ 25 kHz	≥ 25 kHz	
Broadband Resolution(1 to 10,000 Hz)	0.0002 g rms	0.002 m/s² rms	[1]
Non-Linearity	≤ 1 %	≤ 1 %	[2]
Transverse Sensitivity	≤ 5 %	≤ 5 %	
Environmental			
Overload Limit(Shock)	± 5000 g pk	± 49,050 m/s² pk	
Temperature Range(Operating)	-65 to +170 °F	-54 to +77 °C	
Temperature Response	See Graph	See Graph	[1]
Base Strain Sensitivity	0.001 g/με	0.01 (m/s²)/με	[1]
Electrical			
Excitation Voltage	18 to 30 VDC	18 to 30 VDC	
Constant Current Excitation	2 to 20 mA	2 to 20 mA	
Output Impedance	≤ 200 ohm	≤ 200 ohm	
Output Bias Voltage	8 to 12 VDC	8 to 12 VDC	
Discharge Time Constant	1 to 3 sec	1 to 3 sec	
Settling Time(within 10% of bias)	<10 sec	<10 sec	
Spectral Noise(1 Hz)	50 µg/√Hz	490 (µm/sec²)/√Hz	[1]
(10 Hz)	20 µg/√Hz	196 (µm/sec²)/√Hz	[1]
(100 Hz)	5 µg/√Hz	49 (µm/sec²)/√Hz	[1]
(1 kHz)	2 µg/√Hz	19.8 (µm/sec²)/√Hz	[1]
(10 kHz)	1 µg/√Hz	9.8 (µm/sec²)/√Hz	[1]
Physical			
Sensing Element	Ceramic	Ceramic	
Sensing Geometry	Shear	Shear	
Housing Material	Titanium	Titanium	
Sealing	Hermetic	Hermetic	
Size (Height x Length x Width)	0.45 in x 0.45 in x 0.45 in	11.4 mm x 11.4 mm x 11.4 mm	[1]
Weight(without cable)	0.19 oz	5.4 gm	
Electrical Connector	Integral Cable	Integral Cable	
Electrical Connection Position	Side	Side	
Cable Termination	1/4-28 4-Pin Jack	1/4-28 4-Pin Jack	
Cable Length	5 ft	1.5 m	
Cable Type	034 4-cond Shielded	034 4-cond Shielded	
Mounting Thread	5-40 Female	5-40 Female	

CE [3]

Typical Sensitivity Deviation vs Temperature

All specifications are at room temperature unless otherwise specified.
In the interest of constant product improvement, we reserve the right to change specifications without notice.
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OPTIONAL VERSIONS

Optional versions have identical specifications and accessories as listed for the standard model except where noted below. More than one option may be used.

A - Adhesive Mount
Supplied Accessory : Model 034G05 4-cond. shielded cable, 5 ft (1.5M), 4-pin plug to (3) BNC plugs (1)
Supplied Accessory : Model 080A109 Petro Wax (1)
Supplied Accessory : Model 080A90 Quick Bonding Gel (1)

J - Ground Isolated

Electrical Isolation(Base)	≥ 10^8 ohm	≥ 10^8 ohm
Size - Height x Length x Width	0.49 in x 0.45 in x 0.49 in	12.5 mm x 11.4 mm x 12.5 mm
Weight(without cable)	0.23 oz	6.5 gm [1]
Mounting	Adhesive	Adhesive

Supplied Accessory : Model 034G05 4-cond. shielded cable, 5 ft (1.5M), 4-pin plug to (3) BNC plugs (1)
Supplied Accessory : Model 080A109 Petro Wax (1)
Supplied Accessory : Model 080A90 Quick Bonding Gel (1)

T - TEDS Capable of Digital Memory and Communication Compliant with IEEE P1451.4
TLB - TEDS LMS International - Automotive Format

Frequency Range(± 5 %)	0.5 to 4000 Hz	0.5 to 4000 Hz
(± 10 %)	0.4 to 5000 Hz	0.4 to 5000 Hz
Output Bias Voltage	8.5 to 13 VDC	8.5 to 13 VDC
Weight(without cable)	0.23 oz	6.4 gm [1]
Mounting	Adhesive	Adhesive

Supplied Accessory : Model 034G05 4-cond. shielded cable, 5 ft (1.5M), 4-pin plug to (3) BNC plugs (1)
Supplied Accessory : Model 080A109 Petro Wax (1)
Supplied Accessory : Model 080A90 Quick Bonding Gel (1)

W - Water Resistant Cable

Electrical Connector	Sealed Integral Cable	Sealed Integral Cable

NOTES:
[1] Typical.
[2] Zero-based, least-squares, straight line method.
[3] See PCB Declaration of Conformance PS023 for details.

SUPPLIED ACCESSORIES:
Model 034G05 4-cond. shielded cable, 5 ft (1.5M), 4-pin plug to (3) BNC plugs (1)
Model 080A Adhesive Mounting Base (1)
Model 080A109 Petro Wax (1)
Model 081A27 Mounting Stud (5-40 to 5-40) (1)
Model 081A90 Mounting stud, 10-32 to 5-40 (1)
Model ACS-1T NIST traceable triaxial amplitude response, 10 Hz to upper 5% frequency. (1)
Model M081A27 Metric mounting stud, 5-40 to M3 x 0.50 long (1)

Entered: LH	Engineer: BM	Sales: JDC	Approved: KB	Spec Number:
Date: 6-1-10	Date: 5-26-10	Date: 5-26-10	Date: 5-27-10	9281

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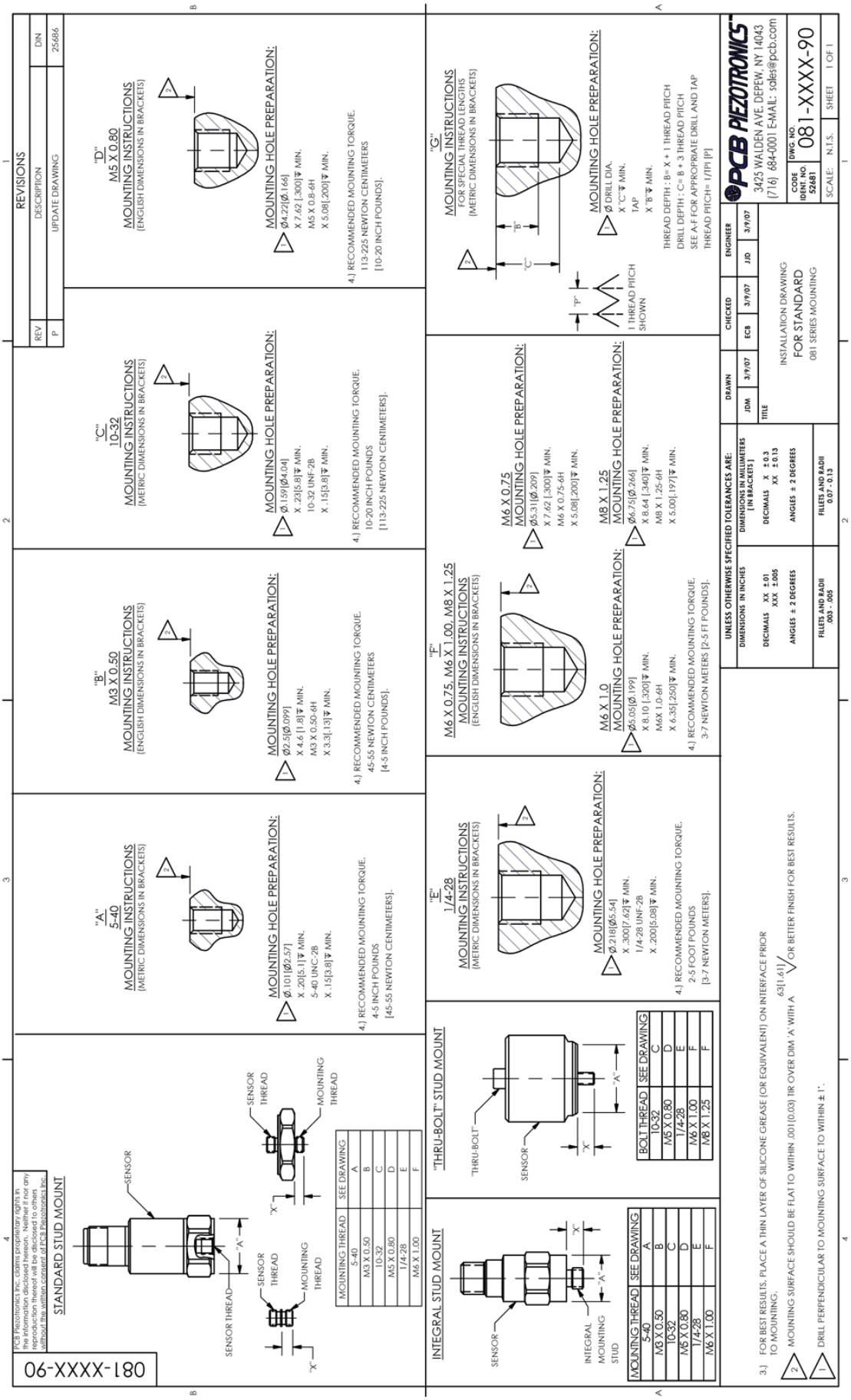
081-XXXX-90
STANDARD STUD MOUNT
SENSOR
SENSOR THREAD
"A"
SENSOR THREAD
MOUNTING THREAD
"X"
MOUNTING THREAD
SEE DRAWING
5-40 A
M3 X 0.50 B
10-32 C
M5 X 0.80 D
1/4-28 E
M6 X 1.00 F
INTEGRAL STUD MOUNT
SENSOR
INTEGRAL MOUNTING STUD
"X"
"A"
MOUNTING THREAD
SEE DRAWING
5-40 A
M3 X 0.50 B
10-32 C
M5 X 0.80 D
1/4-28 E
M6 X 1.00 F
"THRU-BOLT" STUD MOUNT
"THRU-BOLT"
SENSOR
"X"
"A"
BOLT THREAD
SEE DRAWING
10-32 C
M5 X 0.80 D
1/4-28 E
M6 X 1.00 F
M8 X 1.25 F
"A" 5-40 MOUNTING INSTRUCTIONS (METRIC DIMENSIONS IN BRACKETS)
MOUNTING HOLE PREPARATION:
Ø.101 [Ø2.57]
X .20[5.1] ▼ MIN.
5-40 UNC-2B
X .15[3.8] ▼ MIN.
4.) RECOMMENDED MOUNTING TORQUE.
4-5 INCH POUNDS
[45-55 NEWTON CENTIMETERS].
"B" M3 X 0.50 MOUNTING INSTRUCTIONS (ENGLISH DIMENSIONS IN BRACKETS)
MOUNTING HOLE PREPARATION:
Ø2.5[Ø.099]
X 4.6 [1.8] ▼ MIN.
M3 X 0.50-6H
X 3.3[.13] ▼ MIN.
4.) RECOMMENDED MOUNTING TORQUE.
45-55 NEWTON CENTIMETERS
[4-5 INCH POUNDS].
"C" 10-32 MOUNTING INSTRUCTIONS (METRIC DIMENSIONS IN BRACKETS)
MOUNTING HOLE PREPARATION:
Ø.159[Ø4.04]
X .23[5.8] ▼ MIN.
10-32 UNF-2B
X .15[3.8] ▼ MIN.
4.) RECOMMENDED MOUNTING TORQUE.
10-20 INCH POUNDS
[113-225 NEWTON CENTIMETERS].
"D" M5 X 0.80 MOUNTING INSTRUCTIONS (ENGLISH DIMENSIONS IN BRACKETS)
MOUNTING HOLE PREPARATION:
Ø4.22[Ø.166]
X 7.62 [.300] ▼ MIN.
M5 X 0.8-6H
X 5.08[.200] ▼ MIN.
4.) RECOMMENDED MOUNTING TORQUE.
113-225 NEWTON CENTIMETERS
[10-20 INCH POUNDS].
"E" 1/4-28 MOUNTING INSTRUCTIONS (METRIC DIMENSIONS IN BRACKETS)
MOUNTING HOLE PREPARATION:
Ø.218[Ø5.54]
X .300[7.62] ▼ MIN.
1/4-28 UNF-2B
X .200[5.08] ▼ MIN.
4.) RECOMMENDED MOUNTING TORQUE.
2-5 FOOT POUNDS
[3-7 NEWTON METERS].
"F" M6 X 0.75, M6 X 1.00, M8 X 1.25 MOUNTING INSTRUCTIONS (ENGLISH DIMENSIONS IN BRACKETS)
M6 X 0.75 MOUNTING HOLE PREPARATION:
Ø5.31[Ø.209]
X 7.62 [.300] ▼ MIN.
M6 X 0.75-6H
X 5.08[.200] ▼ MIN.
M6 X 1.0 MOUNTING HOLE PREPARATION:
Ø5.05[Ø.199]
X 8.10 [.320] ▼ MIN.
M6X 1.0-6H
X 6.35[.250] ▼ MIN.
M8 X 1.25 MOUNTING HOLE PREPARATION:
Ø6.75[Ø.266]
X 8.64 [.340] ▼ MIN.
M8 X 1.25-6H
X 5.00[.197] ▼ MIN.
4.) RECOMMENDED MOUNTING TORQUE.
3-7 NEWTON METERS [2-5 FT POUNDS].
"G" MOUNTING INSTRUCTIONS FOR SPECIAL THREAD LENGTHS (METRIC DIMENSIONS IN BRACKETS)
"B"
"C"
"P"
1 THREAD PITCH SHOWN
MOUNTING HOLE PREPARATION:
Ø DRILL DIA.
X "C" ▼ MIN.
TAP
X "B" ▼ MIN.
THREAD DEPTH : B= X + 1 THREAD PITCH
DRILL DEPTH : C= B + 3 THREAD PITCH
SEE A-F FOR APPROPRIATE DRILL AND TAP
THREAD PITCH= 1/TPI [P]
REVISIONS
REV P
DESCRIPTION
UPDATE DRAWING
DIN 25686
UNLESS OTHERWISE SPECIFIED TOLERANCES ARE:
DIMENSIONS IN INCHES
DECIMALS XX ±.01 XXX ±.005
ANGLES ± 2 DEGREES
FILLETS AND RADII .003 - .005
DIMENSIONS IN MILLIMETERS [IN BRACKETS]
DECIMALS X ±0.3 XX ±0.13
ANGLES ± 2 DEGREES
FILLETS AND RADII 0.07 - 0.13
DRAWN JDM 3/9/07
CHECKED ECB 3/9/07
ENGINEER JJD 3/9/07
TITLE
INSTALLATION DRAWING FOR STANDARD 081 SERIES MOUNTING
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3425 WALDEN AVE. DEPEW, NY 14043
(716) 684-0001 E-MAIL: sales@pcb.com
CODE IDENT. NO. 52681
DWG NO. 081-XXXX-90
SCALE: N.T.S. SHEET 1 OF 1
3.) FOR BEST RESULTS, PLACE A THIN LAYER OF SILICONE GREASE (OR EQUIVALENT) ON INTERFACE PRIOR TO MOUNTING.
2.) MOUNTING SURFACE SHOULD BE FLAT TO WITHIN .001 [0.03] TIR OVER DIM 'A' WITH A 63[1.61] OR BETTER FINISH FOR BEST RESULTS.
1.) DRILL PERPENDICULAR TO MOUNTING SURFACE TO WITHIN ± 1°.
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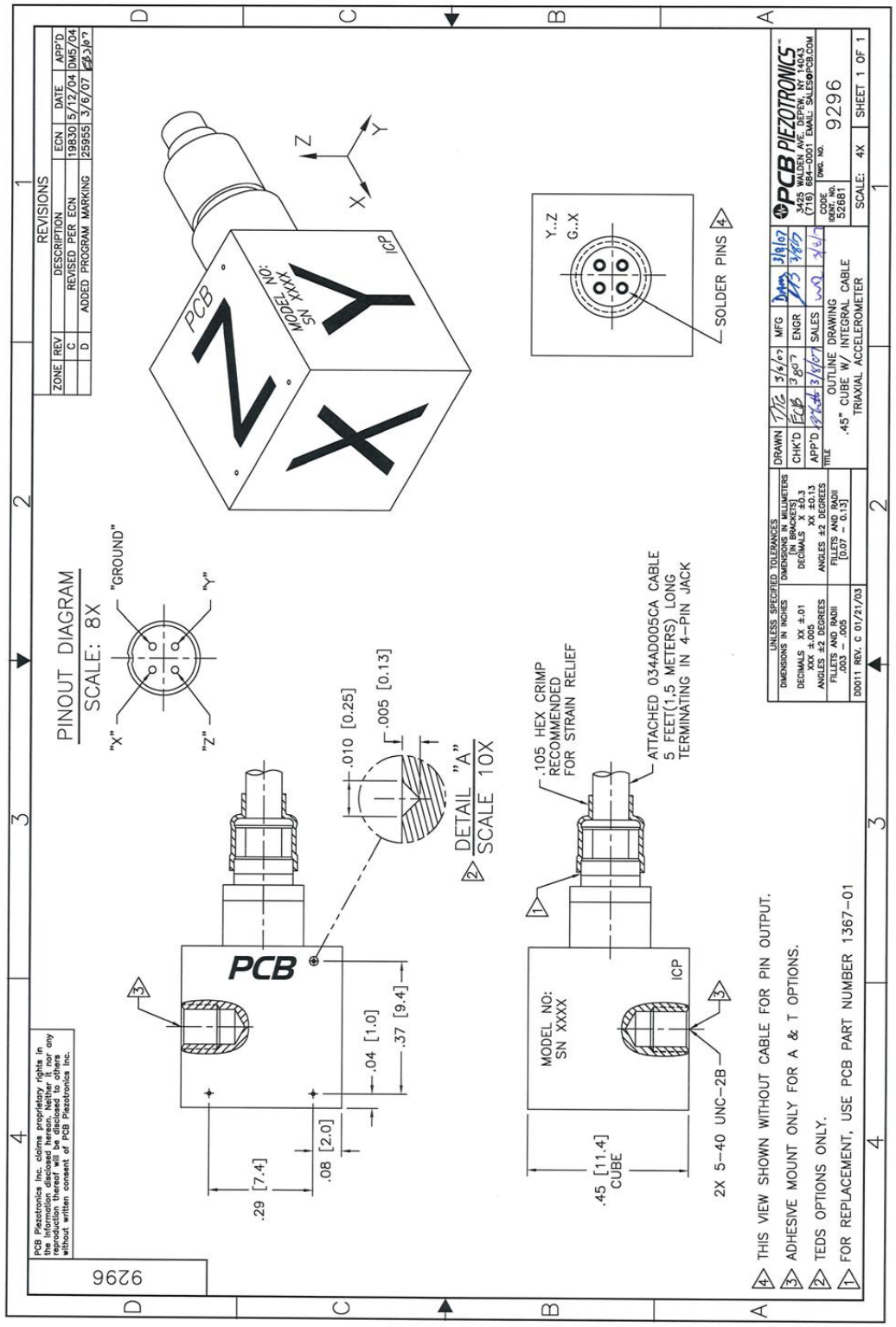
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"GROUND"
"Y"
"X"
"Z"
DETAIL "A"
SCALE 10X
.010 [0.25]
.005 [0.13]
.105 HEX CRIMP
RECOMMENDED
FOR STRAIN RELIEF
ATTACHED 034AD005CA CABLE
5 FEET(1.5 METERS) LONG
TERMINATING IN 4-PIN JACK
PCB
MODEL NO:
SN XXXX
ICP
.29 [7.4]
.08 [2.0]
.04 [1.0]
.37 [9.4]
.45 [11.4]
CUBE
2X 5-40 UNC-2B
SOLDER PINS
Y..Z
G..X
4 THIS VIEW SHOWN WITHOUT CABLE FOR PIN OUTPUT.
3 ADHESIVE MOUNT ONLY FOR A & T OPTIONS.
2 TEDS OPTIONS ONLY.
1 FOR REPLACEMENT, USE PCB PART NUMBER 1367-01
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DRAWN
CHK'D
APP'D
MFG
ENGR
SALES
OUTLINE DRAWING
.45" CUBE W/ INTEGRAL CABLE
TRIAXIAL ACCELEROMETER
CODE IDENT. NO. 52681
DWG. NO. 9296
SCALE: 4X
SHEET 1 OF 1
D0011 REV. C 01/21/03
9296
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Model 832M1 Accelerometer

