A life submerged in sound: Determining if aquaculture sounds induce stress in fish

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Abstract

Fish bioacoustics includes research on sound production and audition in fishes as well as effects of anthropogenic sound on fishes. An environment that is wholly anthropogenic is an aquaculture recirculating system. Fish within this culture system are under unique conditions, as the associated sounds are not 'natural', and the fish are completely confined to this soundscape with no option of escape. This environment is poorly understood in relation to how it affects the biology of cultured species. The limited available information on this type of soundscape and the physiological interaction fish have with it, has presented many unknown potentials, which includes the risk to stock productivity and fish welfare. The lack of understanding helped to create the baseline question for this compilation of work, which was 'are aquaculture sounds, sounds of concern?'

To determine the components of this type of soundscape a survey of an operational recirculating facility was conducted. This evaluation determined the major components of the soundscape (the pump) and the other influences that shape and create this soundscape. Where dominant characteristics (low frequency dominated) and SPLs (min mean SPL of 105 dB re 1µPa^2/Hz and a max SPL 124 dB re 1µPa^2/Hz) at were identified. The information provided by the baseline study created an understanding of acoustic parameters to determine the physiological responses of fish to this type of soundscape.

The acoustic characteristics investigated were further evaluated in combination with information previously published on other species, barramundi was evaluated for physiological stress responses to the introduction of specific sounds at three sound pressure levels (124,130,139 dB re 1µPa^2/Hz, at 187.5Hz). The fish were examined after short-term exposure and long-term exposure to the continuous sounds of an aquaculture soundscape. The results showed a significant correlation between the highest sound level and the initial stress responses of the presence of cortisol which increased over a 24hr period. Due to the increase throughout 24hrs, it was important to determine if this trend continued into the tertiary level of response. Therefore, the fish were evaluated over a long-term duration. The long-term results, displayed high variation among individuals concluding with no significant effect on the growth of the fish across the two-month exposure period. The highest sound level exposure did display the greatest range of variability throughout the treatment.

The last evaluation was determining if transient sounds play a larger role in stress responses of the fish under this type of soundscape. Two interval types were evaluated (random and constant intervals). The data revealed high variability of outcomes between measures of stress concluding that no effect could be determined. Across all physiological evaluations, a limited to no effect was determined, however possible influences associated with the life history of these fish may present levels of pre-adaptation to this type of soundscape. This theory is further investigated and discussed.

Chapter 1. General introduction and literature review

In aquaculture, stress management continually seeks the understanding of behavioural and physiological responses of stress and its effects in cultured conditions (Ashley, 2007). A probable cause of stress in a culture environment is sound (Bart *et al.*, 2001; Smith *et al.*, 2004; Wysocki *et al.*, 2007b; Davidson *et al.*, 2008). Sound is created by the equipment used in the production process (*e.g.* pumps and aerators) and can be amplified by the design and construction of the facility (Bart *et al.*, 2001; Craven *et al.*, 2009). However, the extent of the stress effect in fish to such sounds is relatively unknown (Bart *et al.*, 2001; Smith *et al.*, 2004; Hastings and Popper, 2005; Wysocki *et al.*, 2007b; Davidson *et al.*, 2008; Popper and Hastings, 2009).

Sound in the natural marine environment is well understood since more research has been completed in the field of ocean ambient noise (Hastings and Popper, 2005). The components of the ambient noise includes precipitation and marine animals, as well as localised sources such as surf or ice-cracking (Simpson *et al.*, 2004; Cato, 2006a).

Humans also contribute to ambient sound levels through water traffic, construction, mineral and hydrocarbon exploration and production, naval activities as well as acoustic research (Scholik and Yan, 2001; McCauley *et al.*, 2003; Hastings and Popper, 2005; Popper *et al.*, 2005). It has been demonstrated that these anthropogenic sounds can affect animals under certain conditions and within a certain proximity to the sound source. Many studies have focused on the effects on marine mammals; however, the effects of anthropogenic sounds on fish have only recently been evaluated (McCauley *et al.*, 2003; Hastings and Popper, 2005; Popper *et al.*, 2005). The studies undertaken found that fish can be adversely affected with effects ranging from inner ear damage to death, depending on acoustic parameters such as frequency, duration, duty cycle, rise-time and level (McCauley *et al.*, 2003; Hastings and Popper, 2005; Popper *et al.*, 2005; Popper and Hastings, 2009).

Taking into consideration the current understanding of sound in the environment *how will* sound influence fish within a confined environment, such as an aquaculture facility, where they are *unable* to escape a sound that may be stressful?

Limited research and information has been published on sound in an aquaculture facility (Bart *et al.*, 2001; Davidson *et al.*, 2007; Craven *et al.*, 2009), and less on the effect of sound on fish within cultured conditions. The unknown is: If sound in these facilities causes a stress response, how severe could that response be? This unknown influence is another factor in stress management in aquaculture that is not yet well understood (Hastings and Popper, 2005; Wysocki *et al.*, 2007b; Davidson *et al.*, 2008; Popper and Hastings, 2009). The need to further understand the potential of effects has led to investigations of aquaculture soundscapes and associated effects on fish.

1.1. The basics of the teleost hearing system

The hearing system of fish is typical of most vertebrate hearing sensory structures as it includes both semicircular canals and sensory maculae (Fay and Popper, 1980; Platt and Popper, 1981; Popper *et al.*, 1988). Within the subclass *Actinopterygii* (ray-finned fishes) the structure of the inner ear is highly variable across species (Popper and Lu, 2000). Due to this structural variation other aspects of the hearing system such as mechanisms and function also vary, because of this the structures, mechanisms and functions have been generalised for the majority of teleost characteristics.

1.1.1 Structure, mechanisms and functions

There are two main regions of structures that comprise the inner ear. The first region of interest is the *pars superior*. This region contains the three semicircular canals, which are end-organs (Platt and Popper, 1981) that connect to the ampullary region (Fay and Popper, 1980; Platt and Popper, 1981; Popper *et al.*, 1988). The *pars superior* also includes the utriculus that surrounds one of three otoliths, the lapillus otolith (Popper and Lu, 2000) (Figure 1.1). It is thought that the utriculus is the

most consistent otolith organ among fish species according to Platt and Popper (1981), Fay and Popper (1980), and Popper *et al*. (1988).

The second region is the *pars inferior*, which includes the remaining two otolithic organs, the sacculus and lagena. The sacculus contains the sagitta otolith which is known to vary greatly in size and shape among fish species (Platt and Popper, 1981; Popper and Lu, 2000) (Figure 1.1). The lagena is typically smaller than the sacculus organ and contains the asteriscus otolith (Popper and Lu, 2000).

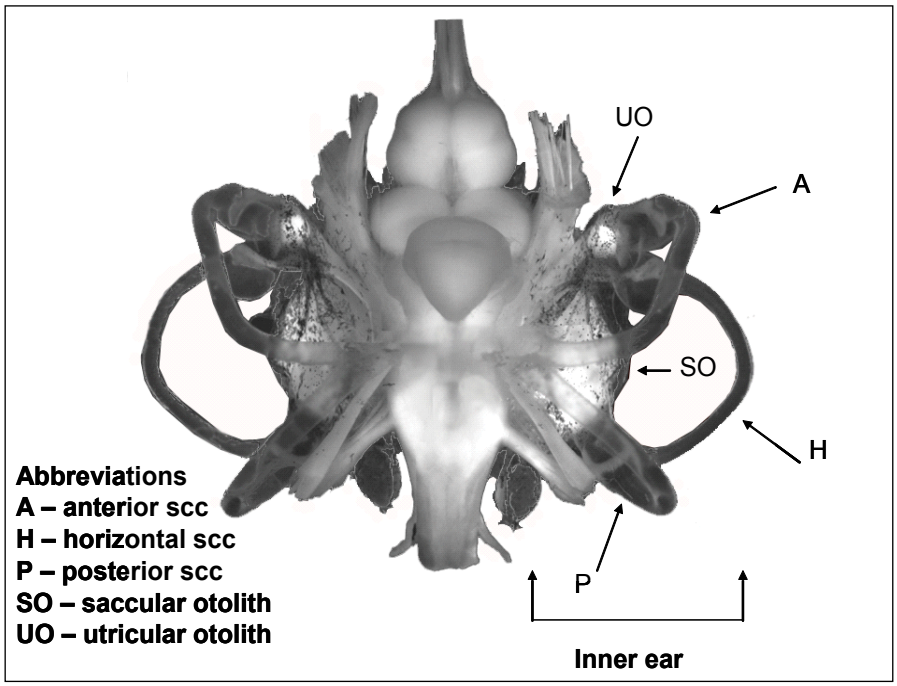

Figure 1.1. Image adapted from Popper (2003) from *Coryphaenoides armatus*. Image shows the dorsal view of the brain (centre) and position of the left and right inner ear. scc (semicircular canals) (Popper, 2003). The pars superior includes A, H, P and UO, where the pars inferior includes the SO and the other otolith the lagena (not shown in this image)

Each of the three otolith organs serves as an end-organ and contains the sensory maculae (or sensory epithelium), which has imbedded ciliary bundles. These bundles of hair cells are connected by the otolithic membrane and protrude toward the calcareous otolith. The bundles consist of a kinocilium, which is a long sensory cilia, and is surrounded by supporting stereocilia cells (Fay and Popper, 1980; Platt and Popper, 1981; Popper *et al.*, 1988; Popper and Lu, 2000) (Figure 1.2). These hair cell bundles have distributions and orientations that serve auditory functional purposes (Popper

and Lu, 2000). The bundle variation is in relation to the length of the kinocilium cells and the length depends upon their location within the inner ear and within the sensory maculae (Platt and Popper, 1981).

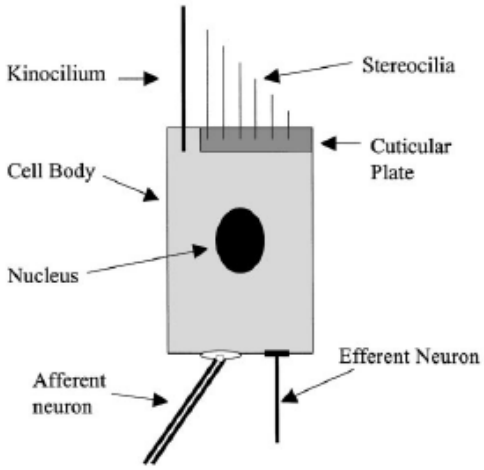

Figure 1.2. The basic structure of a sensory hair cell or ciliary bundle that is found within the sensory maculae (Popper and Lu, 2000).

1.1.2 How fish detect sounds

Sound is received multiple ways. The first is the direct pathway; because the otoliths are three times denser than water and the fish's body, sound signals are able to move the otolith at a different phase and amplitude from the body allowing the signal to be detected (Figure 1.3). The change of movement of the otolith , auditory or directional, is detected by the hair cells, and then transmitted as an electrochemical signal as described previously (Platt and Popper, 1981; Popper and Lu, 2000). Particle movement displacement in water resulting from an auditory signal moves through a fish causing the otolith to oscillate at a different phase than the body of a fish, and in response to the otolith movement induces the hair cells to bend. The bending of the apical bundle of stereocilia by lateral shearing forces either depolarise or hyperpolarise stereocilia, causing an increased release of neurotransmitter from the hair cell changing the firing rate of the fibre. The spontaneous neuron firing activity, the resting rate, is dependent on the stimulus event, the neuron response affects directional sensitivity (Platt and Popper, 1981) and auditory messages.

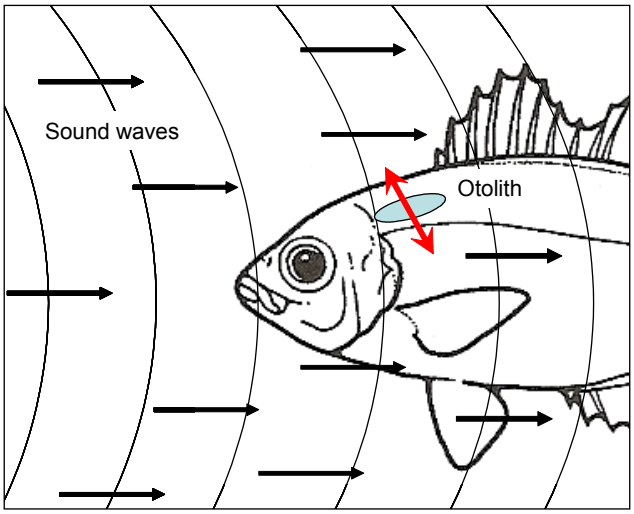

Figure 1.3. As sound waves moves through water in the direction of a fish, sound waves pass through the fish unchanged, but the otolith moves at a different rate because of its density difference from water. That movement is then sensed by the bending of hair cells allowing the sound to be detected.

The second pathway of sound reception, referred to as the indirect method, involves the swimbladder. In contrast to the mechanisms that cause sound to move the otolith, the swimbladder is less dense than water. Therefore when a signal is received it causes the bladder to compress and expand causing a different movement from the body. This motion is then transmitted to the inner ear via Weberian ossicles a bone connection or bullae a tissue connection (Platt and Popper, 1981; Popper *et al.*, 1988; Popper and Lu, 2000).

Fish that lack the swimbladder connection are hearing generalists, and rely on the inner ear method of hearing (Platt and Popper, 1981; Popper and Lu, 2000). These hearing generalists are typically sensitive to sounds in the range of 30 Hz to 600 Hz (Fay, 1988) and include fish within the Order *Perciformes* (Froese and Pauly, 2007).

Fish that have the mechanical connection of the swimbladder to the inner ear have been called hearing specialists also known as ostariophysans. The swimbladder connection allows the detection of other frequencies that are typically not detected by the inner ear and improves a fishes sensitivity to sound (Popper and Lu, 2000). The swimbladder extends the detectable frequencies (Platt and

Popper, 1981; Popper and Lu, 2000), to include a range from 30 Hz to 400 kHz and higher. This variation of detectable sounds is specific to each species (Hawkins, 1981). Hearing specialists belong to the order of *Ostariophysi* include *Gonorynchiformes, Cypriniformes, Characiformes. Siluriformes,* and *Gymnotiformes* (Saitoh *et al.*, 2003).

Until very recently fish were divided into two hearing categories hearing specialists and hearing generalists as mentioned. However, there is now a change in the manner of how fish hearing is categorised. This is due to the large variations in hearing ranges across species and the more species evaluated for hearing sensitivities the more unlikely the hearing will be classified into set categories (Popper and Fay, 2010).

As mentioned fish detect sound from changes in particle displacement caused by sound waves and in addition, some fish detect sound from swimbladder stimulation. Fish are also capable of detecting 'near-field', or very close in proximity, sounds from pressure changes and particle displacement with the use of the lateral line (Webb *et al.*, 2008). Near-field stimulation , the lateral line may assist in determining the source and direction of the stimulus (Shuijf and Buwalda, 1980). According to more recent literature it is currently accepted that a combination of lateral line and inner ear stimulation for near field directional determination is used. This is because the lateral line detects near field low frequency (0-200 Hz) water movement (Weeg and Bass, 2002). Therefore, if a fish is near the source of the sound it is possible for the lateral line to detect the low frequencies exhibited by the sound source (Higgs *et al.*, 2006).

Fish are also able to detect sounds through particle motion within the otolith system (the far field method) often linked to a gas bubble transmitting sound pressure information into mechanical stimuli which drive the otolith system. This is where some of the discrepancies between what is a hearing generalist and hearing specialist as some fish may 'switch' their hearing form detecting

pressure to detecting particle motion or even apply both. This event may explain some species like damsel fish detecting sounds above their established hearing range (Fay *et al.*, 2008).

1.2. The acoustic environment and fish

Water is an ideal medium and environment for the propagation of sound due to its density for the propagation of sound waves (Hawkins, 1993; Cato, 2006b). Water density can be affected by a range of environmental factors and variations in those factors produce differences in the transmission properties of water (Hawkins, 1993; Cato, 2006b).

In water the vibrations of an immersed object produce sound. The vibrational disturbance propagates away from the source, as a compressional wave characterised by repeated regions of compression and rarefaction. During vibrations, acoustic energy is lost into heat. This loss is called absorption and increases with frequency. The absorption of the sound decreases its intensity with distance travelled (Hawkins, 1993; Cato, 2006b). The intensity and frequency of a sound will influence the distance that the sound can travel before being fully absorbed by the water (Jones, 2006).

Sound in water is characterised by a range of attributes; intensity, wavelength, phase, pressure and frequency. The strength of sound in water is measured by a ratio of quantities, specifically in decibels, often relating to the pressure change (*ie.* dB re 1µPa) of a medium. Another important term of sound is in frequency (Hz), which is the rate a sound wave passes over a single point in time (Cato, 2006c). A high frequency will have a higher rate of oscillation then a lower frequency. In relation to pressure and particle displacement, the reference to decibels is a scale of pressure in relation to a logarithmic scale, where a magnitudinal increase is equivalent to 3 dB re 1µPa. Particle displacement occurs where there is water movement; the particles shift when a disruption occurs. In

relation to sound, soundwaves and the energy of a sound will displace and move particles as the wave passes. Together these characteristics are used to describe sound.

Underwater sound is important for fish as it allows for 'acoustic imaging' of the surroundings and is fundamental for communication (Scholz and Ladich, 2006). For fish, ambient noise plays a major role in being able to distinguish and discriminate between sounds. Ambient noise can also impair the detection of a relevant signal or sound, through a phenomenon called masking (Scholz and Ladich, 2006; Wysocki *et al.*, 2007a). Masking interferes with the ability to hear one sound in the presence of another. The louder the ambient noise, the more difficult it is for a fish to detect a specific sound (Scholz and Ladich, 2006).

1.2.1 Sound produced by the environment

Naturally occurring ambient noise produced within the oceans comes from various biotic and abiotic sources. Each source contributes energy over characteristic frequency ranges, as seen in Figure 1.4. Those sources are influenced by the severity of weather, and the activity of animals. Ocean ambient noise sources can range from <1Hz to > 100's kHz depending upon the physical conditions and sources (Cato, 2006a). Biotic sounds are contributed by invertebrates (e.g. snapping shrimp), fish (Sciaenid) and marine mammals (e.g. cetaceans and pinnipeds). The choruses and other sounds produced by these animals can greatly influence ambient noises at a distance (Cato, 2006a).

Ambient noise differs with location and ecosystem. For example, at a coral reef, the accumulation of invertebrates and fish within this habitat and the effects of wave action on a reef create a distinct 'soundscape' or blend of sounds. The ambient frequencies produced range from 0.1 to 10 kHz and levels peak around dusk (Simpson *et al.*, 2004; Cato, 2006a).