Triaxial Piezoelectric Accelerometer
<22μA Current Consumption
Wide Bandwidth to 6kHz
Circuit Board Mountable

The Model 832M1 is a low cost, board mountable triaxial accelerometer. Featuring stable piezo-ceramic crystals, the accelerometer incorporates full power and signal conditioning with a maximum current consumption of 22 micro-amps. The model 832M1 is available in ±25g to ±500g ranges and provides a flat frequency response up to greater than 6kHz. The standard model 832 offers the same envelope with a lower maximum current consumption of 4 micro-amps.

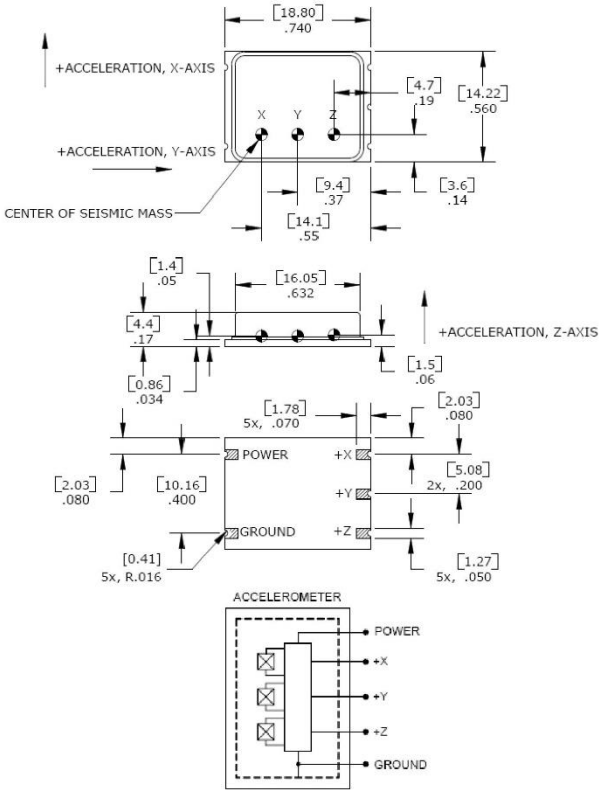
FEATURES

- ±25g to ±500g Dynamic Range
- Low Cost Triaxial
- Hermetically Sealed
- Piezo-ceramic Crystals
- -40° to +125°C Operating Range
- Single Axis Configurations Available

APPLICATIONS

- Asset Monitoring
- Data Loggers
- Impact Monitoring
- Machine Health Monitoring
- System Wake-Up Switch
- Embedded Applications

dimensions



Model 832M1 Accelerometer

performance specifications

All values are typical at +24°C, 100Hz and 3.3Vdc excitation unless otherwise stated. Measurement Specialties reserves the right to update and change these specifications without notice. Standard product parameters are described in PSC-1001 for Embedded AC Accelerometers.

Parameters						
DYNAMIC						**Notes**
Range (g)	±25	±50	±100	±200	±500	
Sensitivity (mV/g)	50.0	25.0	12.5	6.25	2.5	±30%
Frequency Response (Hz)	2-6000	2-6000	2-6000	2-6000	2-6000	±2dB
Natural Frequency (Hz)	>10000	>10000	>10000	>10000	>10000	
Non-Linearity (%FSO)	±2	±2	±2	±2	±2	
Transverse Sensitivity (%)	<10	<10	<10	<10	<10	
Shock Limit (g)	5000	5000	5000	5000	5000	
ELECTRICAL						
Bias Voltage (Vdc)	Exc Voltage / 2	Exc Voltage / 2	Exc Voltage / 2	Exc Voltage / 2	Exc Voltage / 2	
Total Supply Current (µA) [1]	<22	<22	<22	<22	<22	
Excitation Voltage (Vdc) [3]	3.0 to 5.5	3.0 to 5.5	3.0 to 5.5	3.0 to 5.5	3.0 to 5.5	
Output Impedance (Ω)	<100	<100	<100	<100	<100	
Insulation Resistance (MΩ)	>100	>100	>100	>100	>100	@100Vdc
Broadband Noise (µV)	320	160	80	40	30	2Hz-10kHz
Spectral Noise (µg/√Hz)	240	240	240	240	600	@ 10Hz
Spectral Noise (µg/√Hz)	64	64	64	64	160	@ 100Hz
Spectral Noise (µg/√Hz)	24	24	24	24	60	@ 1000Hz
Shielding	100%					
Ground Isolation	Isolated from Mounting Surface					
ENVIRONMENTAL						
Temperature Response (%)	-20/+30 from -40°C to +125°C					
Operating Temperature (°C)	-40 to +125					
Storage Temperature (°C)	-40 to +125					
PHYSICAL						
Sensing Element	Ceramic (shear mode)					
Case Material	Ceramic Base, Nickel Silver Cover					
Weight (grams)	3.0					

[1] A lower current consumption of 4 micro-amps is available on model 832.
[2] The model 832M1 is not to be reflow soldered at high temperature, manual soldering is recommended. See application note.
[3] The model 832M1 can be operated with 2.8V excitation but the full-scale range will be limited.

Calibration supplied: CS-SENS-0100 NIST Traceable Amplitude Calibration at 100Hz

Wiring color code: See schematic

ordering info

PART NUMBERING Model Number+Range

832M1-GGGG
|
|_____ Range (0200 is 200g)

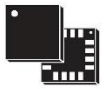
Example: 832M1-0200
Model 832M1, 200g

LIS344ALH

MEMS inertial sensor
high performance 3-axis ±2/±6g ultracompact linear accelerometer

Features

- 2.4 V to 3.6 V single supply operation
- ±2 g / ±6 g user selectable full-scale
- Low power consumption
- Output voltage, offset and sensitivity are ratiometric to the supply voltage
- Factory trimmed device sensitivity and offset
- Embedded self test
- RoHS/ECOPACK® compliant
- High shock survivability (10000 g)



LGA 16L (4x4x1.5 mm)

Description

The LIS344ALH is an ultra compact consumer low-power three-axis linear accelerometer that includes a sensing element and an IC interface able to take the information from the sensing element and to provide an analog signal to the external world.

The sensing element, capable of detecting the acceleration, is manufactured using a dedicated process developed by ST to produce inertial sensors and actuators in silicon.

The IC interface is manufactured using an ST proprietary CMOS process with high level of integration. The dedicated circuit is trimmed to better match the sensing element characteristics.

The LIS344ALH has a dynamically user selectable full-scale of ±2 g / ±6 g and it is capable of measuring accelerations over a maximum bandwidth of 1.8 kHz for all axes. The device bandwidth may be reduced by using external capacitances. The self-test capability allows the user to check the functioning of the system.

The LIS344ALH is available in Land Grid Array package (LGA) manufactured by ST.
It is guaranteed to operate over an extended temperature range of -40 °C to +85 °C.

The LIS344ALH belongs to a family of products suitable for a variety of applications:

- Mobile terminals
- Gaming and virtual reality input devices
- Antitheft systems and inertial navigation
- Appliance and robotics.

Table 1. Device summary

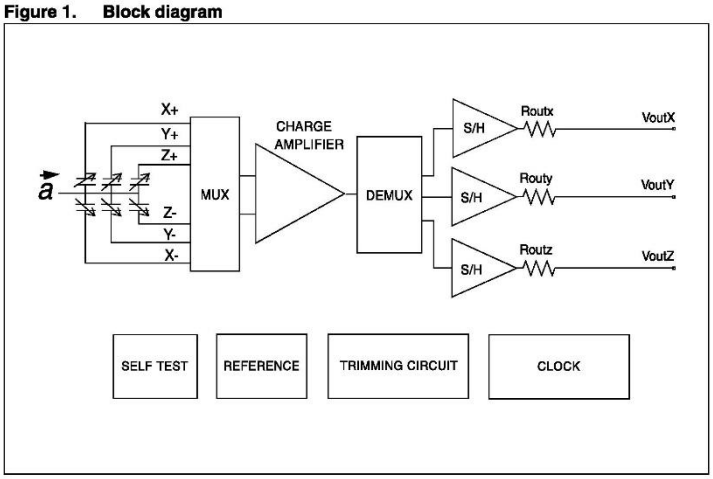
Order codes	Temp range [°C]	Package	Packaging
LIS344ALH	-40 to +85	LGA-16L	Tray
LIS344ALHTR	-40 to +85	LGA-16L	Tape and reel

April 2008 Rev 3 1/19
www.st.com

1 Block diagram and pin description

1.1 Block diagram

Figure 1. Block diagram



1.2 Pin description

Figure 2. Pin connection

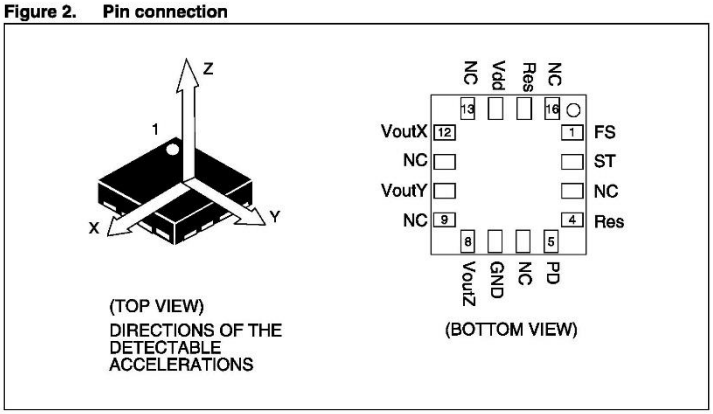


Table 2. Pin description

Pin #	Pin name	Function
1	FS	Full scale selection (logic 0: ±2g full-scale; logic 1: ±6g full-scale)
2	ST	Self test (logic 0: normal mode; logic 1: self-test mode)
3	NC	Internally not connected
4	Res	Leave unconnected or connect to Vdd
5	PD	Power down (logic 0: normal mode; logic 1: power-down mode)
6	NC	Internally not connected
7	GND	0 V supply
8	VoutZ	Output voltage Z channel
9	NC	Internally not connected
10	VoutY	Output voltage Y channel
11	NC	Internally not connected
12	VoutX	Output voltage X channel
13	NC	Internally not connected
14	Vdd	Power supply
15	Res	Connect to Vdd
16	NC	Internally not connected