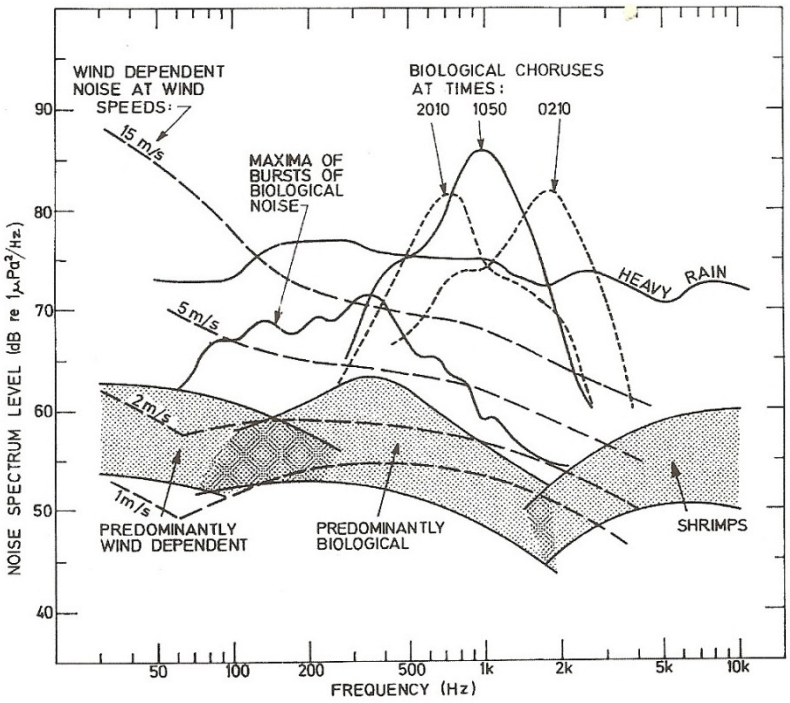

Figure 1.4. Summary of ocean ambient noise components, the shading indicates prevailing background noises and the chorus spectra from biological inputs are shown at the maximum levels (volume) and frequency range observed. Figure also displays variation at time of day and changes in environmental conditions (Cato, 2006b).

1.3. Anthropogenic sounds

Anthropogenic sounds are created by a range of human activities including deterrent systems, water traffic, construction and seismic testing. The inclusion of anthropogenic sounds to natural ambient levels have resulted in increased ambient levels which appear to be increasing (Wysocki and Ladich, 2005). However, this effect is very difficult to quantify due to the diversity of shifting sound sources in the marine environment. The limited data available on underwater ambient sounds in most parts of the world has not been characterised, thus further complicating the ability to quantify the overall effect. Despite the increasing interest of scientists, regulators, and environmental groups in anthropogenic sounds, there is limited experimental data directly addressing how these sources may affect animals (Popper *et al.*, 2005; Wahlberg and Westerberg, 2005).

1.3.1 Airguns and pile driving

Multiple studies have been completed on the evaluation of intense high power sounds caused by airguns and pile drivers (McCauley *et al.*, 2003; Hastings and Popper, 2005; Popper *et al.*, 2005).

Airguns, which are widely used for marine-based seismic exploration, ,inject highly pressurised air into the water creating rapidly expanding bubbles that collapses under the pressure of water, resulting in sharp impulsive waveforms (Popper *et al.*, 2005). Pile drivers are used for creating bridge platform foundations, and for damming purposes (sheet piles). The pile driving sounds, result from a pile driving hammer hitting a pile and an impulse of shear and compressional waves are created (Hastings and Popper, 2005). Both types of sound create high intensity waves with broad frequency ranges, the auditory effects have recently been evaluated on marine mammals and fish (McCauley *et al.*, 2003; Hastings and Popper, 2005; Popper *et al.*, 2005).

1.3.2 Deterrents

In water, animal deterrents are effective methods of discouraging fish and other aquatic organisms away from pumping and power station intakes. Deterrents include visual stimuli (*e.g.* air-bubble screens, lights or strobe lights), water velocity and pressure changes, electrical shocks, mechanical exclusion. Sound producing apparatuses are known as Acoustic Fish Deterrents (AFD) (Maes *et al.*, 2004).

At the Doel Power Plant, Belgium (Maes *et al.*, 2004) an AFD sound projecting unit was tested for effectiveness. This system produced frequencies of 20–600Hz, with a nominal sound pressure output of 174 dB (re 1 μPa). The study established that the frequencies of the sounds used were effective in deterring fish from intake location and reduced occurrences by 59.6 %. However, the reduction in fish was species-specific. Some species such as herring were 99% deterred, but fish such as mullet did not show any avoidance reaction to the AFD system. The AFD effectiveness was attributed to species-specific differences in hearing capabilities.

1.3.3 Water traffic

Water traffic, which includes boats, ships, and other watercraft, are typically more abundant near harbours, marinas, along rivers and bays or areas near large human populations. The noise from boat traffic is caused by the movement of the hull through the water, vibration from machinery

(engine and gearing), pumps, auxiliary machinery and propeller generated noise by cavitations (Norwood, 2006). The frequency of the sounds ranges from < 10Hz to 100kHz depending on the types of machinery and the propulsion used, and the speed at which the boat is moving. The amount of boat traffic will influence the ambient sound levels in a location. Wysocki *et al*. (2006) collected boat traffic noise samples from the Danube River and from the Mondsee and Taunsee Lakes. The average level of noise was 153 dB re 1 µPa and the noise ranged from 128-162 dB re 1 µPa at random intervals of sampling, which varied between locations.

1.4. How sound affects fish

1.4.1 Thresholds and damage

Despite the increasing interest in anthropogenic sounds, minimal experimental data exists that directly address how sound sources may affect fish (Popper *et al.*, 2005; Wahlberg and Westerberg, 2005). However, recent studies have established that exposure to sound of high intensity (*e.g.* airguns and pile driving) can cause inner ear damage in fish (McCauley *et al.*, 2003; Popper and Schilt, 2008). The severity is often reflected in the level fatigue and damage to hair cells and through an effect known as threshold shift. A threshold described by Hastings and Popper (2005), is the lowest recorded signal level an animal will detect in some statistically predetermined response. Threshold shift indicates a change in that detection level and can be temporary or permanent.

Moderate exposure to intense noise can result in temporary hearing loss, and is known as Temporary Threshold Shift (TTS). The potential effect of TTS is the loss of ability to hear important environmental sounds (Smith *et al.*, 2004; 2004a; Popper *et al.*, 2005; Popper *et al.*, 2007). If the source of the acoustic disturbance is powerful enough, a Permanent Threshold Shift (PTS) may result; causing irreversible hair call damage or permanent hearing loss or (Hastings and Popper, 2005).

The severity of TTS depends on the level, duration, exposure regimen (duty cycle) and frequency of the sound. The outcome produced is also dependant on the fish being a hearing specialist or generalist (Hastings and Popper, 2005). Unfortunately only a handful of species have been studied for the effects of TTS and the extent of recovery and adaptation is not well understood (Scholik and Yan, 2001).

Damage caused by noise may also include a range of other effects such as damage to various body tissues (e.g. organ systems, circulatory system and neural tissue), and increase stress levels that could impair or ultimately lead to mortality. In addition there is the possibility that temporary or permanent hearing threshold shift could induce changes in behaviour as animals try to avoid the sound (Knudsen *et al.*, 1992; Maes *et al.*, 2004). While numerous studies have documented the effects of loud sounds on marine mammals, effects of such sounds on fish remain poorly understood (Smith *et al.*, 2004a).

There is evidence that clupeid fish (herring and shad) are capable of hearing ultrasounds, suggesting that some species are capable of identifying dolphin produced ultrasounds (Mann *et al.*, 1997; Remage-Healey *et al.*, 2006). With the acknowledgement that fish can detect dolphin vocalisations, select studies have evaluated the stress response caused by natural acoustics used in predation. A study completed by Remage-Healey et al. (2006) tested the stress response of gulf toadfish to various clicks and whistles of a dolphin. Stress and behavioural response were recorded when the dolphin calls were played. This study suggests that non-anthropogenic sounds are also capable of inducing a stress response in fish.

1.5. Fish stress physiology

A common misconception about ‘stress’ is that it is detrimental to the fish, which is not necessarily the case. The response to stress is considered an adaptive mechanism that allows the fish to cope

with real or possible stressors in order to maintain a normal homeostatic state (Barton, 2002). However, if the intensity of the stressor is overly severe or long-term, the physiological response may compromise the fish's health and well-being, thereby becoming maladaptive (Barton and Iwama, 1991; Barton, 2002).

1.5.1 Stress responses

The stress response process in fish is often referred to as the General Adaptation Syndrome (GAS) theory. Originally adapted from mammals, GAS has since been recognised as an effective tool in describing the stress response in fish (Barton and Iwama, 1991; Barton, 1997; Wendelaar Bogna, 1997). Originally presented by Hans Selye in 1950 (in Barton, 1997), GAS divides the overall stress response into three stages; primary, secondary and tertiary. The primary response is seen as the initial and fast acting response. The secondary response is delayed but initiated by the stimulus of the primary response and the continued exposure to a stressor. Tertiary response is the result of prolonged stress duration that can lead to poor health and even death. Together these three stages form the basis of the stress response process (

Figure 1.5).

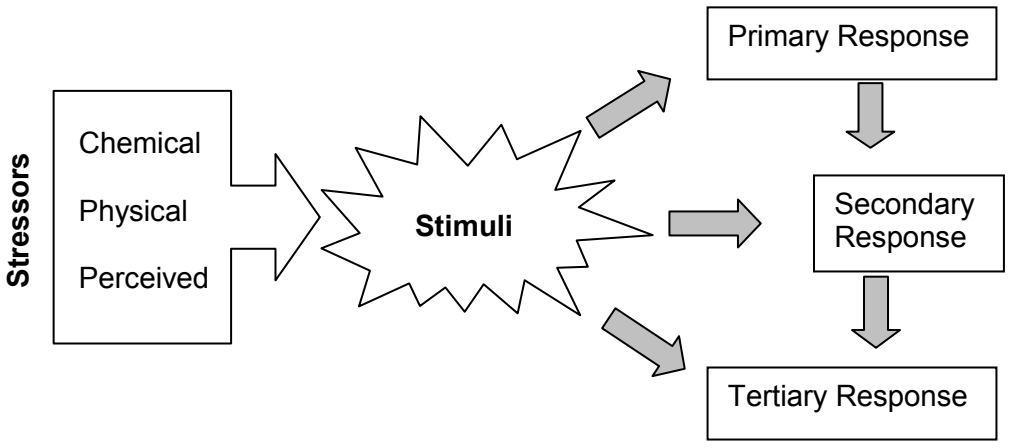

Figure 1.5. This figure describes the possible combination of stressors that can induce the reactions to stress. It also depicts the flow of responses from the primary response through to the tertiary response (adapted from Barton, 2002)

1.5.2 The primary, secondary and tertiary stress responses

Stress stimulus can come from a combination of cues and events. The initial response to acute stressors represents the perception of an altered state, a threat to homeostasis, and initiates the neuroendocrine response. This is the beginning of the generalised chain of reactions across most vertebrates that forms the endocrine stress responses (Sumpter, 1997). When the stress response is received, the body of the fish immediately begins to synthesise glucose to supply required energy to the stress process and responses (Barton, 1997).

In fish the primary stress endocrine response is part of the Hypothalamic-Pituitary-Interrenal axis (HPI). The primary response cascade of this axis initiates the immediate release and increase in plasma concentrations of catecholamines and the cascade for production of cortisol, both of which are hormones (Barton, 2002). Catecholamines combine with dopamine to create noradrenalin and eventually to produce, adrenaline (also referred to as epinephrine) (Sumpter, 1997; Iwama *et al.*, 2006). Adrenaline is the fast response endocrine hormone that helps the animal remove itself from the stressor or to cope with the stressor (Sumpter, 1997).

During the initial endocrine response, the release of the stress hormones may induce a behavioural response. Changes in behaviour may be adaptive and therefore, potentially increase the chances of survival (Schreck *et al.*, 1997; Koolhaas *et al.*, 1999; Overli *et al.*, 2002). The first line of behavioural defence for a fish is to remove or distance itself from the stressor thereby lessening the stressors impact (Barton, 1997; Schreck *et al.*, 1997).

The secondary response is predominately based on the next phase of the endocrine response where production of glucose and maintenance of cortisol levels continues to strive to achieve a level of homeostasis (Barton, 2002; Overli *et al.*, 2002) This phase includes changes in ion concentrations where adaptive and maladaptive response begin (Barton and Iwama, 1991). Since adrenaline is

short-lived in the blood system, a second hormone is initiated during the reduction of adrenaline to maintain a level of stress compensation (Iwama *et al.*, 2006). The hormone that substitutes for the effect of adrenaline is cortisol. The synthesis of cortisol is part of the HPI axis, a cascade of hormonal cues that first initiates the production of adrenaline, this is then followed by the production of cortisol. Typically in fish the measure of plasma concentrations of cortisol is the most widely used indicator of stress, and is present around peak levels in plasma within a few minutes to a couple of hours post stress (Wendelaar Bogna, 1997; Barton, 2002). But in the case of acute stress, cortisol can be present in the plasma for prolonged periods but below peak levels (Wendelaar Bogna, 1997).

In aquaculture and other fields the primary and secondary responses (adrenaline and cortisol) are important indicators of stress. These indicators are commonly used to determine if there is a stress reaction to a stimulus, to establish whether a particular stimulus may have negative effects, and to gauge the severity of the stressor (Barton and Iwama, 1991; Thomas and Robertson, 1991; Pickering, 1993; Sumpter, 1997; Schreck *et al.*, 2001; Ashley, 2007).

Tertiary responses in fish are considered "whole-animal" effects, which in-turn can affect the population as whole (Wedemeyer and McLeay, 1981; Barton and Iwama, 1991). The "whole-animal" may experience functional changes in body processes such as shut-down of organs and functions, behavioural influences (eg. aggression, feeding), impaired growth, and changes in development (smoltification) and reproduction (Wedemeyer and McLeay, 1981; Barton and Iwama, 1991; Rottmann *et al.*, 1992; Pickering, 1993; Schreck *et al.*, 1997; Wendelaar Bogna, 1997; Koolhaas *et al.*, 1999; Schreck *et al.*, 2001; Barton, 2002). During this time the fish may experience further damage to functional processes or be recovering from the primary and secondary responses (Schreck *et al.*, 1997). Throughout this period of prolonged chronic stress or recovery, survival is inevitably threatened.

1.5.3 Auditory and non-auditory effects of sound stress

As previously discussed, specific levels and frequencies can cause TTS and PTS. When exposure to sound causes damage to the ear hair cells, it may cause behavioural changes, leading to disorientation, vulnerability to predation, and possibly death. It has been documented by Smith et al. (2004a), that the change in threshold shifts coincided with increased levels of stress (indicators used: glucose and cortisol) in goldfish (*Carassius auratus*; a hearing specialist). This study demonstrated that corticosteroid response levels were higher during short term exposure (0.1-10kHz at 160-170 dB re 1 µPa) and that there was no statistically significant change in the long term (Smith *et al.*, 2004). Other studies have shown elevated auditory thresholds (0.5-4.0 kHz at 142 dB re 1 µPa) (Scholik and Yan, 2002) and cortisol levels (128 to 162 dB re 1 µPa) (Wysocki *et al.*, 2006) caused by exposure to boat and engine noise. In addition, there is some evidence that sound has detrimental effects on eggs and larval development (Banner and Hyatt, 1973; Lagardère, 1982) suggesting that growth is suppressed by sounds. Smith et al. (2004, 2004a) and Hastings and Popper (2005) have concluded more studies need to be completed regarding sound and its threshold effects on stress. Direct damage from sound (e.g. hair cells, TTS) has been documented, but the impact of sound on stress levels is much more difficult to define because it is difficult to quantify this measure in fish due to the limited range of studies (Hastings and Popper, 2005).

The lack of knowledge on how anthropogenic sound influences fish in terms of stress leaves much to be learnt (Hastings and Popper, 2005; Popper *et al.*, 2005; Popper and Hastings, 2009). The outcomes are assumed to induce responses like other known causes of stress, such culture stresses (eg. handling, crowding, transport, and water quality) (Barton and Iwama, 1991; Smith *et al.*, 2004; Hastings and Popper, 2005; Popper *et al.*, 2005; Iwama *et al.*, 2006) and other stress studies on vertebrates, predominantly mammals (including humans)(Hastings and Popper, 2005).

Peer-reviewed publications on the non-auditory influences of sound suggest that there is a high possibility of a detrimental stress effect caused by anthropogenic sounds, but the limited quantity and scope of such publications invites further study and analysis of that suggestion.

1.6. Aquaculture and fish: life submerged in an anthropogenic environment

1.6.1 Stress in aquaculture

The idea that stress is an important concern for the successful husbandry of finfish is well accepted among aquaculturists (Barton, 1997), as stress management is a key factor to maintaining a quality stock. In aquaculture particularly, measures of stress showing that the fish may be experiencing negative effects are considered an important determination of animal welfare. Commonly associated causes of stress in aquaculture are: containment, water quality and condition (dissolved gases, temperature, and chemical constituents), handling, transport, crowding (influences competition, carrying capacity of the water), and diet (Wedemeyer, 1997; Mosig and Fallu, 2004). Aquaculturists are most concerned with the initiation of primary and secondary effects since they could lead to detrimental tertiary effects of stress such as a reduction in growth, suppression of reproductive function, diminished immune function or disease resistance (Ashley, 2007). How these elements interact and impact fecundity, fitness and health of the stock can greatly influence the overall productivity and value of the stock and eventually affect the profitability and capabilities of the facility.

1.6.2 The acoustic environment in aquaculture

An aquaculture environment is one that is purely anthropogenic. Recirculating facilities are designed to maximise the potential of their stock by creating a highly controlled environment (Mosig and Fallu, 2004). In systems like sea cages and open facilities (e.g. ponds), the systems are more exposed to external environmental conditions and fluctuations. All facilities, nonetheless, are exposed to a

range of anthropogenic sounds. The sources of the sounds are dependent on the requirements of the facility (Bart *et al.*, 2001).

Sounds levels produced in various facilities have been evaluated by Bart *et al.* (2001) and Craven *et al.* (2009). These works surveyed and determined the intensity of various culture containments. The maximum sound pressure levels were reported in the recirculating aquaculture facility at 153 dB re 1 µPa (Bart *et al.*, 2001) ; Clark *et al.* (1996: in *Bart et al.*, 2001) found sound pressure levels as high as 160 dB re 1 µPa; however in other facilities more moderate levels were recorded at 125 dB re 1 µPa/Hz^2 (Craven *et al.*, 2009)[1]. Those studies suggested that the containment of sound and the materials used in a recirculating facility influence the overall sound levels produced in a facility.

Table 1.1. Lists of possible equipment that produce sounds in various types of systems and how the sound is affected within the system. The higher dB readings occurred at lower frequencies.

System	*Possible Equipment used*	*Environment*	*Influences on sound*	*Known acoustic levels*
Cages	Boats, engines, pumps, aerators, automated feeders, maintenance, harvesting equipment	Open water	Sediment, water density, locality to shore, boat activity	unknown
Ponds	Pumps, filtration aerators, airguns, vehicles, feeders (automated or semi automated), maintenance, harvesting equipment	On land, in open air	Sediment, water density	Aerator on: 90-125 dB re 1 µPa Aerator off: 65-90 dB re 1 µPa (Bart *et al.*, 2001)
Raceways	Pumps, filtration, aerators, automated feeders, maintenance, harvesting equipment	On land, open air or semi enclosed	Raceway material, *e.g.* concrete, wood fibreglass	Concrete: 84-120 dB re 1 µPa Wood frame: 73-119 dB re 1 µPa (Bart *et al.*, 2001)
Fibreglass (recirculating)	Pumps, filtration, aerators, automated feeders, maintenance, harvesting equipment	Completely enclosed	Shape, tanks material: fibreglass, enclosed	Circular fibreglass: 70-130 dB re 1 µPa (Bart *et al.*, 2001). 105-125 dB re 1 µPa/Hz^2

[1] To maintain consistency between published work (Chapter 3, Craven, *et al.* 2009) and this thesis, units of dB are expressed as dB re 1 µPa/Hz^2 . This unit cannot be directly compared with dB re 1 µPa, but it can be

			surroundings; tank size	(Craven *et al.*, 2009)

1.6.3 Fish, stress and sound in aquaculture

Few studies have investigated the effects of noise on stress, especially in regard to aquaculture species, where the sounds produced may elicit a stress response in fish (Davidson *et al.*, 2007; Wysocki *et al.*, 2007b; Davidson *et al.*, 2008). An important fact prompting additional studies is that fish exposed to an irritating sound in their natural environment can most likely escape from that location, but in an aquaculture setting the animals are contained to their culture environment and cannot escape the sound.