2 Mechanical and electrical specifications

2.1 Mechanical characteristics

Table 3. Mechanical characteristics @ Vdd =3.3 V, T = 25 °C unless otherwise noted(1)

Symbol	Parameter	Test condition	Min.	Typ.(2)	Max.	Unit
Ar	Acceleration range(3)	FS pin connected to GND	±1.8	± 2		g
		FS pin connected to Vdd	±5.4	± 6		
So	Sensitivity(4)	Full-scale = ±2 g	Vdd/5 - 5%	Vdd/5	Vdd/5 + 5%	V/g
		Full-scale = ±6 g	Vdd/15 - 10%	Vdd/15	Vdd/15 + 10%	
SoDr	Sensitivity change Vs Temperature	Delta from +25 °C		± 0.01		%/°C
Voff	Zero-g level(4)	Full-scale = ±2 g T = 25 °C	Vdd/2 - 5%	Vdd/2	Vdd/2 + 5%	V
OffDr	Zero-g level change Vs Temperature	Delta from +25 °C		±0.4		mg/°C
NL	Non linearity(5)	Best fit straight line Full-scale = ±2 g		±0.5		% FS
CrossAx	Cross-axis(6)			±2		%
An	Acceleration noise density	Vdd = 3.3 V; Full-scale = ±2 g		50		$\mu g/\sqrt{Hz}$
Vt	Self test output voltage change(7),(8),(9)	X axis T = 25 °C; Vdd=3.3 V	80	140	200	mV
		Y axis T = 25 °C; Vdd=3.3 V	-200	-140	-80	mV
		Z axis T = 25 °C; Vdd=3.3 V	100	230	350	mV
Fres	Sensing element resonant frequency(10)	X,Y,Z axis	1.8			KHz
Top	Operating temperature range		-40		+85	°C
Wh	Product weight			0.040		gram

1. The product is factory calibrated at 3.3 V. The operational power supply range is from 2.4 V to 3.6 V. Voff, So and Vt parameters will vary with supply voltage.
2. Typical specifications are not guaranteed.
3. Guaranteed by wafer level test and measurement of initial offset and sensitivity.
4. Zero-g level and sensitivity are essentially ratiometric to supply voltage at the calibration level ±8%.
5. Guaranteed by design.
6. Contribution to the measuring output of an inclination/acceleration along any perpendicular axis.
7. "Self test output voltage change" is defined as $Vout_{(Vst=Logic1)}$-$Vout_{(Vst=Logic0)}$.
8. "Self test output voltage change" varies cubically with supply voltage.
9. When full-scale is set to ±6 g, "Self test output voltage change" is one third of the specified value at ±2 g.
10. Minimum resonance frequency Fres=1.8 kHz. Sensor bandwidth=1/(2*π*110kΩ*Cload), with Cload>1 nF.

2.2 Electrical characteristics

Table 4. Electrical characteristics @ Vdd =3.3 V, T = 25 °C unless otherwise noted(1)

Symbol	Parameter	Test condition	Min.	Typ.(2)	Max.	Unit
Vdd	Supply voltage		2.4	3.3	3.6	V
Idd	Supply current	Normal mode		680	850	µA
		Power-down mode		1	5	
Vfs Vst Vpd	Full-scale input Self-test input Power-down input	Logic 0 level	0		0.3*Vdd	V
		Logic 1 level	0.7*Vdd		Vdd	V
Rout	Output impedance of VoutX, VoutY, VoutZ		90	110	130	KΩ
Cload	Capacitive load drive(3) for VoutX, VoutY, VoutZ		1			nF
Ton	Turn-on time at exit of Power-down mode	Cload expressed in µF		550*Cload+ 0.3		ms
Top	Operating temperature range		-40		+85	°C

1. The product is factory calibrated at 3.3 V.
2. Typical specifications are not guaranteed.
3. Minimum resonance frequency Fres=1.8 kHz. Device bandwidth=1/(2*π*110 kΩ*Cload), with Cload>1 nF.

2.3 Absolute maximum ratings

Stresses above those listed as "Absolute maximum ratings" may cause permanent damage to the device. This is a stress rating only and functional operation of the device under these conditions is not implied. Exposure to maximum rating conditions for extended periods may affect device reliability.

Table 5. Absolute maximum ratings

Symbol	Ratings	Maximum value	Unit
Vdd	Supply voltage	-0.3 to 7	V
Vin	Input voltage on any control pin (FS, ST, PD)	-0.3 to Vdd +0.3	V
A_{POW}	Acceleration (any axis, powered, Vdd = 3.3 V)	3000 g for 0.5 ms	
		10000 g for 0.1 ms	
A_{UNP}	Acceleration (any axis, not powered)	3000 g for 0.5 ms	
		10000 g for 0.1 ms	
T_{STG}	Storage temperature range	-40 to +125	°C
ESD	Electrostatic discharge protection	4 (HBM)	KV
		1.5 (CDM)	KV
		400 (MM)	V

This is a mechanical shock sensitive device, improper handling can cause permanent damages to the part

This is an ESD sensitive device, improper handling can cause permanent damages to the part

Appendix B

Plate layouts for biochemical and genetic experiments in sub-chapter 4.2 and 4.4 respectively. Included are the plates for SOD, TBARS, Bradford correction, GSH, GPX, qPCR HSP70 efficiency, qPCR HSP70, and qPCR 18S.

TBARS

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	25	25	25	27	27	27	44	44	44	45	45	45
B	15	15	15	48	48	48	33	33	33	1	1	1
C	5	5	5	43	43	43	17	17	17	25	25	25
D	2.5	2.5	2.5	38	38	38	9	9	9	15	15	15
E	0.5	0.5	0.5	35	35	35	3	3	3	31	31	31
F	5	5	5	18	18	18	36	36	36	41	41	41
G	10	10	10	11	11	11	34	34	34	4	4	4
H	19	19	19	2	2	2	16	16	16	30	30	30

PLATE 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	25	25	25	13	13	13	6	6	6			
B	15	15	15	22	22	22	20	20	20			
C	5	5	5	39	39	39	47	47	47			
D	2.5	2.5	2.5	23	23	23	32	32	32			
E	0.5	0.5	0.5	8	8	8	7	7	7			
F	46	46	46	26	26	26	21	21	21			
G	14	14	14	37	37	37	28	28	28			
H	24	24	24	42	42	42	40	40	40			

SOD

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	B1	B1	13	13	13	15	15	15	22	22	22
B	B2	B2	B2	3	3	3	30	30	30	11	11	11
C	B3	B3	B3	24	24	24	25	25	25	10	10	10
D	7	7	7	4	4	4	19	19	19			
E	1	1	1	20	20	20	14	14	14			
F	8	8	8	5	5	5	2	2	2			
G	16	16	16	17	17	17	6	6	6			
H	18	18	18	9	9	9	21	21	21			

PLATE 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	B1	B1	39	39	39	35	35	35	41	41	41
B	B2	B2	B2	45	45	45	46	46	46			
C	B3	B3	B3	40	40	40	37	37	37			
D	36	36	36	47	47	47	23	23	23			
E	48	48	48	27	27	27	34	34	34			
F	44	44	44	33	33	33	31	31	31			
G	38	38	38	28	28	28	32	32	32			
H	42	42	42	43	43	43	26	26	26			

October

Control replicate 1	
Mussel 1	28
Mussel 3	30
Mussel 4	33
Mussel 5	32
Mussel 6	31

Noise replicate 1	
Mussel 1	42
Mussel 2	41
Mussel 3	40
Mussel 4	45
Mussel 5	44
Mussel 6	43

Control replicate 2	
Mussel 1	34
Mussel 2	35
Mussel 3	36
Mussel 4	27
Mussel 5	26
Mussel 6	25

Noise replicate 2	
Mussel 1	46
Mussel 2	47
Mussel 3	48
Mussel 4	37
Mussel 5	38
Mussel 6	39

November

Control replicate 1	
Mussel 1	18
Mussel 2	17
Mussel 3	16
Mussel 4	24
Mussel 5	23
Mussel 6	22

Noise replicate 1	
Mussel 2	11
Mussel 3	10
Mussel 4	6
Mussel 5	5
Mussel 6	4

Control replicate 2	
Mussel 1	15
Mussel 2	14
Mussel 3	13
Mussel 4	21
Mussel 5	20
Mussel 6	19

Noise replicate 2	
Mussel 1	3
Mussel 2	2
Mussel 3	1
Mussel 4	7
Mussel 5	8
Mussel 6	9

Calibration

BRADFORD

PROTIEN PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	19	19	19	19	19		
B	0.8	0.8	0.8	0.8	0.8	27	27	27	27	27		
C	0.6	0.6	0.6	0.6	0.6	48	48	48	48	48		
D	0.4	0.4	0.4	0.4	0.4	43	43	43	43	43		
E	0.2	0.2	0.2	0.2	0.2	38	38	38	38	38		
F	0	0	0	0	0	35	35	35	35	35		
G	5	5	5	5	5	18	18	18	18	18		
H	10	10	10	10	10	11	11	11	11	11		

PROTIEN PLATE 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	33	33	33	33	33		
B	0.8	0.8	0.8	0.8	0.8	17	17	17	17	17		
C	0.6	0.6	0.6	0.6	0.6	9	9	9	9	9		
D	0.4	0.4	0.4	0.4	0.4	3	3	3	3	3		
E	0.2	0.2	0.2	0.2	0.2	36	36	36	36	36		
F	0	0	0	0	0	34	34	34	34	34		
G	2	2	2	2	2	16	16	16	16	16		
H	44	44	44	44	44	45	45	45	45	45		

PROTIEN PLATE 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	15	15	15	15	15		
B	0.8	0.8	0.8	0.8	0.8	31	31	31	31	31		
C	0.6	0.6	0.6	0.6	0.6	41	41	41	41	41		
D	0.4	0.4	0.4	0.4	0.4	4	4	4	4	4		
E	0.2	0.2	0.2	0.2	0.2	30	30	30	30	30		
F	0	0	0	0	0	46	46	46	46	46		
G	1	1	1	1	1	14	14	14	14	14		
H	25	25	25	25	25	24	24	24	24	24		

PROTIEN PLATE 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	39	39	39	39	39		
B	0.8	0.8	0.8	0.8	0.8	23	23	23	23	23		
C	0.6	0.6	0.6	0.6	0.6	8	8	8	8	8		
D	0.4	0.4	0.4	0.4	0.4	26	26	26	26	26		
E	0.2	0.2	0.2	0.2	0.2	37	37	37	37	37		
F	0	0	0	0	0	42	42	42	42	42		
G	13	13	13	13	13	6	6	6	6	6		
H	22	22	22	22	22	20	20	20	20	20		

PROTIEN PLATE 5

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	7	7	7	7	7		
B	0.8	0.8	0.8	0.8	0.8	21	21	21	21	21		
C	0.6	0.6	0.6	0.6	0.6	28	28	28	28	28		
D	0.4	0.4	0.4	0.4	0.4	40	40	40	40	40		
E	0.2	0.2	0.2	0.2	0.2							
F	0	0	0	0	0							
G	47	47	47	47	47							
H	32	32	32	32	32							

October

Control replicate 1	
Mussel 1	28
Mussel 3	30
Mussel 4	33
Mussel 5	32
Mussel 6	31

Noise replicate 1	
Mussel 1	42
Mussel 2	41
Mussel 3	40
Mussel 4	45
Mussel 5	44
Mussel 6	43

Control replicate 2	
Mussel 1	34
Mussel 2	35
Mussel 3	36
Mussel 4	27
Mussel 5	26
Mussel 6	25

Noise replicate 2	
Mussel 1	46
Mussel 2	47
Mussel 3	48
Mussel 4	37
Mussel 5	38
Mussel 6	39

November

Control replicate 1	
Mussel 1	18
Mussel 2	17
Mussel 3	16
Mussel 4	24
Mussel 5	23
Mussel 6	22

Noise replicate 1	
Mussel 2	11
Mussel 3	10
Mussel 4	6
Mussel 5	5
Mussel 6	4

Control replicate 2	
Mussel 1	15
Mussel 2	14
Mussel 3	13
Mussel 4	21
Mussel 5	20
Mussel 6	19

Noise replicate 2	
Mussel 1	3
Mussel 2	2
Mussel 3	1
Mussel 4	7
Mussel 5	8
Mussel 6	9

Calibration

GSH PLATE 1

GSH	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	3	3	3	11	11	11	19	19	19
B	5	5	5	4	4	4	12	12	12	20	20	20
C	10	10	10	5	5	5	13	13	13	21	21	21
D	15	15	15	6	6	6	14	14	14	22	22	22
E	20	20	20	7	7	7	15	15	15	23	23	23
F				8	8	8	16	16	16	24	24	24
G				9	9	9	17	17	17	25	25	25
H				10	10	10	18	18	18	26	26	26

GSH BRADFORD PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	3	3	3	11	11	11	19	19	19
B	0.2	0.2	0.2	4	4	4	12	12	12	20	20	20
C	0.4	0.4	0.4	5	5	5	13	13	13	21	21	21
D	0.6	0.6	0.6	6	6	6	14	14	14	22	22	22
E	0.8	0.8	0.8	7	7	7	15	15	15	23	23	23
F	1	1	1	8	8	8	16	16	16	24	24	24
G				9	9	9	17	17	17	25	25	25
H				10	10	10	18	18	18	26	26	26

GPx PLATE 1

GPx	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	3	3	3	11	11	11	19	19	19
B	20	20	20	4	4	4	12	12	12	20	20	20
C	40	40	40	5	5	5	13	13	13	21	21	21
D	60	60	60	6	6	6	14	14	14	22	22	22
E	80	80	80	7	7	7	15	15	15	23	23	23
F	100	100	100	8	8	8	16	16	16	24	24	24
G	P	P	P	9	9	9	17	17	17	25	25	25
H	B	B	B	10	10	10	18	18	18	26	26	26

NADPH TEST

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	20	40	60	80	100			1	2	P	B
B	0	20	40	60	80	100			1	2	P	B
C	0	20	40	60	80	100			1	2	P	B
D												
E												
F												
G												
H												