Wysocki *et al.* (2007) recently evaluated rainbow trout (*Oncorhynchus mykiss*) for effects of a recirculating culture production sound on hearing, growth and diseases resistance using TTS and cortisol, chloride and glucose levels. The evaluation concluded that there was no effect of background noise on hearing thresholds, growth, and disease resistance. However, the study also concluded that since this species is from a habitat that has a relatively high ambient sound environment, the species might therefore be adapted to higher ambient sounds. The authors also emphasised that species-specific responses will most likely vary, and that more research is needed on the subject to draw definite conclusions on the effect of sound in a culture system.

In many areas of their natural environment, including aquaculture facilities, fish are exposed to higher sound levels as a result of anthropogenic noise that may negatively affect normal behavioural and physiological processes (Bart *et al.*, 2001; Smith *et al.*, 2004a; Davidson *et al.*, 2007). Sound levels and frequencies recorded in commercial-scale aquaculture systems are within the hearing range of fish, including the less sensitive hearing generalists and range from 125 to 135 dB re 1 µPa at 25–1000 Hz, and from 100 to 115 dB re 1 µPa at 1– 2 kHz (Bart *et al.*, 2001; Davidson *et al.*, 2007). Other variables of interest that may influence the effects of sound include: differences among

species, the type of system in which they are kept, the ability to adjust the environment, and the age at which they are most at risk (Wysocki *et al.*, 2007b).

Until further investigation is completed only suggestions of possible effects can be made. The understanding of the fish auditory system, what responses stem from various stressor including acoustics and the ability of fish to detectable aquaculture sounds (Bart *et al.*, 2001; Wysocki *et al.*, 2007b), it is highly likely that acoustic sounds produced in the aquaculture environment could induce an acute level of stress. Nevertheless, it is difficult to conclude whether there is a detrimental effect caused by ambient anthropogenic sounds. Although, the effects of sound on fish are expectedly variable from one species to another, but a generalised approximation can be developed regarding what sounds and how sounds will affect fish (Hastings and Popper, 2005).

1.7. Thesis direction and aims

Anthropogenic sounds have been shown to have negative effects on fish, including irreversible damage to the auditory system, confusion, and an inability to interpret biological sounds. Exposure to anthropogenic noise in an aquaculture environment is unavoidable and inescapable for a fish, yet the extent to which sounds may affect them remains unidentified. Understanding individual components and the linkages between them will develop a general knowledge basis that will bring forth further explanations and questions to assist in answering these questions.

Understanding this field has the potential to improve current aquaculture species production, and could be crucial in introducing culture sensitive species to aquaculture production. The progress of improvement is delayed because of the lack of peer-reviewed material on sound and stress. Nonetheless awareness and interest in this field is growing, as suggested by the most relevant

literature having very recent publication dates, for example Wysocki *et al.* (2007) and Davidson *et al*. (2008).

The purpose of this thesis was to first evaluate a functional recirculating facility to form a well rounded understanding of a 'typical' aquaculture facility and increase the understanding of the aquaculture soundscape. Then apply that knowledge to ascertain if physiological stress responses could be identified from exposure of Barramundi (*Lates calcarifer*) to real soundscape recordings in continuous and transient sound environments, and to establish short-term and long-term physiological effects, if such effects exist, as well as introduce a second species in the knowledge base of acoustical stress in an aquaculture environment. With this understanding the physiological responses of barramundi is important because the species is a highly valued and regarded cultured species within Australia and across the world, as well as determining if these sounds should be of concern.

Chapter 2. A lesson in bioacoustics: an introduction to thesis methods

2.1. Introduction

In the subject of acoustics there are two fields of understanding. The first is the field of physics, constructed around the mathematics and physical properties of the acoustic world. The second is that of biology, which is concerned with the interaction of sounds with the biotic environment, in particular the physiological and behavioural responses of the whole organism. These ideas combined have lead to the multidisciplinary field of bioacoustics. Of interest is the branch of fish bioacoustics, an interdisciplinary field combining understanding in psychology, biology, evolution, population biology, biomechanics, physical acoustics and mathematical modelling (Fay *et al.*, 2008).

Fish bioacoustics is unique in its applications, and is expanding continuously to new areas of interest. The key characteristics that contribute to its uniqueness are the way fish are receptive to the acoustic environment as they hear and 'feel' the sound via two sensory pathways.
A branch of fish bioacoustics has become very focused on the influences of the anthropogenic sounds on the behavioural and physiological response of fish. With the rise of aquaculture, part of this branch has concentrated on fish stress in aquaculture environments. In the evaluation of aquaculture acoustic environments', specifically recirculating facilities, the fish are completely isolated from their natural conditions and fluctuations. This environment presents parameters and opportunities that are unlike that of attempting to evaluate natural conditions in a laboratory setting. As the environment is completely anthropogenic the characteristics are much more easily replicated, modified and tested. However working within 'small' contained environments also creates new considerations and restrictions that will influence the properties of the soundscape and experimental design.

For the work conducted in Chapter 4-Chapter 6 a baseline set of parameters was determined prior to running the experiments. This chapter will introduce the more technical aspects of the

experimental methods in relation to acoustics, and will also describe the designed parameters and their applications.

2.2. Absolute SPL and unit determination

Sounds can be measured in multiple ways and by a continually increasing range of equipment that is persistently improving the accuracy of recording and analysis capabilities. In particular, in underwater acoustics there are basic pieces of equipment that are required. The first, hydrophones are the sensors used to detect the sound. The sounds are received as changes in pressure, for instance as a sound wave passes over the hydrophone the change of pressure vibrates a piezoelectric disk. The magnitude of vibration represents the amplitude of the sound and the rate of vibration represents the frequency of sound. Measurements of decibels (scale of sound) in underwater pressure are referred to in sound pressure levels (SPL).

Following the hydrophone is a preamplifier, which amplifies the signal from the piezoelectric disk to take advantage of the voltage available; many hydrophones have the preamp included within the physical construction of the hydrophone. After the amplification process, the analogue input signal is converted to a digital signal in order to be stored or analysed by a computer, this is achieved in this case through a soundcard. The digital signal is then passed to a logging device, such as a computer or a specific data logging apparatus.

Specifically for this thesis during the period of replaying the sounds, the sounds were analysed using a High Tech 94-SSQ series (a hydrophone with in-built pre amps with a frequency response range of 2 Hz to 30 kHz, hydrophone sensitivity -165 dB re: 1 V/µPa ±1 dB), a soundcard (DirectMix USB) which digitalised the signal and passed it to a laptop computer (Dell Inspiron 600m). The sounds were recorded and evaluated using Adobe Audition 2.0 software and saved for records.

Once the equipment is obtained there is a requirement for establishing the actual values of what the particular combination of equipment is reading. This is where the relative values, or the arbitrary values established by the software output can be converted to absolute values, or values that are accurate and consistent. Often the setup is connected to an oscilloscope, a piece of equipment that reads the sound signal in terms of direct voltage. The voltage can then be converted to a measure of pressure. This method is used in Chapter 3 to calibrate the sound sampling equipment.

In Chapter 4-Chapter 6, a hydrophone calibrated using a G.R.A.S. Pistonphone was used for recording data. A pistonphone is a battery-operated, precision sound source for accurate and reliable calibration of hydrophones and other sound measuring equipment. It allows a value for system gain to be calculated, which can be used to correct the relative value displayed in the Audition software. The pistonphone and the way the hydrophone is inserted are shown in Figure 2.1. When the pistonphone is turned on, with the hydrophone in the adaptor, the tone shown in Figure 2.2 is recorded.

Figure 2.1. G.R.A.S. Pistonphone with HTI-96 hydrophone and adaptor

This recording can then be analysed by a program that was written by JASCO Applied Science Ltd, with the specific purpose of calculating the system gain of a recording system, through the use of the pistonphone signal (Figure 2.3). The output of this program, Figure 2.4, shows the system gain, which can be applied to the relative levels seen in Adobe Audition, to calculate the actual SPL in the tanks. This conversion is shown in Table 2.1, which illustrates the importance of using the correct units.

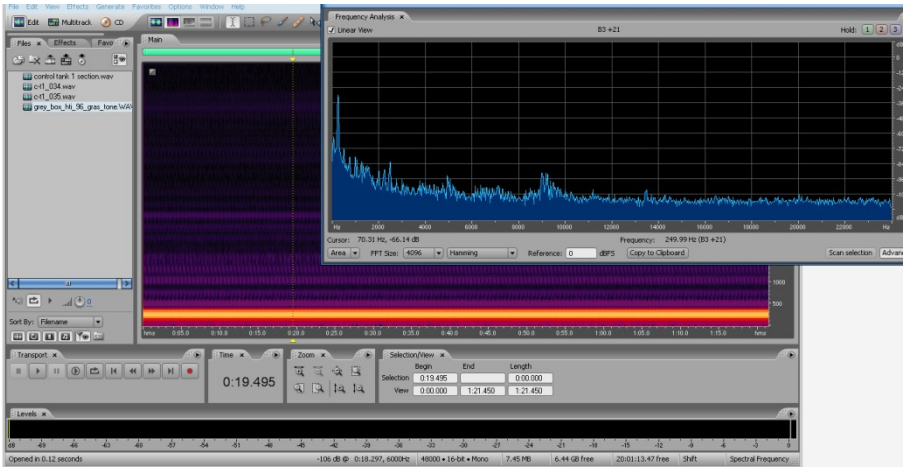

Figure 2.2. Tone recorded from pistonphone, shown in Adobe Audition, with the frequency domain in the top right and the bottom image is of the time domain.