Control replicate 1	
Mussel 1	3
Mussel 2	7
Mussel 3	10
Mussel 4	11
Mussel 5	15
Mussel 6	26

Noise replicate 1	
Mussel 1	8
Mussel 2	9
Mussel 3	14
Mussel 4	21
Mussel 5	23
Mussel 6	24

Control replicate 2	
Mussel 1	5
Mussel 2	13
Mussel 3	17
Mussel 4	19
Mussel 5	22
Mussel 6	25

Noise replicate 2	
Mussel 1	4
Mussel 2	6
Mussel 3	12
Mussel 4	16
Mussel 5	18
Mussel 6	20

Test	
Control	1
Noise	2
Calibration	

qPCR Hsp70

Plate 1

	1	2	3	4	5	6	7	8
A	Noise 2 - 2	Noise 2 - 2	Noise 2 - 2	Noise 0 - 1	Noise 0 - 1	Noise 0 - 1	Noise 0 - 2	Noise 0 - 2
B	Noise 0 - 2	Noise 0 - 3	Noise 0 - 3	Noise 0 - 3	Noise 0.5 - 1	Noise 0.5 - 1	Noise 0.5 - 1	Noise 0.5 - 2
C	Noise 0.5 - 2	Noise 0.5 - 2	Noise 0.5 - 3	Noise 0.5 - 3	Noise 0.5 - 3	Noise 1 - 1	Noise 1 - 1	Noise 1 - 1
D	Noise 1 - 2	Noise 1 - 2	Noise 1 - 2	Noise 1 - 3	Noise 1 - 3	Noise 1 - 3	Noise 2 - 1	Noise 2 - 1
E	Noise 2 - 1	Noise 2 - 3	Noise 2 - 3	Noise 2 - 3	Noise 3 - 1	Noise 3 - 1	Noise 3 - 1	Noise 3 - 2
F	Noise 3 - 2	Noise 3 - 2	Noise 3 - 3	Noise 3 - 3	Noise 3 - 3	NTC	NTC	H_2O

Plate 2

	1	2	3	4	5	6	7	8
A	Noise 2 - 2	Noise 2 - 2	Noise 2 - 2	Noise 5 - 1	Noise 5 - 1	Noise 5 - 1	Noise 5 - 2	Noise 5 - 2
B	Noise 5 - 2	Noise 5 - 3	Noise 5 - 3	Noise 5 - 3	Control 0 - 1	Control 0 - 1	Control 0 - 1	Control 0 - 2
C	Control 0 - 2	Control 0 - 2	Control 0 - 3	Control 0 - 3	Control 0 - 3	Control 0.5 - 1	Control 0.5 - 1	Control 0.5 - 1
D	Control 0.5 - 2	Control 0.5 - 2	Control 0.5 - 2	Control 0.5 - 3	Control 0.5 - 3	Control 0.5 - 3	Control 1 - 1	Control 1 - 1
E	Control 1 - 1	Control 1 - 2	Control 1 - 2	Control 1 - 2	Control 1 - 3	Control 1 - 3	Control 1 - 3	Control 2 - 1
F	Control 2 - 1	Control 2 - 1	Control 2 - 2	Control 2 - 2	Control 2 - 2	NTC	NTC	H_2O

qPCR Hsp70 Efficiency

Plate 1

	1	2	3	4	5	6	7	8
A	Noise 2 - 1	Noise 2 - 1	Noise 2 - 1	1/100	1/100	1/100	1/1000	1/1000
B	1/1000	1/10000	1/10001	1/10002				
C								
D								
E								
F						NTC	NTC	H_2O

qPCR Hsp70

Plate 3

	1	2	3	4	5	6	7	8
A	Noise 2 - 2	Noise 2 - 2	Noise 2 - 2	Control 2 - 3	Control 2 - 3	Control 2 - 3	Control 3 - 1	Control 3 - 1
B	Control 3 - 1	Control 3 - 2	Control 3 - 2	Control 3 - 2	Control 3 - 3	Control 3 - 3	Control 3 - 3	Control 5 - 1
C	Control 5 - 1	Control 5 - 1	Control 5 - 2	Control 5 - 2	Control 5 - 2	Control 5 - 3	Control 5 - 3	Control 5 - 3
D								
E								
F						NTC	NTC	H_2O

Noise
Control
Non-Template Control
Water only blank

qPCR 18S

Plate 1

	1	2	3	4	5	6	7	8
A	1/100	1/100	1/100	1/1000	1/1000	1/1000	1/10000	1/10000
B	1/10000	Control 0 - 1	Control 0 - 1	Control 0 - 1	Control 0 - 2	Control 0 - 2	Control 0 - 2	Control 0 - 3
C	Control 0 - 3	Control 0 - 3	Control 0.5 - 1	Control 0.5 - 1	Control 0.5 - 1	Control 0.5 - 2	Control 0.5 - 2	Control 0.5 - 2
D	Control 0.5 - 3	Control 0.5 - 3	Control 0.5 - 3	Control 1 -1	Control 1 -1	Control 1-1	Control 1 - 2	Control 1 - 2
E	Control 1 - 2	Control 1 - 3	Control 1 - 3	Control 1 - 3	Control 2 - 1	Control 2 - 1	Control 2 - 1	Control 2 - 2
F	Control 2 - 2	Control 2 - 2	Control 2 - 3	Control 2 - 3	Control 2 - 3	NTC	NTC	H_2O

Plate 2

	1	2	3	4	5	6	7	8
A	Control 3 - 1	Control 3 - 1	Control 3 - 1	Control 3 - 2	Control 3 - 2	Control 3 - 2	Control 3 - 3	Control 3 - 3
B	Control 3 - 3	Control 5 - 1	Control 5 - 1	Control 5 - 1	Control 5 - 2	Control 5 - 2	Control 5 - 2	Noise 0 - 1
C	Noise 0 - 1	Noise 0 - 1	Noise 0 - 1	Noise 0 - 1	Noise 0 - 1	Noise 0 - 2	Noise 0 - 2	Noise 0 - 2
D	Noise 0 - 3	Noise 0 - 3	Noise 0 - 3	Noise 0.5 - 1	Noise 0.5 - 1	Noise 0.5 - 1	Noise 0.5 - 2	Noise 0.5 - 2
E	Noise 0.5 - 2	Noise 0.5 - 3	Noise 0.5 - 3	Noise 0.5 - 3	Noise 1 - 1	Noise 1 - 1	Noise 1 - 1	Noise 1 - 2
F	Noise 1 - 2	Noise 1 - 2	Noise 1 - 3	Noise 1 - 3	Noise 1 - 3	NTC	NTC	H_2O

Plate 3

	1	2	3	4	5	6	7	8
A	Noise 3 - 1	Noise 3 - 1	Noise 3 - 1	Noise 3 - 2	Noise 3 - 2	Noise 3 - 2	Noise 3 - 3	Noise 3 - 3
B	Noise 3 - 3	Noise 5 - 1	Noise 5 - 1	Noise 5 - 1	Noise 5 - 2	Noise 5 - 2	Noise 5 - 2	Noise 5 - 3
C	Noise 5 - 3	Noise 5 - 3	Control 0 - 1	Control 0 - 1	Control 0 - 1	Noise 5 - 3	Noise 5 - 3	Noise 5 - 3
D	Noise 2 - 1	Noise 2 - 1	Noise 2 - 1	Noise 2 - 2	Noise 2 - 2	Noise 2 - 2	Noise 2 - 3	Noise 2 - 3
E	Noise 2 - 3							
F						NTC	NTC	H_2O

Noise
Control
Non-Template Control
Water only blank

Appendix C

Standard operating procedures for experiments performed in sub-chapter 4.2 and 4.4. Included are directions for the GPx kit, SOD kit, RNA extraction, Reverse Transcriptase, PCR, Gel Electrophoresis, and qPCR.

BioVision

rev. 12/14

For research use only

Glutathione Peroxidase Activity Colorimetric Assay Kit

(Catalog #K762-100; 100 reactions; Store kit at –20°C)

I. Introduction:

Glutathione Peroxidase (GPx, EC 1.11.1.9) family of enzymes play important roles in the protection of organisms from oxidative damage. GPx converts reduced glutathione (GSH) to oxidized glutathione (GSSG) while reducing lipid hydroperoxides to their corresponding alcohols or free hydrogen peroxide to water. Several isozymes have been found in different cellular locations and with different substrate specificity. Low levels of GPx have been correlated with free radical related disorders. In BioVision's Glutathione Peroxidase Activity Assay, GPx reduces Cumene Hydroperoxide while oxidizing GSH to GSSG. The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH (easily measured at 340 nm) is proportional to GPx activity. The assay can be used to measure all of the glutathione dependent peroxidases in plasma, erythrocyte lysates, tissue homogenates, and cell lysates with a detection sensitivity of ~ 0.5 mU/ml of GPx in samples.

II. Kit Contents:

Components	K762-100	Cap Code	Part Number
GPx Assay Buffer	25 ml	WM	K762-100-1
NADPH (lyophilized)	1 vial	Blue	K762-100-2
Glutathione Reductase	1 vial	Green	K762-100-3
Glutathione (GSH; lyophilized)	1 vial	Brown	K762-100-4
Cumene Hydroperoxide	1 vial	Yellow	K762-100-5
GPx Positive Control (lyophilized)	1 vial	Red	K762-100-6

III. Storage and Handling:

Store the kit at -20°C, protect from light. Warm the assay buffer to room temperature before use. Briefly centrifuge vials before opening. Read the entire protocol before performing the assay.

IV. Reagent Reconstitution and General Consideration:

NADPH: Reconstitute with 0.5 ml dH_2O to get a 40 mM NADPH solution.
GR: Dilute with 0.22 ml Assay Buffer.
GSH: Reconstitute with 0.22 ml Assay Buffer.
Cumene Hydroperoxide: Dilute with 1.25 ml Assay Buffer. Mix well
GPx Positive Control: Reconstitute with 100 µl Assay Buffer.

All the solutions are stable for at least 1 week at 4 °C and 1 month at -20 °C.
Ensure that the assay buffer is at room temperature before use. Keep samples, GR mix solution and GPx Positive Control on ice during the assay.

V. Glutathione Reductase Activity Assay:

1. Sample Preparations:

Homogenize 0.1 g tissues, 10^6 cells, or 0.2 ml erythrocytes on ice in 0.2 ml cold assay buffer; Centrifuge at 10,000 x g for 15 min at 4 °C; Collect the supernatant for assay and store on ice. Serum can be tested directly. Keep samples at -80 °C for storage. Add 2 - 50 µl of the samples into a 96-well plate; bring the volume to 50 µl with Assay Buffer. We suggest testing several doses of your sample to make sure the readings are within the standard curve range.

2. NADPH Standard Curve:

Dilute 25 µl of the 40 mM NADPH solution into 975 µl dH_2O to generate 1 mM NADPH standard. Add 0, 20, 40, 60, 80, 100 µl of the 1 mM NADPH Standard into 96-well plate in duplicate to generate 0, 20, 40, 60, 80,100 nmol/well standard. Bring the final volume to 100 µl/with Assay Buffer. Measure O.D. 340 nm to plot the NADPH Standard Curve.

3. Positive Control (optional) and Reagent Blank:

For Positive Control use 5 - 10 µl of the GPx Positive Control into the desired well(s) and adjust to 50 µl with Assay Buffer. Add 50 µl of Assay Buffer into a well (s) as a Reagent Control (RC).

4. Reaction Mix: For each well, prepare 40 µl Reaction Mix:

33 µl Assay Buffer
3 µl 40 mM NADPH solution
2 µl GR solution
2 µl GSH solution

Add 40 µl of the Reaction Mix to each test samples, Positive Control (s) and RC(s) mix well, and incubate for 15 minutes to deplete all GSSG in your sample. Add 10 µl Cumene Hydroperoxide Solution to start GPx reaction. Mix well. Measure OD 340 nm at T1 to read A1, measure OD 340 nm again at T2 after incubating the reaction at 25 °C for 5 min (or longer if the GPx activity is low) to read A2, protect from light. $\Delta A_{340\,nm}$ = [(Sample_A1-Sample_A2) – (RC_A1 – RC_A2)]

Notes:

A. Measure the OD 340 nm before adding Cumene Hydroperoxide. Add more NADPH if the Sample OD at 340 nm is lower than 1.0 to ensure there is enough NADPH in the reaction system. 1 µl of 40 mM NADPH will give ~ 0.5 OD at 340 nm.

B. If A1 reading is too low (< 0.7), it means either too much GPx or too much GSSG presence in the sample. You may need to dilute the samples, or remove GSSG from your sample using methods, such as dialyzing the sample or using spin filters (BioVision Cat.# 1997-25) to remove GSSG.

C. It is essential to read A1 and A2 in the reaction linear range. It will be more accurate if you read the reaction kinetics. Then choose A1, A2, in the reaction linear range.

5. Calculation: Plot NADPH standard Curve. Apply the ΔA_{340nm} to the NADPH standard curve to get NADPH amount B.

$$\text{GPx Activity} = \frac{B}{(T2 - T1) \times V} \times \text{Sample dilution} = \text{nmol/min/ml} = \text{mU/mL}$$

Where: **B** is the NADPH amount that was decreased between T1 and T2 (in nmol).
T1 is the time of first reading (A1) (in min).
T2 is the time of second reading (A2) (in min).
V is the pretreated sample volume added into the reaction well (in ml).

Unit Definition: One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to $NADP^+$ under the assay kit condition per minute at 25°C.

Figure: (a) NADPH Standard Curve. (b) Measurement of GPx activity using purified enzyme. (c) GPx Activity was measured using rat liver lysate (23 µg), human serum (1 µl) and HeLa cell lysate (16 µg). Assays were performed following the kit protocol.

RELATED PRODUCTS:

Glutathione Reductase Assay Kit
Colorimetric Glutathione Detection Kit
GST Assay Kit
NAD(P)/NAD(P)H Quantification Kit
Catalase Assay Kit
Glutathione Kit (GSH, GSSG and Total)
Triglyceride and Fatty Acid Assay Kit
Hydrogen Peroxide Assay Kit

FOR RESEARCH USE ONLY! Not to be used on humans.

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GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures until use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		

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Product Information

19160 SOD determination kit

Application

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion (O2.-) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In order to determine the SOD activity, several direct and indirect methods have been developed. Among these methods, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use. However, there are several disadvantages to the NBT method, such as poor water solubility of the formazan dye and the interaction with the reduced form of xanthine oxidase. **SOD Assay Kit-WST** allows very convenient SOD assaying by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O2 are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, as shown in Figure 1. Therefore, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method.

Figure 2 shows the absorption spectrum of WST-1 formazan. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 440 nm.

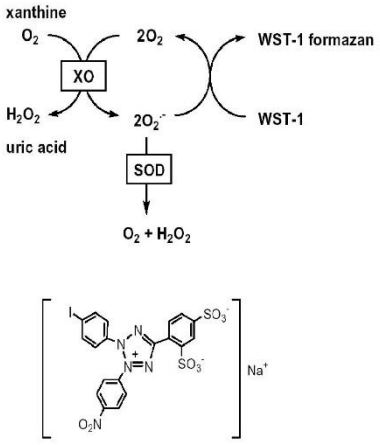

Figure 1: Principle of the SOD Assay Kit

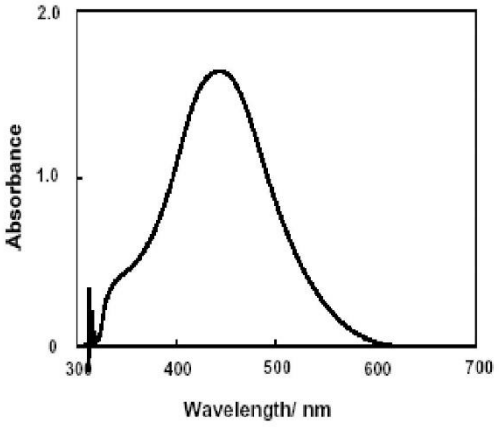

Figure 2. Absorption spectrum of WST-1 formazan.

Kit Content

a) WST Solution 5 ml x 1
b) Enzyme Solution 100 µl x 1
c) Buffer Solution 100 ml x 1
d) Dilution Buffer 50 ml x 1
e) Manual

Equipment required

a) Plate reader (450 nm filter)
b) 96-well microplate
c) 10 µl & 100-200 µl pipettes and a multi-channel pipette
d) Incubator
e) Superoxide dismutase (SOD), if necessary for the preparation of an inhibition curve

Preparation of working solutions

a) WST working solution: Dilute 1 ml of WST Solution with 19 ml of Buffer Solution.
b) Enzyme working solution: Centrifuge the Enzyme Solution tube for 5 sec. Mix by pipeting, and dilute 15 µl of Enzyme Solution with 2.5 ml of Dilution Buffer.
c) SOD Solution (for assay monitoring, if necessary): Dilute SOD with Dilution Buffer to prepare SOD Standard Solution as follows:
200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml, 0.01 U/ml, 0.001 U/ml

Methods

Concentrated protein assay
Refer to Table 1 for the amount of solutions in each well. If you are using a SOD standard, set up wells for it in the same manner as the sample.

1) Add 20 µl of sample solution to each sample and blank 2 well, and add 20 µl of ddH2O (double distilled water) to each blank 1 and blank 3 well.
2) Add 200 µl of WST Working Solution to each well, and mix.
3) Add 20 µl of Dilution Buffer to each blank 2 and blank 3 well.
4) Add 20 µl of Enzyme Working Solution to each sample and blank 1 well, and then mix thoroughly*.
5) Incubate the plate at 37 °C for 20 min.
6) Read the absorbance at 450 nm using a microplate reader.
7) Calculate the SOD activity (inhibition rate %) using the following equation:
SOD activity (inhibition rate %) = {[(Ablank 1 - Ablank 3) – (Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} x 100

	Sample	Blank 1	Blank2*	Blank 3
Sample solution	20 µl		20 µl	
ddH2O		20 µl		20 µl
WST working solution	200 µl	200 µl	200 µl	200 µl
Enzyme working solution	20 µl	20 µl		
Dilution buffer			20 µl	20 µl

Table 1: Amount of each solution for sample, blank 1, 2 and 3
*If Sample Solution has visible color, set up separate "blank 2" lane.

Notes

1) For an accurate measurement, the use of multiple wells per sample is recommended (see Fig. 3).
2) Since superoxide will be released immediately after the addition of Enzyme Working Solution to a well, use a multi-channel pipette to avoid the reaction time lag of each well.
3) Inhibition activity can also be determined by a kinetic method. Please determine an incubation time range that has a linearity of the slope before the assay. A good linearity should be observed up to 20 min. For the calculation, use the following equation:
   SOD activity (inhibition rate %) = {[(S1 - S3) - (SS - S2)] / (S1 – S3)} x 100
   S1: slope of blank 1
   S2: slope of blank 2
   S3: slope of blank 3
   SS: slope of sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	SOD 200 U/ml			Blank 1			Blank 2			Blank 3		
B	SOD 100 U/ml			SOD 0.05 U/ml			SOD 0.01 U/ml			SOD 0.001 U/ml		
C	SOD 50 U/ml			Sample 1			Sample 7			Sample 13		
D	SOD 20 U/ml			Sample 2			Sample 8			Sample 14		
E	SOD 10 U/ml			Sample 3			Sample 9			Sample 15		
F	SOD 5 U/ml			Sample 4			Sample 10			Sample 16		
G	SOD 1 U/ml			Sample 5			Sample 11			Sample 17		
H	SOD 0.1 U/ml			Sample 6			Sample 12			Sample 18		

Figure 3:Sample and blank arrangement on a 96-well plate including SOD standard solutions.

Interfering agents

Reducing agents, such as ascorbic acids and reduced forms of glutathiones, interfere with the SODassay. The following are the concentrations of materials that cause 10% increase in the O.D. value. Please note that since the increase in the O.D. values can be subtracted as the O.D. of blank 2, these materials do not interfere with the actual SOD assay.

Bovine Serum Albumin: 5% w/v
Ascorbic acid: 0.1 mM
Glutathione, reduced form: 5 mM

Inhibition curve

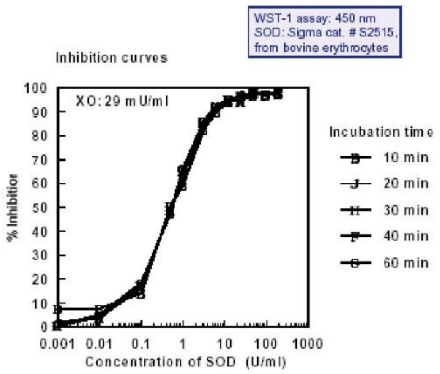

Figure 4: Inhibition curves prepared by WSZ-1 assay with different incubation time

Storage
Please store at 0-5℃. WST Working Solution is stable for 2 months at 4℃. Enzyme Working Solution is stable for 3 weeks at 4℃. Protect WST Solution and WST Working Solution from light.

Precautions and Disclaimer:
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Standard Operating Procedure	SOP:238
Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory (HWU/UTK)	Date:07/10/15
Dr. TB Henry (T.Henry@hw.ac.uk)	

RNA EXTRACTION FROM MUSSEL TISSUE

Authors of SOP: Julianna Measures, jm17@hw.ac.uk
Ana Catarino, a.catarino@hw.ac.uk

Objectives: Protocol for extracting total RNA from mussel (*Mytilus edulis*) gill and digestive gland tissue

Reference: Based on the Qiagen RNA easy mini kit (cat#74106, Qiagen) protocol and SOP 107 (For extracting total RNA from Zebrafish larvae). These procedures were further modified to improve efficiency, quantity and quality of RNA extracted.

Required buffers/reagents and eppendorfs/minicolumns (pink) for RNA extraction up to samples:

Kit Buffer/Reagent	Total Quantity # / Volumes required (µl) (Stocks)									
	1 sample	2 samples	3 sample	4 samples	5 samples	6 sample	7 samples	8 sample	9 samples	10 samples
Eppies	1	2	3	4	5	6	7	8	9	10
Eppies	1	2	3	4	5	6	7	8	9	10
RLT	350	700	1,050	1,400	1,750	2,100	2,450	2,800	3,150	3,500
70% EtOH	350	700	1,050	1,400	1,750	2,100	2,450	2,800	3,150	3,500
RNeasy minicolumn (pink)	1	2	3	4	5	6	7	8	9	10
RW1 buffer (x2)	350	700	1,050	1,400	1,750	2,100	2,450	2,800	3,150	3,500
	350	700	1,050	1,400	1,750	2,100	2,450	2,800	3,150	3,500
	700	1,400	2,100	2,800	3,500	4,200	4,900	5,600	6,300	7,000
RDD	70	140	210	280	350	420	490	560	630	700
DNase	10	20	30	40	50	60	70	80	90	100
RPE buffer (x2)	500	1,000	1,500	2,000	2,500	3,000	3,500	4,000	4,500	5,000
	500	1,000	1,500	2,000	2,500	3,000	3,500	4,000	4,500	5,000
Eppies	1	2	3	4	5	6	7	8	9	10
RNase-free water	30	60	90	120	150	180	210	240	270	300

NOTE: During centrifugation of odd number of samples, make sure to use a dummy eppie with same (tap) water volume as your sample, or provided minicolumn.

1. Set up work space and equipment:

- Gloves
- Ethanol (for cleaning)
- Wipes/tissues
- Sonicator (S1, JM)
- Ice container
- Ice
- 50 mL plastic beaker (with ice)
- Pipettes for 80, 350, 500, 700 µl
- Tips with filter
- Tips waste container
- Eppendorf racks
- Eppendorf tubes
- Qiagen RNA easy mini kit (cat#74106, Qiagen)
- Centrifuges (S4, JM)
- NanoDrop (3.18, WP)
- Clean benchtop and sample rack (ethanol 70%)

2. Storage and initial steps:

2.1. After dissection, tissue samples (e.g digestive gland and gills) should be stored at -80°C

2.2. Before starting the RNA extraction, cut a very small piece of the target tissue and place in new labelled Eppendorf. Store back the original sample at -80°C
2.3. Allow new sample to thaw
2.4. **Keep samples on ice throughout the procedure.** Work on samples on ice as far as possible/unless otherwise stated

3. Preparation of DNase solution:

3.1. Remove metal shield from lyophilized vial
3.2. Add 550 µl of molecular water using a syringe to lyophilized vial (trough the rubber lid)
3.3. Mix thoroughly
3.4. Ensure all powder is dissolved
3.5. Remove rubber lid carefully
3.6. Using a pipette, carefully collect all liquid remaining in the lid and mix with main solution
3.7. Aliquote 50 µl into clean 0.5 ml eppie tubes
3.8. Label DNase (date)
3.9. Store in a labelled plastic bag (content, person in charge, date) at -80°C (or fridge for 4-6 weeks)
3.10. When ready to use, thaw one of the stock in a 0.5 ml eppie

4. Homogenization using sonication:

4.1. Add 350 µl RLT to each sample
4.2. Place eppendorf sample tube in a beaker of ice
4.3. Clean the sonicator with 70% EtOH before each sample. Wipe with tissue to dry
4.4. Insert the sonication probe in the eppendorf tube with the tip in the middle of the sample
4.5. Secure into position using the clamp
4.6. Close cabinet doors
4.7. Sonicate for 5s on power medium, amplitude 4, check and leave to rest for 10s (ensure the sample does not overheat, this may damage the sample)
4.8. Repeat for up to five times: 5s, power medium, amplitude 4, check
Note: It is ok if tissue is still visible but the liquid should be yellowed
4.9. Centrifuge: 3 min, max speed
4.10. Transfer the supernatant (top) (350µl), leave the pellet (bottom) into a new eppendorf tube
4.11. Add equal volumes of 70% ethanol (c. 350-340µl), mix well by pipetting

5. RNeasy minicolumn (pink):

5.1. Pipette the 700 µl sample (all, including any precipitate) onto an RNeasy minicolumn (pink)
5.2. Centrifuge: 15s, max speed
5.3. Discard the flow through (keep the collection tube)

5.4. Pipette 350 µl RW1 buffer onto the RNeasy minicolumn (pink)
5.5. Centrifuge: 15s, max speed
5.6. Discard the flow through (keep the collection tube)

5.7. Pipette 70 µl of RDD and add of 10 µl DNase stock directly onto the RNeasy gel membrane (make sure it does not stay in tube walls)
5.8. Leave out of ice for 15 min

5.9. Pipette 350 µl RW1 buffer onto the RNeasy minicolumn (pink)
5.10. Centrifuge: 15s, max speed
5.11. Discard the flow through (keep the collection tube)

5.12. Pipette 500 µl RPE buffer onto the RNeasy minicolumn (pink)
5.13. Centrifuge: 15s, max speed
5.14. Discard the flow through (keep the collection tube)
5.15. Pipette a second 500 µl RPE buffer onto the RNeasy minicolumn (pink)
5.16. Centrifuge: 15s, max speed
5.17. Discard the flow through (keep the collection tube)
5.18. Centrifuge: 2 min, max speed (to dry to RNeasy gel membrane)

5.19. Transfer the RNeasy minicolumn (pink) to a new 1.5 mL eppendorf tube (label for storage)
5.20. Pipette 30 µl of RNase-free water directly onto the RNeasy gel membrane
5.21. Let sit for 5 min
5.22. Centrifuge: 1 min, max speed (take care when placing eppies, not to break tops while centrifuging)
5.23. Discard the RNeasy minicolumn (pink) and close the eppendorf tube
5.24. Return to ice

6. NanoDrop test for quality control and RNA quantification:

6.1. Turn on the computer
6.2. Log in using the password 'WP318'
6.3. Clean with soft wipe
6.4. Open software NanoDrop
6.5. Add 1 µl RNase-free water on "reader"
6.6. Double click Nucleic Acid
6.7. Click OK - read
6.8. Clean with wipe
6.9. Change sample type to RNA
6.10. Add 1 µl RNase-free water
6.11. Click blank - read
6.12. Clean with wipe

6.13. Mix your sample by gently pipetting
6.14. Add 1 µl of sample
6.15. Name the sample i.e. JME5:1A
6.16. Click measure - read
6.17. Clean with wipe

6.18. Add a second 1 µl of the same sample
6.19. Name the sample i.e. JME5:1B
6.20. Click measure - read
6.21. Clean with wipe

6.22. Once all samples have been measured (twice) click report
6.23. Highlight all samples and save
6.24. Export to excel
6.25. Using the excel file calculate the amount of RNase-free water to add to 28 µl (or remaing volume, depending on number of reads) of the sample to dilute it to 100 ng / µl
6.26. Store at -80°C or leave on ice (fridge) if reverse transcription is done on same day

Notes:
Optimum quantity > 100 ng / µl (in mussel tissue usually above 500 ng / µl)
Optimum ratios: 260/280 at 1.8-2.1 and 260/230 above 1.5
If samples are not of high enough quantity or quality discard and repeat RNA extraction on another tissue sample.