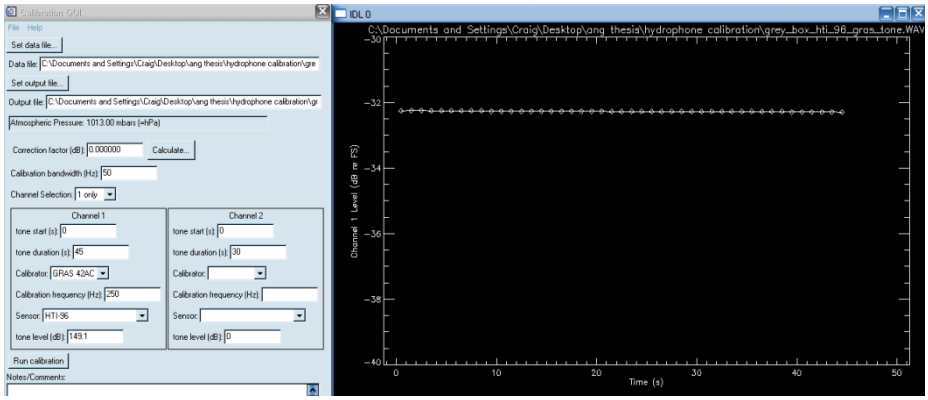

Figure 2.3. User Interface of software used to determine the system gain, and the relative level of the pistonphone tone

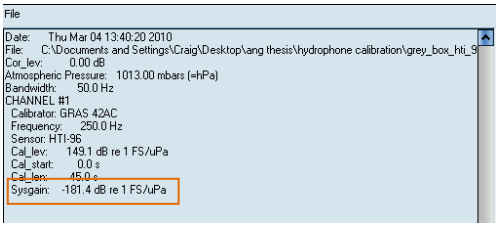

Figure 2.4. Output of system gain calculation software

Table 2.1. Conversion of Audition relative levels to actual SPL levels

	Audition relative SPL (dB re FS)	System Gain Correction (dB re FS/ µPa)	System Gain Correction (dB re 1 µPa^2/Hz)	Output SPL (dB re 1 µPa^2/Hz)
Level 1	-40	-181.4	-164.45	124.45
Level 2	-34	-181.4	-164.45	130.45
Level 3	-25	-181.4	-164.45	139.45
Control	-60	-181.4	-164.45	104.45

The units used throughout the thesis follow those used in Craven *et al.* (2009) (published version in Chapter 3), specifically the units used were dB re: 1 µPa^2/Hz, FFT (Fast Fourier Transformation[2]) with resolution of 46.9Hz. The sampling frequency was 48 kHz, and viewed in a 1024 point FFT Blackman window and therefore the bin widths (bandwidth) were 46.9Hz. The unit of dB re: 1 µPa^2/Hz is used as it is representative of the narrowband bin width of the FFT analysis, and can be converted to dB re 1 µPa by adding 10 log10 (bandwidth)(McCauley, 1998).

2.3. Experimental and sound considerations

For Chapter 4-Chapter 6 the experiments were conducted within the Marine Aquarium Research Facility Unit (MARFU) at James Cook University. The availability of space within the facility placed restrictions on the experimental design, however where possible the design worked to make the most of the available space.

For these experiments there are two enclosed rooms, a treatment room and a control room. Both rooms were housed within the same facility and were run on the same recirculating system. The rooms were adjacent maintaining close proximity and a level of sound isolation. The separation of the control from the treatments was required as it was very difficult to isolate the treatment sounds from the controls if they were located within the same room.

The control room was considered a quiet lab environment, and while not devoid of sound, was substantially quieter then the treatment room. The sound levels in the treatment room varied dependent on the current experiment, while the control room was relatively consistently around 80 dB re: 1 µPa ^{2}/Hz. Also, both the control room and the treatment room were in temperature and

[2] An FFT is an efficient algorithm used to compute the Discreet Fourier Transformation (DFT). A DFT decomposes any complex valued series into components of different frequencies.

photoperiod controlled rooms, with maintaining a 27 °C water temperature and 12:12LD photoperiods.

The treatment room layout consisted of three groups of tanks and each group was composed of three 250L tanks. The size of the available control room reduced the number of tanks that could fit within the room, therefore the control room contained only one group of three tanks. Between each group was a sound source device. A cost effective method for replaying the sounds of an aquaculture soundscape was using a speaker set (Logitech x-240) with satellites and a subwoofer, and the sounds were stored and replayed using iPods (iPod classic 8 G). Both the speaker sets and the iPods were individually controlled allowing for specific sound adjustments per group, such as base levels. The use of compact playback equipment meant the equipment could be stored in a sound directional box. This box allowed for the output sounds to be channelled from the subwoofer to the three tanks of a group (**Error! Reference source not found.**), it also protected the electronic equipment from the hostile room environment. The satellites were protected from the environment by being wrapped in a thin plastic. The satellites were suspended above the tanks to distribute their contribution to the sound in the tanks equally (Figure 2.6).

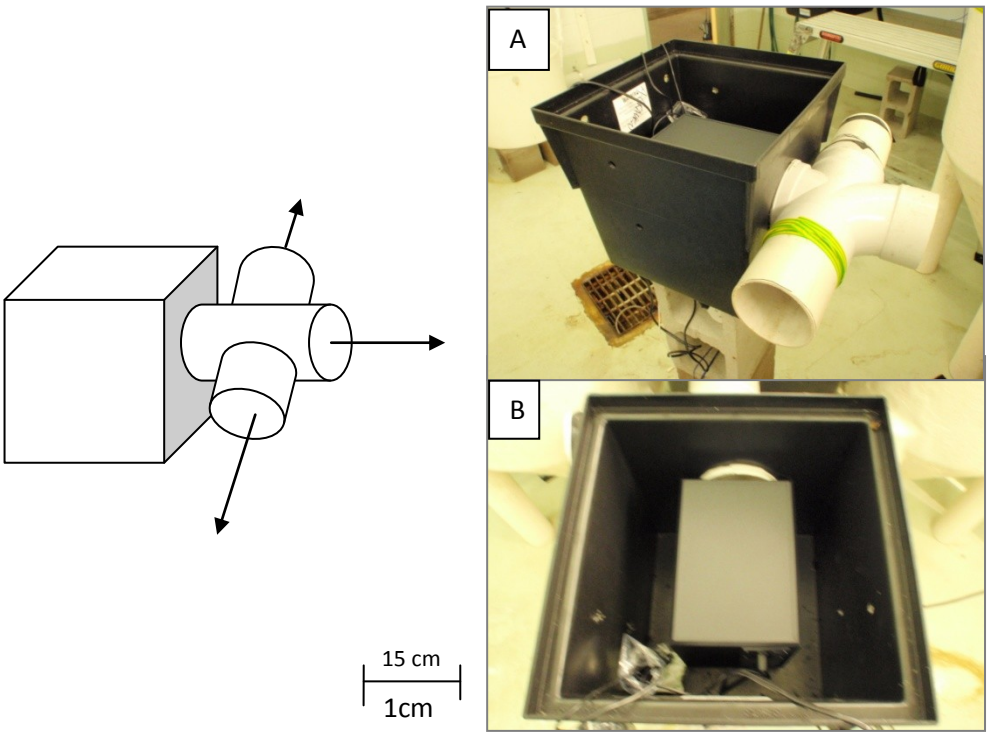

Figure 2.5. This image shows a representation of the sound transmission case, with photos A and B showing the actual setup, with the subwoofer positioned in the middle (B) of the sound case and the four way PVC pipe (A). The four way PVC connection allowed for the directional control of the sound of the sub woofer out of the case.