Standard Operating Procedure	SOP:240
Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory (HWU/UTK)	Date: FEB 2015
Dr. TB Henry (T.Henry@hw.ac.uk)	

REVERSE TRANSCRIPTASE REACTION TO GENERATE cDNA FROM EXTRACTED RNA
MUSSEL TISSUE (DIGESTIVE GLAND AND GILLS)
(Preparation for gene expression assays)

Authors of SOP: Danai Patsiou, Dr. TB Henry
Adapted by Ana Catarino, a.catarino@hw.ac.uk, for mussel tissues

Objective: Establish a protocol for reverse transcription of RNA for assessment of gene expression (see original SOP #128 for further detail)

Reference: This SOP was developed in part from resources provided by Primer Design. Complete manual on file: Precision nanoScript ™2 Reverse Transcription kit, Instruction for cDNA synthesis using up to 2 µg of RNA. Handbook HB05.09.02

Kit Contents: (Enough reagents are supplied to complete up to 50 Reverse Transcription reactions)

- Oligo-dT primer (YELLOW)
- Random nonamer primer (RED)
- dNTP mix (10mM of each) (ORANGE)
- nanoScript 2 enzyme (WHITE)
- nanoScript 2 4X reaction buffer (BLACK)
- RNAse/DNAse free water (WHITE)
- (Optional) Gene specific primer, lyophilised (GREEN) re-suspend in 990ul Water Reagents

Equipment (supplied by user)

- Thermocycler
- Pipettors
- Tips with filters
- Ice and container
- Vortex and centrifuge
- RNA Template (It is recommended RNA be DNAse treated prior to use to reduce the potential for real-time PCR detection of contaminating genomic DNA)
- Thin walled 0.5 ml tubes (or 0.2 ml PCR tubes)

Required buffers/reagents to prepare stock solutions and total volumes according to the number of samples:

Step 1: RNA (sample) + RT primer (yellow)	x 2 per sample										
RNA (sample)	9 µl	Always 9 in total but this can be diluted with water (i.e. 4.5 RNAse-free water, 4.5 RNA)									
RT primer (yellow)	1 µl	Always 1									
Total	10 µl	Always 10									
Step 2: RT Mix											
	1	2	3	4	5	6	7	8	9	10	reactions
nanoScript2 buffer (black)	5	10	15	20	25	30	35	40	45	50	µl
dNTP mix (orange)	1	2	3	4	5	6	7	8	9	10	µl
nanoScript2 enzyme (white)	1	2	3	4	5	6	7	8	9	10	µl
Rnase free water (white)	3	6	9	12	15	18	21	24	27	30	µl
Total	10	20	30	40	50	60	70	80	90	100	µl
Add Step 2 to Step 1											
Step 1	10 µl										
Step 2	10 µl										
Total	20 µl										

Annealing Step:

1. Allow samples from RNA extraction to be thawed (if previously stored at -80°C)
2. Pipette 9 µl to new labelled small eppie (0.5 ml)
3. For each RNA sample add the following reagents:

RNA (sample)	9	µl
RT primer (yellow)	1	µl
Total	10	µl

4. Run Step 1 (annealing) in the thermocycler (room: 3.18 WP)
5. Apply a lid to each sample and then heat to 65 °C for 5 min
6. Immediately cool the tubes in an ice bath

For optimal results, samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice

Extension step:

1. Prepare RT Mix stock:

Component	1 Reaction (sample)
nanoScript2 4x Buffer (BLACK)	5.0 µL
dNTP mix 10mM (ORANGE)	1.0 µL
RNase/DNase free water (WHITE)	3.0 µL
nanoScript2 enzyme (WHITE)	1.0 µL
Final Volume	**10 µL**

2. Add 10 µL of this mix to each of the samples on ice.
3. Using the thermocycler, run Step 2
4. Analyse cDNA quality using nanoDrop (WP 3.18)
5. Store cDNA samples at -20°C until use

Standard Operating Procedure	SOP:131
Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory (HWU/UTK)	Date: 26 Feb 2015
Dr. TB Henry (T.Henry@hw.ac.uk)	

STANDARD OPERATING PROCEDURES

FOR CONDUCTING PCR

Author of SOP: Nicola Foster, TB Henry

Objective: Establish a protocol for routine PCR with samples obtained from fish tissue

Reference: This SOP was developed in part from a kit provided by Sigma: ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Catalogue number P4600)

Materials: For kit see attached sheet from Sigma.

Notes:

- The PCR reaction on the thermocycler takes ~2 h. Be sure you have enough time to run the reaction because the machine cannot be left to run overnight.
- Forward and reverse primers obtained from the manufacturer are ordered separately and based on specific design criteria. They are shipped as lyophilized vials and must be reconstituted with nuclease-free water. The amount of primer is given on the literature sent with the primer. Following the direction for reconstituting primers, make a 100 µmole/L (=100 pmol/µl) stock solution and also prepare at least four vials of primer working solution (this is to prevent too much freezing and thawing of primers). Working solution is prepared by taking 5 µL of stock solution and diluting to 500 µL with nuclease-free water. This results in a primer working solution concentration of 1 µmole/L (1 µM).
- The concentration of primers in the final reaction volume is important for the PCR reaction. If PCR does not work well, primer concentrations may need to be optimized to improve the success.
- The procedure below is designed for a final primer concentration of 300 nM. Thus, 10 µL of primer working solution will be added for each 50 µL reaction (each sample requires 10 µL).
- The DNA template concentration is important. If the sample is cDNA obtained from reverse transcription of RNA see the procedure for the reverse transcription of RNA. The reverse transcription reaction of RNA is performed on 800 ng (obtained from total RNA extracted from sample and diluted to 100 ng/µL; and final volume of RT reaction of 24 µL) of total RNA and the final concentration of DNA is ~33.3 ng/µL.
- The procedure below is designed for a final DNA template concentration of ~165 ng in the 50 µL reaction volume. Thus, 5 µL of the cDNA template (concentration 33.3 ng/ µL) will be added for each reaction.

Procedure:

1. Obtain a new 0.5 mL nuclease-free tube and label as PCR Master Mix. Keep tube on ice and add the following (where S = the number of samples + 1):
    a. S×(25 µL) ReadyMix (from kit)
    b. S×(10 µL) Forward primer
    c. S×(10 µL) Reverse primer
2. Obtain new 0.2 mL PCR tubes and label for each of your samples and for the no-template control
    a. Keep all tubes on ice.
    b. Add 45 µL of PCR Master Mix (prepared in step 1) to each tube
    c. Add 5 µL (~165 ng) of the sample cDNA (DNA template) to the correct tube.
    d. Add 5 µL of water to the tube labeled "no template control"
    e. Total final volume is 50 µL.
3. PCR cycling parameters (use GeneAmp PCR system 9700 machine in room 428):
    a. Select User > Sheren > Run > Exp001. Parameters should be as below
        i. Heat Lid at 94 °C for 5 min
        ii. Denature the template at 94 °C for 45 s
        iii. Anneal primers at 55 °C for 45 s
        iv. Extension at 72 °C for 45 s
        v. 30 cycles of amplification
        vi. Hold at 72 °C for 7 min
        vii. Hold at 4 °C
        viii. Total running time is ~ 2 h
    b. Remove samples when PCR machine has cooled to 4 °C and store at -80 °C or proceed directly to evaluate product on gel.

Do **NOT** leave the PCR machine to remain at 4 °C overnight.

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Product Information

ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$

Catalog Number **P4600**

TECHNICAL BULLETIN

Product Description
ReadyMix Taq PCR Reaction Mix with $MgCl_2$ is Sigma's high quality Taq DNA polymerase in a 2× concentrate containing all the nucleotides and reagents necessary to perform a standard PCR reaction. ReadyMix Taq PCR Reaction Mix with $MgCl_2$ saves preparation time, reduces the risk of contamination from multiple pipetting steps and provides consistent reaction-to-reaction performance.

For a typical PCR reaction, mix 25 µL of ReadyMix Taq PCR Reaction Mix with $MgCl_2$ with 25 µL of a mixture containing template DNA, primers, and water. Reaction volumes can be scaled down, if desired.

Reagents
- ReadyMix Taq PCR Reaction Mix with $MgCl_2$, Catalog Number P0476. 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.002 % gelatin, 0.4 mM dNTP mix (dATP, dCTP, dGTP, TTP), stabilizers, 0.06 units Taq DNA Polymerase/µL. Provided as 20 reactions, 5 × 20 reactions
- Water, PCR Reagent, Catalog Number W1754 Provided as a 1.5 ml vial

Reagents and equipment required, not provided
- Primers
- DNA template
- Dedicated pipets
- PCR pipet tips
- 0.2 ml or 0.5 ml thin-walled PCR tubes, Catalog Numbers P3114 and P3364 or plates for specific thermal cycler
- Thermal cycler
- Mineral Oil, Catalog Number M8662 (optional)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.
When radioactive tracers are used, standard procedures for safely handling radioactive materials should be followed.

Storage/Stability
Store ReadyMix Taq PCR Reaction Mix with $MgCl_2$ at 2-8 °C so there is no waiting for reaction components to thaw. It can also be stored at –20 °C. Repeated freeze thaws do not affect the activity of the reactions.

Procedure
The optimal conditions for template DNA, primers, and cycling parameters will depend on the system being utilized. Sigma offers a separate PCR Optimization Kit, Catalog Number OPT2, that contains a variety of buffers and adjuncts for optimizing the specificity, fidelity, and yield of a PCR product.

1. Add the following reagents to a 0.2 or 0.5 ml PCR tube in the following order:

Volume	Reagent	Final Concentration
25 µL	2× ReadyMix Taq PCR Reagent Mix	1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, stabilizers
1 µL	Forward Primer	0.1–1.0 µM (15-30 bases in length)
1 µL	Reverse Primer	0.1–1.0 µM (15-30 bases in length)
x µL	Template DNA	
q.s.	Water	
50 µL	**Total Volume**	

2. Mix gently and briefly centrifuge to collect all components to the bottom of the tube.

3. Add 50 µL of mineral oil to the top of each tube to prevent evaporation if using a thermal cycler without a heated lid.

4. The amplification parameters should be optimized for individual primers, template, and thermal cycler.

   Common cycling parameters:
   a. Denature the template at 94 °C for 1 minute
   b. Anneal primers at 55 °C for 2 minutes
   c. Extension at 72 °C for 3 minutes

   25-30 cycles of amplification are recommended.

5. The amplified DNA can be evaluated by agarose gel electrophoresis and subsequent ethidium bromide staining. Mineral oil overlay may be removed by a single chloroform extraction (1:1), recovering the aqueous phase.

AF,CR,PHC 07/09-1

Standard Operating Procedure	SOP:130
Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory (HWU/UTK)	Date: 15 04 2015
Dr. TB Henry (T.Henry@hw.ac.uk)	

STANDARD OPERATING PROCEDURES FOR PERFORMING AGAROSE GEL ELECTROPHORESIS ON RT-PCR PRODUCTS FROM ZEBRAFISH

Author of SOP: Valentina Ricottone, Dalia A. Sabrei and TB Henry.

Objective: To establish a protocol for identification of the sizes and relative quantities of the products of RT-PCR from mRNA.

Notable Hazards: Take care when handling hot solutions. Risk of superheating solution, caused if boils over without warning. Swirl regularly when heating and hold at arm's length.

Materials:

- EDTA disodium salt (E5134-500g) SIGMA
- Agarose (A9539SIGMA)
- Sucrose (S1888 SIGMA)
- Bromophenol blue (114405 SIGMA)
- Tris base (T1503 SIGMA)
- Boric acid (B7901 SIGMA)
- TrackIt™ 10 bp DNA Ladder (10488-019- Life technologies)
- GelRed Nucleic Acid Gel Stain, 10,000X in water (BT41003-Biotium)

Equipment:

- 1-10 µl and 20-100 µl pipettes and correspondent filtered tips.
- Ice
- RNA-free microcentrifuge tubes
- Flask
- Electrophoresis tank
- Power supply
- Tray for staining gel
- UV DOC (room:S17)
- Gloves
- RNase ZAP (R2020 SIGMA)

Reagent Preparation

Prepare 10xTBE buffer (or 10xTAE-see end protocol) as per the manufacturer's instructions, or from the following recipes.

10xTBE buffer (1l)

- 109g Tris base
- 55g Boric acid
- 4.8g EDTA (disodium salt)
- Dissolve in 900ml deionised water, adjust pH to 8.3 with NaOH, and then make up to 1L with water.

Loading dye (5ml)

- 40% Sucrose
- 0.25% bromophenol blue
- Dissolve in 5ml of deionised water

Staining solution (500ml)

Dissolve 50µl of gel red in 500ml of deionized water.

- Note: This solution can be used up to 5-7 times. There is already a made one in S4 fridge. If you use it, please write it on the bottle (USE:…)

Procedure:

Preparing Agarose Gel

1. Prepare the gel mould by pressing along the edges the rubber red leads to ensure a good seal. Set on a level surface.
2. Make in a conical flask, a 2% agarose in 50ml or 300ml TBE respectively for a mini or wide gel.
3. Heat on full power in a microwave oven for 1-6 min, depends the volume. Swirling after 45s, the gel will be ready when the solution is clear. Check that all the agarose is dissolved.
4. Take the flask out the microwave very carefully and slowly pour the molten gel into the mould. When finished, use a disposable pipette tip to sweep any air bubbles to the sides of the gel.
    - **Notes:** You can stain the gel now by adding 5µl of gel red X 50ml of the small gel or 30µl of gel red X 300ml of the wide gel.
    - **Note:** You can post-stain the small or the wide gel after run the electrophoresis using the staining solution as described above (just put the gel in a try and covered with the staining solution in room temperature around 1 h.
5. Add the gel comb on the tray's notches.

Leave the gel to set for 30 min to 1 h, depends the dimensions.

Preparing Electrophoresis Tank

1. Prepare 1X TBE buffer. This is the running buffer. There should be enough to cover the gel in the tank.
2. Carefully remove the gel comb, making sure that the wells aren't torn, and do not contain air bubbles.

3. Remove the rubber leads from the ends of the gel mould and place it on the platform in the tank, ensuring that the comb is closer to the negative (black) terminal.
    - Note: your sample will run from the black end (negative) to the red one (positive).
4. Pour on the running buffer, ensuring the gel is well covered.

Loading Samples

1. Prepare a clean microcentrifuge tube for each sample. Add 10µl of PCR product to each tube, and then add 2µl of gel loading buffer to each tube.
2. Depending on the primers that you have, there are 2 kinds of ladders (10bp, and 1Kbp). You need to take 2µl ladder mix well with 10µl 1X TBE and load in the first well 10µl.
3. Transfer the entire contents of the microcentrifuge tubes to one of the wells in the agarose gel. Take care not to pierce the gel with the pipette tip and **do not** depress the plunger completely, to prevent ejecting air into the gel.
4. Record each well's contents for reference after the gel has run.

Run the Gel

1. Place the lid on the gel tank and connect to the power supply, ensuring the negative (black) terminal is closer to the sample wells.
2. For the small gel set 100 Volt, 250 mA, 25 W, and run for 52 min.in the other hand the wide gel need 180 Volt, 350 mA, 65 W, and run for 75 min. A stream of bubbles coming from the terminals indicates that a current is flowing.
3. Leave the gel to run until the blue loading dye has moved between ⅔ and ¾ of the length of the gel.
4. Turn off the power supply and disconnect the tank. Remove the lid and carefully extract the tray.
5. If you have already loaded the gel red, proceed to the step 19, otherwise continue as follow. Pour the staining solution in a tray. Place the gel in it, ensuring that the solution is enough to cover the gel. Leave it from 30 min to 1 h, depends the gel's dimension.
6. Take the tray and bring it to S17 for imaging.

Read the gel (J.M. lab S17) - ALWAYS WEAR GLOVES, THE AREA IS CONTAMINATED WITH ETHIDIUM BROMIDE.

1. Switch on the UV illumination DOC.
2. Open the drawer and place the gel in it. The gel should be placed with the wells on the back side of the machine.
3. Switch on the computer. Create a folder on the desktop with your name.
4. Click on the program Image Lab on the desktop.
5. Click on protocol→New→ Application→ Select→ nucleic acid→ gel red
6. Select the size of the gel
7. Click on Position gel. It will appear the image of your gel, minimize and if necessary open the up-door of the machine and reposition the gel.
8. Click on Run protocol

9. Once you have your image, you can customize it. You can save the entire protocol or only the image in your folder.
10. Turn off the computer and the UV DOC.
    IMPORTANT: Remember to throw away the gel and to wipe the surface afterward!!

Tips and Alternative Methods

For best results, the running buffer and gel buffer should be the same concentration.

If possible, avoid using the wells at the sides of the gels, as these can run unevenly.

Here is used TBE as buffer but you could use TAE (below recipe)
10xTAE buffer (1l)

- 1.86g EDTA (disodium salt)
- 48.4g Tris Base
- 1.1ml glacial acetic acid
- Add 900ml deionised water, adjust pH to 8.5 with NaOH, and then make up to 1l with water.

Standard Operating Procedure	SOP:243
Ecotoxicology and Pathophysiology of Aquatic Organisms Research Laboratory (HWU/UTK)	Date: 3rd/May/2016
Dr. TB Henry (T.Henry@hw.ac.uk)	

STANDARD OPERATING PROCEDURES

FOR qPCR USING PRIMER DESIGN METHOD USING cDNA FROM REVERSE TRANSCRIPTASE REACTION IN ORDER TO ANALYSE GENE EXPRESSION.

Author of SOP: J. Middlemiss, Dalia Sabrei and TB. Henry

Objective: Establish a protocol for the quantitative analysis of gene expression in zebrafish *Danio rerio* embryos and larvae exposed to stress-inducing chemicals through following the Primer Design Mastermix for qPCR.

Reference: This SOP was developed at Heriot-Watt University from the procedure outlined in the Primer Design hand book "Instructions for use of Primer Design PrecisionPlus Mastermix and PrecisionFAST Mastermix".

Kit Contents:

- 8 X 1.6ml aliquots of Mastermix:
  1. PrecisionPLUS / PrecisionFAST
  2. Primer/Probe mix
  3. Template (25ng)
  4. RNAse/DNAse free water

Materials required but not supplied in kit:

- Real-Time PCR Instrument
- Pipettes and Tips
- Vortex

Recommended Accompanying Products:

- Primer Design custom designed real-time PCR primers
- Primer Design Precision nanoscript2 Reverse Transcription kit for production of cDNA template

Procedure:

1. To set up the master mix, as shown below, for each 20µl real-time PCR reaction add the following to each reaction tube (see Table 1):

Components	**Volume for 1 Reaction**
PrecisionPLUS	10 µL
Primer (forward)	1 µL
Primer (reverse)	1 µL
Template (cDNA) (25 ng)	5 µL

RNAse/DNAse free water	3 µL
Final Volume	**20 µL**

Table-1 the PrimerDesign kit components

2. Following RNA extraction (SOP 107), high quality samples with a high quantity of RNA must be chosen, for example those samples >100ng/µl with a ratio reading of 260/280 which is around 1.9 to 2.2 and 260/230 which around 1.5 to 2.2. It is often safe to assume that cDNA has been produced in a 1:1 ration after a RT reaction protocol (SOP 128) has taken place.
3. Each qPCR reading plate has 48 wells, which hold a final volume of 20 µl. This volume is composed of the sum of the components found in Table 1.
   Note: Well plates and 'qPCR experiment planning pads' can be found in the drawer below the qPCR machine in laboratory JM S.13.
4. When preparing samples for qPCR reactions, it is advised to set up a stock solution of the master mix for each primer used, due to the fact that such small quantities are used. When setting up the stock solution for a master mix, the number of wells required will determine the amount of master mix that should be prepared. For example if 10 wells are to be used 150 µL of mastermix should be prepared as follows: 100 µL of PrecisionPLUS, 10 µL of forward primer, 10 µL of reverse primer, and 30 µL of RNAse/DNAse-free water. To each well on the plate, 15 µL of mastermix will be added and 5 µL of the specific cDNA template that you want to amplify. It is wise to prepare enough for one extra well.
5. It is strongly advised that the Rv and Fw primers/probes should be made up into separate working stock solutions for repeated use, so that the main stock is not defrosted and frozen repeatedly. This prevents possible degradation.
   Note: The qPCR Mastermix must be kept on ice and wrapped in tin foil due to its photodegrading properties.
6. The final volume for our samples after doing the RT protocol is 20 µl.
7. Before run any primer for our samples, we need to check the efficiency for it to make sure that the efficiency must be between 90 – 110, so we need to make a serial dilution from 1:10 dilution to 1:100000 for one of the experimental samples that we nearly sure that we will get an amplification for it to the specific primer.
8. It is important to use the 'qPCR experiment planning pad' in order to note down which wells contain what, and they are numbered from 1A to F8. This not only ensures accuracy whilst pipetting out samples, but also gives a guide for how to enter data into the Step1 Software used to analyse samples later on.
   For example (where the gene of interest is Cyp-1, tested in sample A, and the reference gene β-actin, tested in sample B, and NTC refers to Non Template Control (na adding cDNA):

	1	2	3	4	5	6	7	8
A	A	A	A	A/10	A/10	A/10	A/100	A/100
B	A/100	A/1,000	A/1,000	A/1,000	A/10,000	A/10,000	A/10,000	B
C	B	B	B/10	B/10	B/10	B/100	B/100	B/100

D	B/1,000	B/1,000	B/1,000	B/10,000	B/10,000	B/10,000		
E								
F				NTC Cyp-1A	NTC Cyp-1A	NTC β-actin	NTC β-actin	Water

9. As seen above, all samples tested must be done so in duplicate or triplicate where possible. In wells A1 to F3 where experimental samples are being tested, the components from table- 1 are required (15µl) along with the reference or specific gene of interest.

Note: When carrying out qPCR to measure the presence of a specific gene sequence, it is important to use a second primer relating to a reference gene which is always stably expressed in the organism of whom their RNA you are testing. An appropriate reference gene to use for zebrafish larvae can be β-actin, but others should be considered depending on the experiment.

10. In well F4 to F7, it is conventional to run a negative control for the qPCR reaction for both the reference gene and the experimental gene used. These controls are made up of the usual 15µl of stock solution, but instead of a test sample, 5µ of RNAse/DNAse free water is added.

11. In the final well, F8, 20µl of RNAse/DNAse free water is added to test the evaporation of the qPCR instrument.

12. Once the well plate has been set up for testing, the qPCR machine and accompanying computer (located in JM S.13) must be switched on with the log in details:
Username:Administrator
Password: Administrator
And the program 'Step1 Software' opened up.

13. See Figs – 1 to 5 to set up the qPCR plate through following the numbered red arrows:

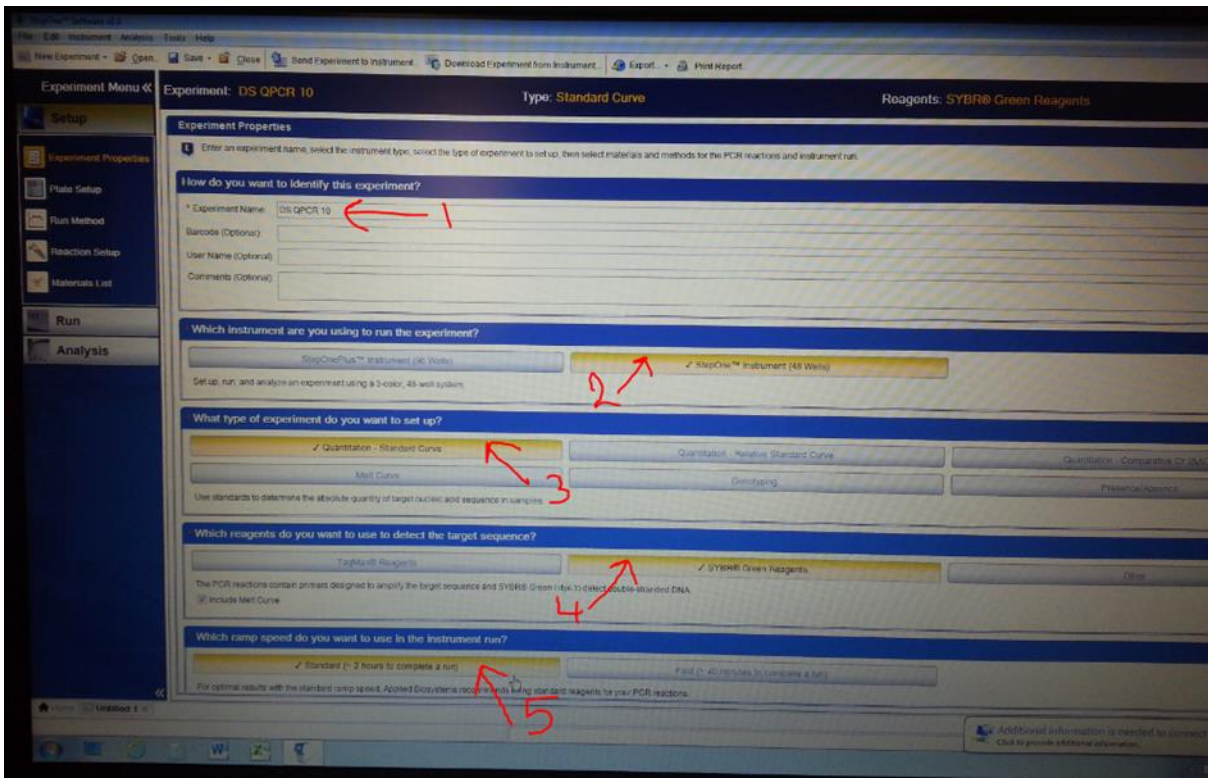

Fig-1: In the tab labelled 'Experimental Properties' in Advanced set up, the experiment name should be entered and the correct experimental properties selected (i.e. 48wells, standard curve, SYPR green, ~ 2hours run).

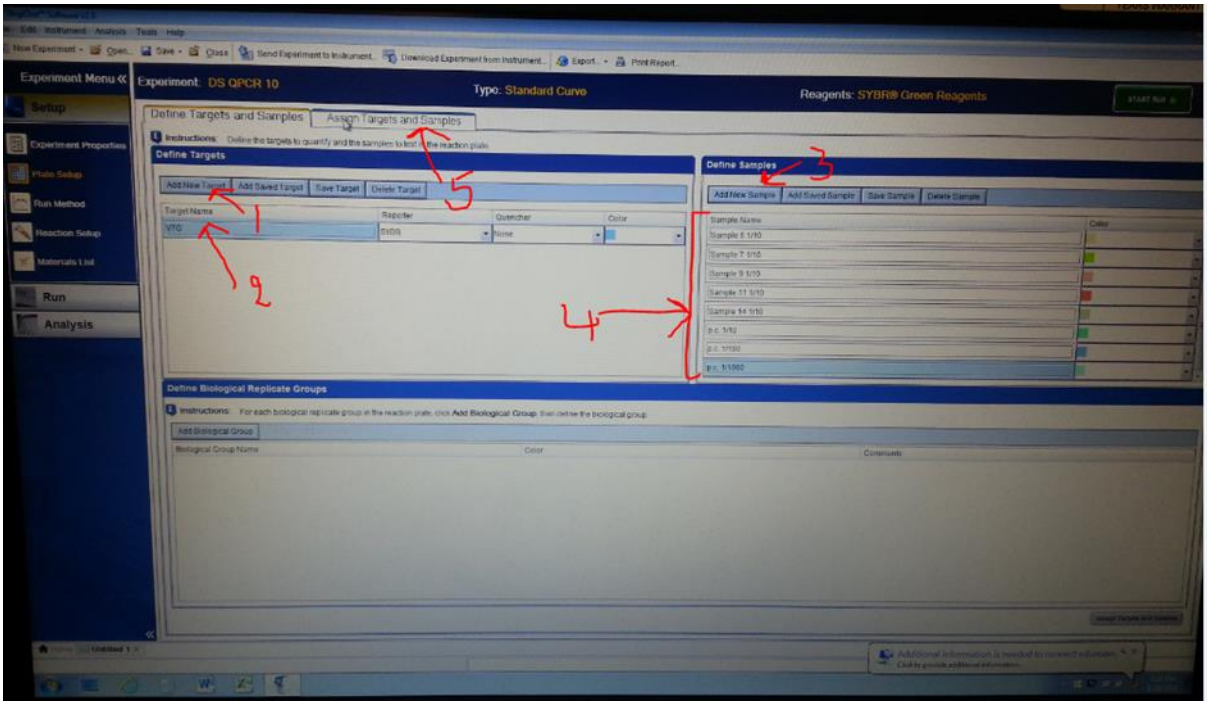

Fig- 2: Then the tab 'Targets' in plate set up should be selected, and where necessary the targets of this experiment should be outlined. For example, the gene of interest, the reference gene, the NTC's for both genes and the control. Then the tab 'Sample name' should be selected and assigned, for example A/1, A/10, A/100 etc. The corresponding targets and sample names should be assigned to the 48 well plate graphic on the program to allow for ease of reading the data.