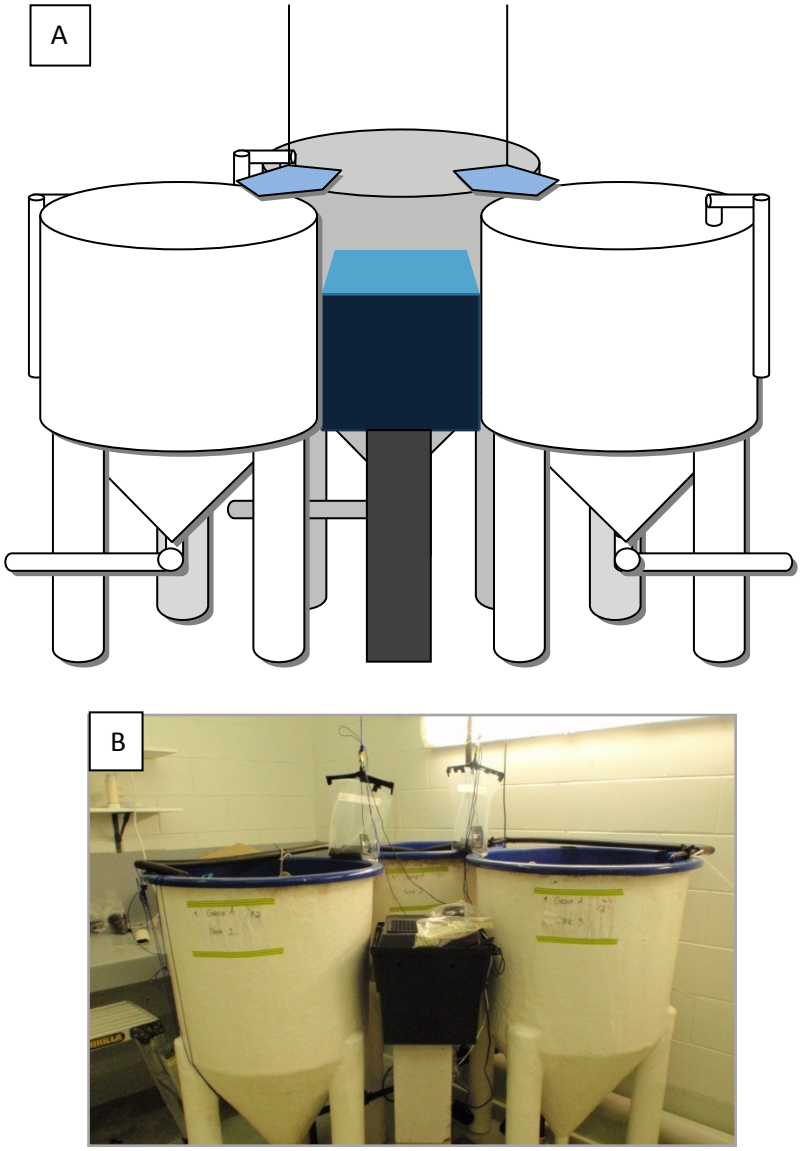

Figure 2.6. This figure (A) depicts the 250L tanks setup within each group. With the satellite speakers suspended from above (patterned with hatching) and the subwoofer within the sound transmission case between the tanks. The figure also depicts the tank design with tall supporting legs. In the image (B) displays the actual set up. While an experiment was in progress the sound transmission case would be covered in a sheet of plastic to protect the electrical devices.

When replaying the sounds and setting treatment soundscapes, it is important to mention that the small tank environment can be very difficult to control and predict (Hawkins, 1993), in particular the exact matching of sounds across tanks. This necessitated the creation of a set of guidelines to maintain consistency across the tanks within a group, but also when needed across groups.

The guidelines consisted of acceptable frequency and sound level variations for each tank. The variation in frequency was allowed to extended ± one bin width (46Hz) and the source level was

allowed to be within ± 1 dB (Table 2.2). The frequency of interest is 187.5Hz, a value identified in the work completed in Chapter 3 as it was the main contribution to the soundscape evaluated. This frequency was used as it is based upon real measurements, but also as it falls within the hearing range of most fish species, and is detectible by the lateral line.

Table 2.2. Sound determination guidelines for tank soundscapes.

	SPL	Frequency	Between tanks/ groups
Allowed variation range	+/- 1 dB of identified peak SPL	Ideal 187.5 Hz ; however allowed for SPL peak to occur +/- 50 Hz either side. Based on distribution of the FFT bins	+/- 1dB and within +/- 50Hz from 187

Adjustments needed to meet the set guidelines were accomplished by typically minor changes to sound output level, position and the physical connections with the tanks (Figure 2.7). After every sound level change, the tanks were re-evaluated for specific characteristics (the peak, the shape of the sound recording in the frequency spectrum, etc.) (Figure 2.8). Once this was completed, required adjustments were made to individual tanks. However if any position changes were to one tank in a group, all other tanks in the group would require re-evaluation to maintain consistencies.

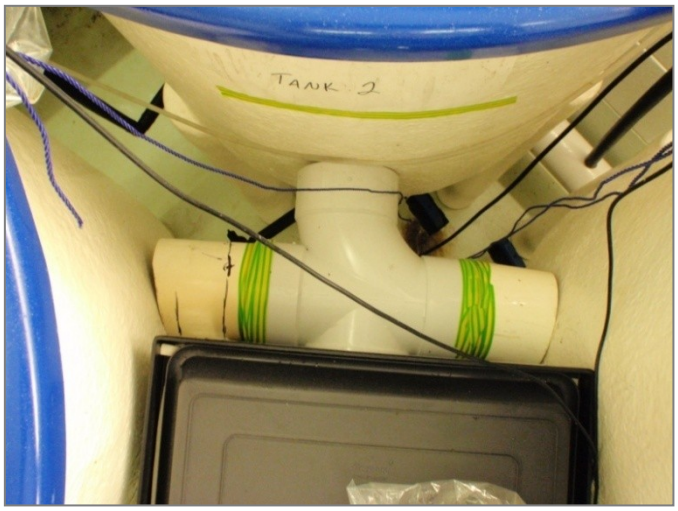

Figure 2.7. This image depicts the physical connections made with the sound transmission case. Adjustments would be made by dampening the sound by inserting a small square of rubber mat between the tube and the tank wall reducing the vibration carry over and creating a tighter physical connection.

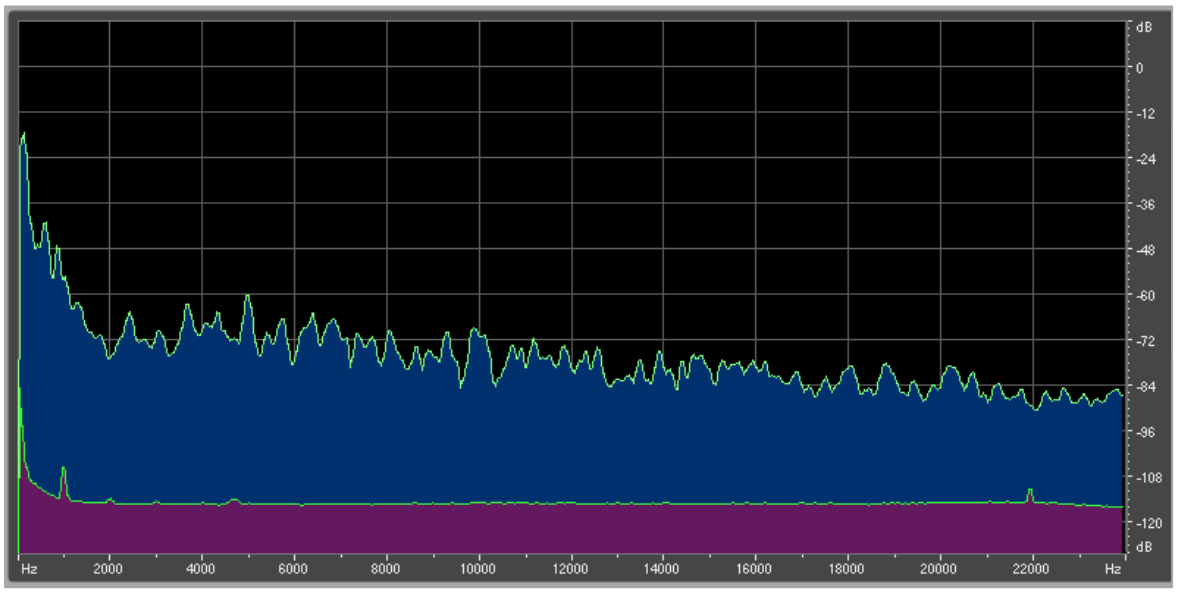

Figure 2.8. This is the ideal spectrum of a soundscape recorded from one of the test tanks. The peak sound intensity lies at the frequency of interest followed by a decline in sound strength into the higher frequencies. The SPL is expressed in Audition relative values.

2.4. Conclusions

The combination of equipment and guidelines is part of the strategy that achieved consistency throughout a single experiment but to also maintain consistencies across all experiments. The ability to control the sound so efficiently simplified the process of resetting and changing experiments. Removing any complications of resetting the sound systems as well as allowing the sound system to be preset prior to an experiment became a time efficient and cost effective solution.

Within the following Chapter 4-6, more specific detail will be provided on the variations in methods for the specific experiment, for example; number of fish, exposure periods, and sound levels. These chapters will also evaluate the biological side of the effects more specifically the physiological response of the various tested environments. The following chapters will develop and discuss ideas and results of each of the experiments which will be further discussed.

Chapter 3. Determining and quantifying components of an aquaculture soundscape

This chapter was published as:

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3.1. Introduction

There is an increasing focus on both the welfare and ethical treatment of aquaculture finfish (Conte, 2004; Ashley, 2007). The potential sources of stress in aquaculture facilities can be many and varied, the effects of which are often amplified as the stressor is frequently anthropogenic in origin and the fish is unable to escape the stressor. Although stressors in an aquaculture setting are unavoidable (Ashley, 2007), the fundamental goal for successful growth and production is the development of optimal strategies and practices that effectively manage or mitigate both acute (transient) and chronic (continuous) stressors. In order to achieve this goal both the nature of the stressor and its consequent affects must be known and understood (Ashley, 2007).

A possible although often overlooked source of stress in an aquaculture facility is water borne sound(s) (Wysocki et al., 2007). Culture systems produce noise from multiple sources, which can either be continual, such as the sound produced by water pumps and aerators (Bart et al., 2001) or transitory, such as sounds that originate from the activities of personnel managing the facility. A key difference between these types of sounds is that the former is constantly generated and therefore becomes predictable (expected), while the latter is not. These characteristics of continuous and

transitory sounds would be considered quite different from naturally occurring ambient environments as the soundscape is anthropogenically created and dominated.

The extent to which water borne sounds common to an aquaculture facility stresses fish remains largely unknown. However as demonstrated Wysocki et al (2007) that hearing sensitivity, growth, survival and disease susceptibility in rainbow trout are not negatively impacted by noise levels found in a recirculating aquaculture system. The authors highlighted that such findings cannot be generalized to all cultured fish species, as hearing sensitivity and tolerance to potential stressors will ultimately differ across species.

The impact of water borne sound in an aquaculture facility will depend on the species being cultured as well as the facility itself. Different facilities are equipped with different machinery based on the types of production and species-specific requirements. There is also the influence of how sound may be carried, transmitted and displayed in regards to the facility design and purpose. For instance the facility housing, equipment, substrate, location, personal activities and the material of the tanks used will all influence how the sound appears within a facility. Therefore all facilities will present an individualized soundscape.