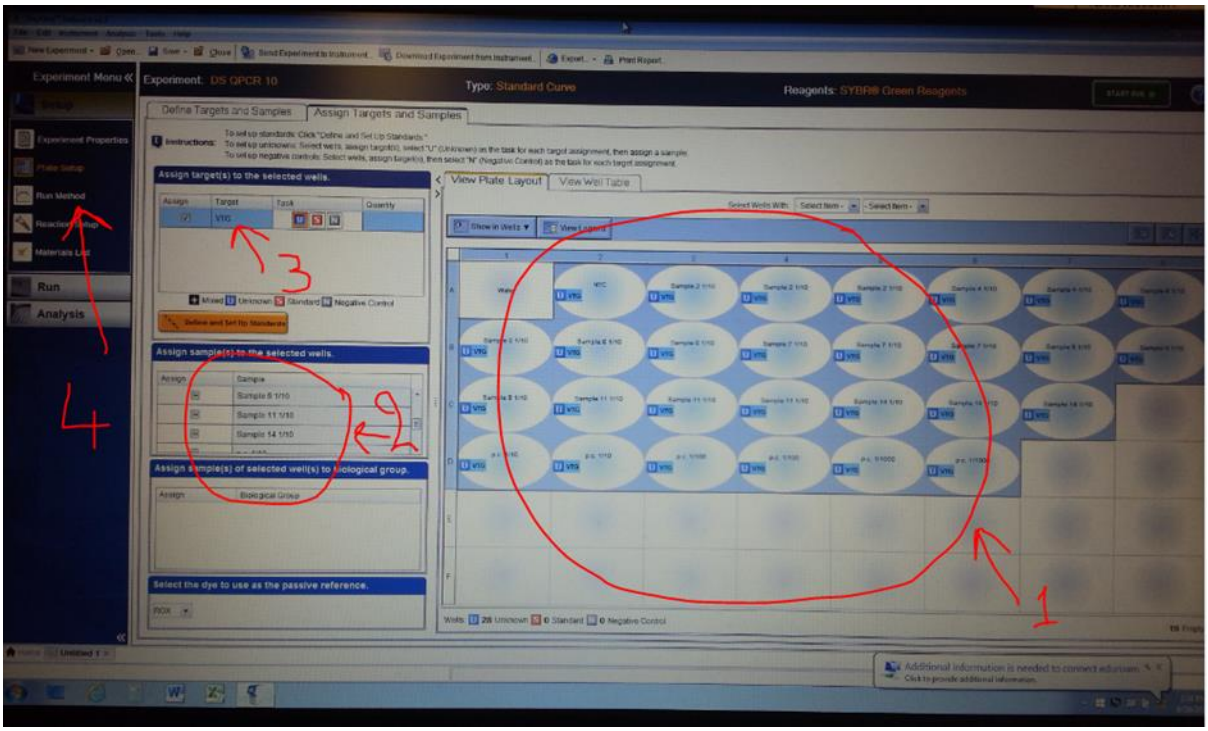

Fig-3: In the ''Assign Targets and Samples'' and through following the qPCR's pad plan which needs to apply in the 48 wells plate by picking the specific wells with their specific number (see red arrows 1&2), then highlighted all the wells and choose the target gene (red arrows no. 3), Finally, before beginning the qPCR process, 'Run Method' must be selected and the appropriate Annealing temperature inputted.

Note: Theory states that the annealing temperature for qPCR should be 5°C below the calculated primer melting temperature; however it has been found that the use of different software will use different temperature calculations and thus the values may widely differ. To confirm the correct annealing temperature, one should run regular PCR at different temperatures, run an electrophoresis gel and then determine which band is brightest at which temperature. Once the optimum temperature has been identified, it is then possible to carry out the qPCR experiments with your primer. However, if the required genes had been determined there annealing temperature by the previous researcher in the lab, so no need to do the PCR and the electrophoresis gel AGAIN!

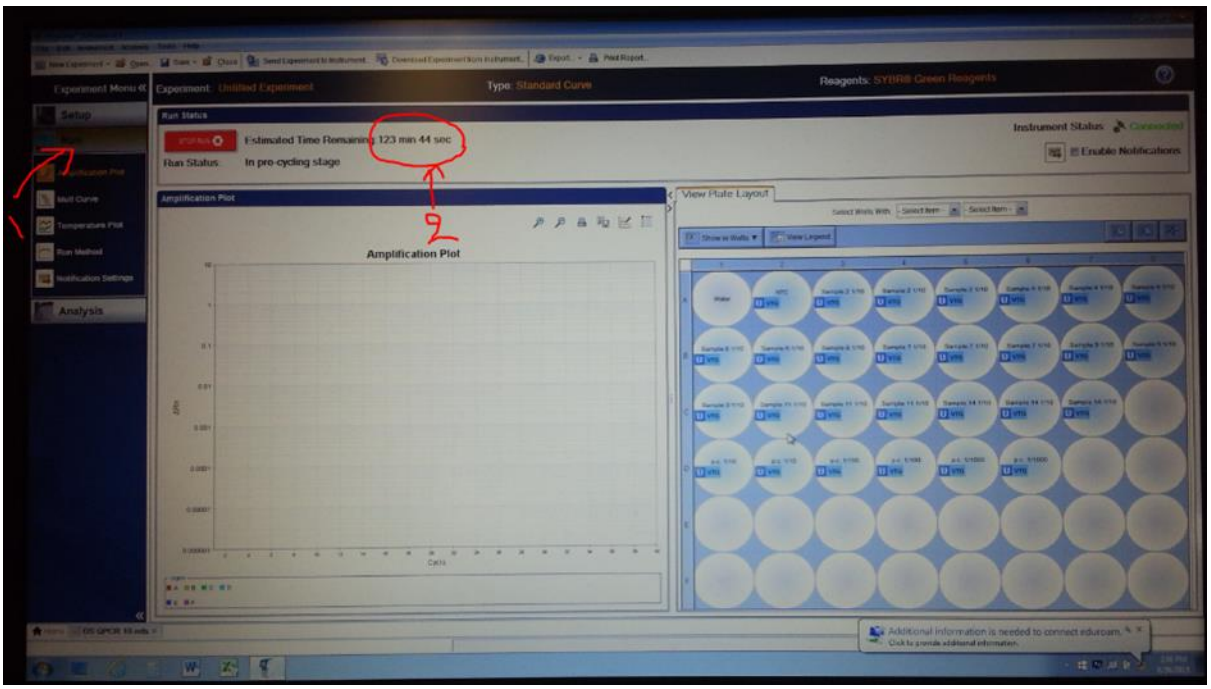

Fig-4: After finishing named all the wells, then press Run (red arrow number 1), then the qPCR machine start to work and there is a dialogue box comes in the screen ask you to save your plate in you folder and then the qPCR machine starts to work and the stage goes up and start running through seeing the time appears in the screen (see red arrow number 2).

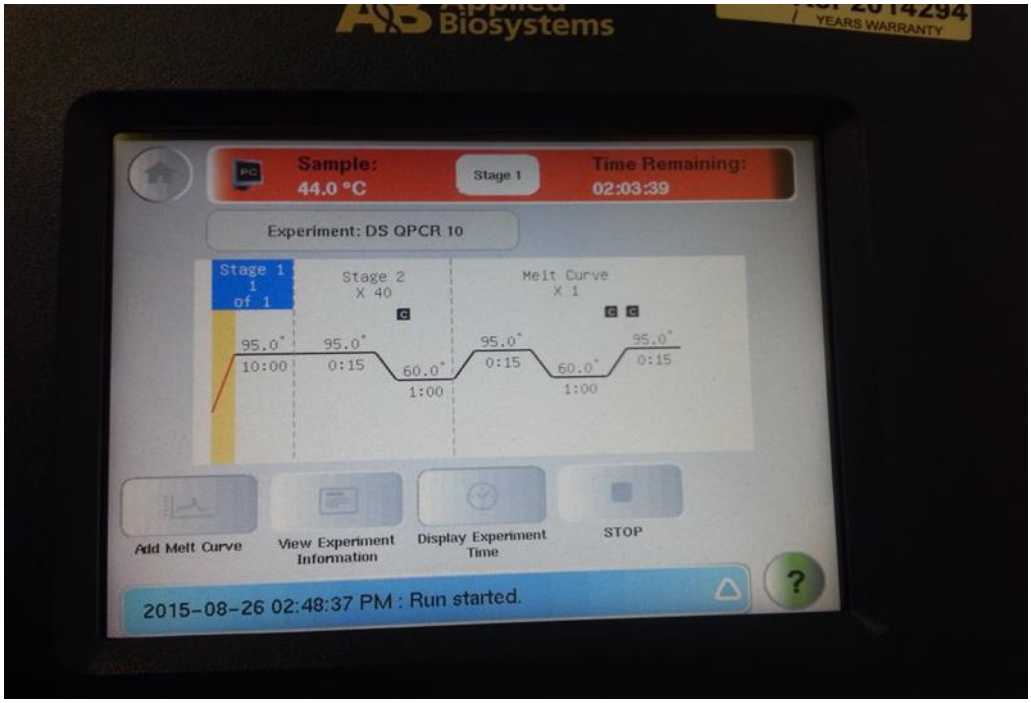

Fig-5: Finally, this the screen that will be papered in the qPCR machine itself after pressing the RUN bottom.

Analysing the qPCR Data:

Note: The information of this part of the SOP was taken from the module: ((A11PL Practical Skills in Marine Biotechnology)).

After finishing run the qPCR we copy paste the results to the Excel sheet and label then with the same experiment name and number. Then, **analysing the qPCR Data** comes through as below example:

- These results were obtained was designed to assess changes in expression of the gene transcript cytochrome p450 1A (*cyp1A*) in larval zebrafish according to exposure to different concentrations of a toxicant, and according to different durations of exposure. The output of the Q-PCR machine will be provided as the dataset for analyses, and your first step should be to examine the data set and make sure you understand the treatments and the arrangement of the data set. Your reference gene transcript is β-actin (*β-actin*), and your target gene is *cyp1A*. Determine the efficiency of the PCR reaction by comparing the change in C_T value for the gene transcript relative to the concentration of the standard, which has dilutions of 1, 1/10, 1/100, and 1/1000 (note: convert dilutions to Log_{10} for plotting) (Figure-6). The efficiency of the PCR reaction is computed from the following equation: Efficiency $= 10^{(-1/\text{slope})} - 1$

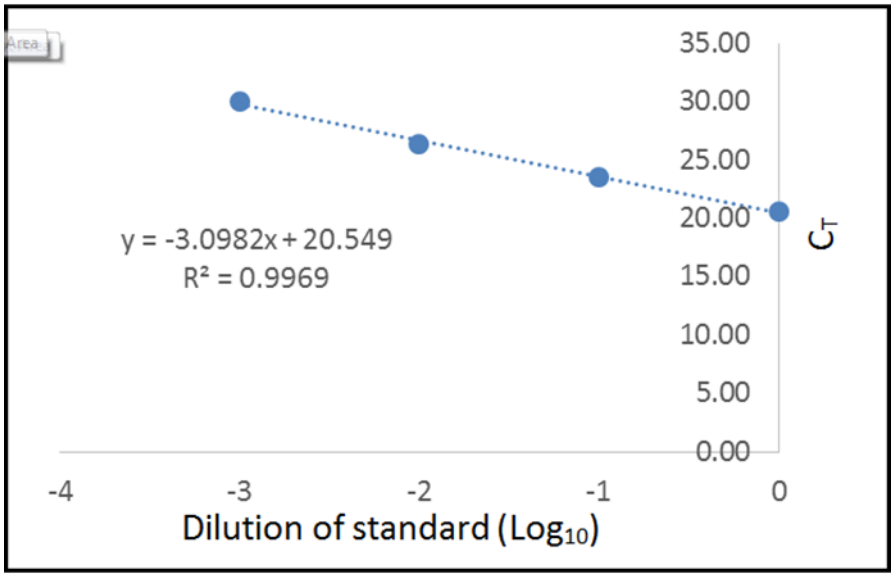

Figure-6: Efficiency of Q-PCR indicated by the C_T of target gene relative to dilution (plotted as Log_{10} of the dilution) of the standard. The computed efficiency for this example was 110%.

- Plot the change in expression of the reference gene and determine whether the selected reference gene is appropriate for comparison to the target gene (Figure-7).

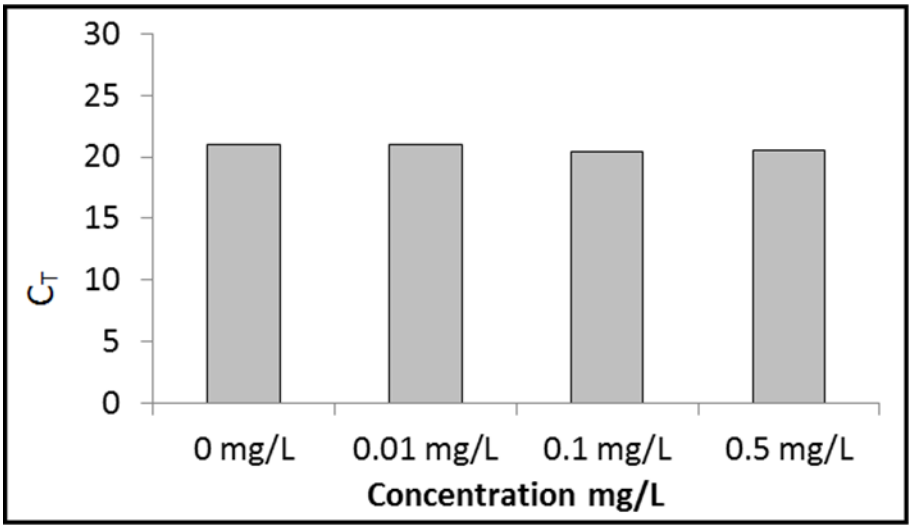

Figure-7: Change in C_T value relative to concentration of toxicant exposure. By observation there is no appreciable change in C_T relative to the experimental treatments (i.e., toxicant concentration).

- Change in expression of target gene is assessed by the $\Delta\Delta C_T$ (delta-delta C_T) method, which enables computation of the fold change in expression to be determined for a target gene relative to a reference gene. In words, this is computed by subtracting the C_T value of the target gene from the C_T of the reference gene within each sample (to obtain the ΔC_T value), then subtracting the ΔC_T for each treatment sample from the ΔC_T of the control (to obtain the $\Delta\Delta C_T$ value), and then raising 2 to the exponent of the $-\Delta\Delta C_T$. (i.e., $2^{-\Delta\Delta Ct}$). The fold change in expression of *cyp1A* is $2^{-\Delta\Delta Ct}$ of the sample (Figure-8).

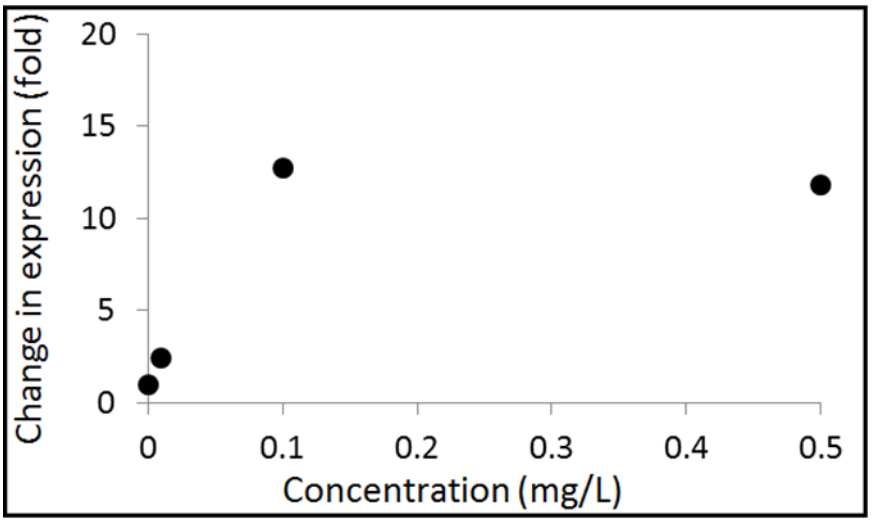

Figure-8: Change in expression of target gene in response to experimental treatment (e.g., toxicant concentration).

Appendix D

Raw data collected for chapters 3 – 5. This appendix is included on the supplied CD.