Water borne sounds generated by an aquaculture facility were previously explored by Bart et al (2001), who measured the frequency distribution and sound pressure levels (SPL) in fibreglass circular tanks, raceways and earthen ponds. Bart et al (2001) noted that the sound in recirculating tanks had both low (25-250 Hz) and high (630-2000 Hz) frequency components and SPLs upwards of 153 dB (re 1µPa), with average sound levels ranging between 125 and 135 dB (re 1µPa). This work characterized a range of aquaculture sounds based on types of facility type but did not resolve the frequency distribution and SPLs of the individual mechanical components involved in the culturing process or the spatial distribution of sound within culturing tanks. Determining these characteristics

is crucial as this information can then be used to develop and evaluate the effectiveness of sound reduction strategies in the culturing process.

Although the mechanical equipment involved in production and the coupling of the tank to substrata borne vibrations are the dominant contributors to the aquaculture soundscape, the impact of facility personnel activities involved in the culturing process may also be significant, although this has not been demonstrated. The sounds generated by facility personnel are presumably different from those generated by mechanical equipment in two important ways. Firstly, the sound generated by personnel will be largely limited to diurnal periods, and secondly the sounds generated are likely to be transient in nature (e.g. accidental dropping of tools, momentary impacts on tank walls, and other facility/location related tonal sounds), and will therefore be unanticipated by the fish.

The purpose of this work was to obtain a thorough understanding of the nature and sources of water borne sounds that construct this 'soundscape', defined by Genuit and Bray (2006) as a variety of sounds with individual and collective attributes, that may potentially act as stressors in a large scale commercial aquaculture recirculation facility. In order to do this we focused on characterizing and quantifying four main attributes; 1) the dominant sounds and sound characteristics of tanks used during various production stages, 2) the contribution of individual components of mechanical equipment, and 3) the contribution of facility personnel to the aquaculture soundscape.

3.2. Methods and Materials

3.2.1 Location and facility

Sampling was undertaken at a large-scale commercial broodstock facility located in North Queensland, Australia. This enclosed facility contains a range tank sizes for the purposes of larval and juvenile rearing and housing of mature wild caught broodstock fish.

The facility houses broodstock in two different size tanks, both 2.2m in depth. Large broodstock tanks have a diameter of 6m (~62,000 L), while the smaller broodstock tanks have a diameter of 4m (~28,000 L). Juvenile rearing tanks range in size from 2.2-2.7m diameter with depths of approximately 0.6m (~2,800 L). All tanks are sited on large concrete slabs which that are part of the facility's foundations.

Water flow through both large and small broodstock tanks are achieved via independent recirculating filtration systems, employing an Onga Sta-Rite Dura-Glas II three phase pump operating at 2800 RPM, which are situated on the same concrete slabs as the tanks. Rearing tanks were run on a flow through system. These tanks were fed water directly from the pump station which is located a considerable distance (~800m), from the aquaculture facility.

3.2.2 Sound Recording Equipment

Collection of acoustic data was accomplished using an EDIROL R-4 four channel data logger/ recorder (frequency response 20 Hz – 40kHz) and up to four High Tech 94-SSQ series hydrophones with built in pre amps (frequency response range of 2 Hz to 30 kHz , hydrophone sensitivity -165 dB re: 1 V/µPa ±1 dB within response range (information provided by High Tech), were used simultaneously.

The acoustic samples were acquired using a sampling rate of 48 kHz with 16-bit resolution. The R-4 data recorder was powered by a remote 12V battery to remove the possibility of AC power induced low frequency noise being introduced into the recording, and the hydrophones were supplied with 'phantom power' from individual 9V batteries.

3.2.3 Sampling Protocol

3.2.3.1. *Spatial distribution of tank sound*

Three hydrophones were positioned at three depths within the large and small broodstock tanks (~40cm above the bottom, middle ~1.1m from surface and 40cm below the surface). Hydrophones were stationed ~40cm from the tank wall. The distance from the wall was decided based upon the

hatch locations in the hoods that allowed access into the tanks when the hoods were in the down position, therefore 40 cm became the set sampling distance from the wall for all tank samples. Unless otherwise stated all other recordings in the study used the same hydrophone array. Sampling was conducted over a normal operational week so as to include any daily variations in facility activities. A total of four large broodstock and six small broodstock tanks were sampled continuously for a period of 5 minutes, this was repeated on three separate occasions and at random times (between 09:00-16:00), during the week.

3.2.3.2. *Influence of hood position*

Broodstock tanks within the facility were equipped with movable hoods that cap the top of the tank, thereby forming a temperature and light controlled environment. These hoods are used in the 'down position' when broodstock are under photothermal manipulation for the purposes of advancing or delaying reproductive maturation. To investigate the influence of hood position on the sound characteristics of tanks, broodstock tanks were sampled with the hood in the down (sealed), and up (unsealed), positions and sound samples compared between the two treatments.

3.2.3.3. *Identification of individual components and airstone evaluation*

During each evaluation three minute samples were recorded using all four hydrophones positioned across the diameter of the tank at 2 depths, with two approximately 70cm above bottom and two 70 cm below the surface. This layout varies from the other hydrophone array to maximize sound recordings from the individual components and air stones simultaneously and also these recordings were not used in any comparison with other collected sound samples.

3.2.3.3.1. Individual component identification

To identify the contribution of individual system components to the tank soundscape, a single tank was recorded under the following conditions individually; 1) entire tank system off, 2) the water pump operating, 3) the aeration system operating, 4) the pump and heater operating, and 5) all system components operating at once. Three minute samples were deemed to be sufficient as the sound produced by each system component was consistent and constant. To identify the signature

and contribution of each system component the frequencies and SPLs were compared across the five different operating conditions outlined above.

3.2.3.3.2. Aeration recordings

Sound recordings of two different airstone types at the same air pressure (ceramic Sweetwater Air Diffusers: coarse: 1-3 mm bubble size and fine: 0.5-2 mm bubble size), was undertaken to isolate the effect of air bubble size on the soundscape within the large broodstock tanks. Sound samples were recorded for 3 minutes and the samples analysed for differences.

3.2.3.4. ***Comparison between day and night***

Large and small broodstock tanks were sampled during the course of the night to determine if any difference existed between night and day sound characteristics. Tanks were deployed with two hydrophones placed at two depths (40 cm above bottom and 40cm below surface) which allowed for the comparison of Shallow and Deep recordings from the day time samples). Recording began when staff vacated the facility (17:00), and continued until the battery powering recorder was exhausted (~ 8+ hours). The comparison between day and night recordings was intended to isolate the contribution of facility personnel to water borne sound.

3.2.3.5. ***Generation of simulated transient sounds***

Impacts on the outside surface of tanks often occur during routine operation of an aquaculture facility. These impacts are random transient events unlike the sounds generated through operation of system machinery. This means that the sounds are unexpected and not predicted by the fish. In order to quantify the sound characteristics of these events we simulated tank impacts by dropping a hard shelled object with a ridged shell and a spherical shape (representative of a tool used in regular maintenance), and a dense soft shelled object consisting of a leather shell and a spherical shape (representative of an object such as a boot), against the tank wall. The aim was to simulate transient impacts in a reproducible manner. Object impacts were created at two vertical positions on the tank, the hydrophones were situated in the same array as the component recordings (outline in 2.3.3), and impacts were repeated fifteen times with 10 second intervals between impacts.

3.2.3.6. *Juvenile rearing tanks*

Due to the shallow depth of the juvenile rearing tanks, sampling consisted of a single hydrophone placed mid-depth (~0.3m), in the centre of the tank. Juvenile rearing tanks were sampled as outlined above, three times during the week.

3.2.4 Data analysis

Raven 1.3 Beta: interactive acoustic analysis software (Cornell Lab of Ornithology) was used to analyse all sound recordings. This program provided a range of visual aids and outputs with which to analyse the properties of recorded sound from the various treatments. When required, larger sound files were segmented using Cool Edit Pro 2.0 (Adobe), prior to analysis in Raven.

Sound Pressure levels (SPLs) were measured to survey the inter-tank soundscape. The method of using SPL may be perceived as a coarse evaluation; however the likelihood of establishing exact measures of acoustic conditions becomes difficult and unpredictable. This is because in the tank environment, which is surrounded by reflecting walls, as described by Hawkins (1993), the environment becomes very complex and no longer possible to easily predict the particle velocities. Also the tanks within this system have continuous high flow rates, vertical and horizontal water flow due to inlet/outlet and air bubble movement further influencing particle motion within the tank. SPL is commonly used as a unit of displaying sounds within aquaculture facilities (Bart *et al*., 2001; Davidson *et al*., 2007; Wysocki *et al*., 2007a). SPL are also commonly used when describing hearing thresholds in auditory threshold determinations in fish. It is discussed elsewhere that particle motion in regards to fish hearing is more important than SPLs (Hastings and Popper, 2004), as the fish hearing system is designed more like an accelerometer, but it is difficult to measure accurately in small tanks. Despite this difficulty, particle velocity components of intensity around the dominate frequency were likely. American National Standards Institute (ANSI) S1.20-2003 standard indicates that particle velocity augmentation at the dominant frequency (187.5 Hz) within 1.2 m of a source

would be in the order of 3 dB. This augmentation is minimal; therefore the purpose of this work the evaluation of SPL gives the overall impression of what the tank environment resembles.

3.2.5 Hydrophone calibration

The recording system based on the EDIROL R-4 and hydrophones was standardized in order to convert to absolute sound pressure levels and correct any input gain from the EDIROL R-4. This was achieved through a Picoscope ADC-212 in FFT mode, using the same 46.9 kHz FFT bin width (resulting from a 1024 point FFT (implemented with a Blackman window)) as used in the Raven analysis software. The Picoscope was used with a hydrophone to record the same acoustic environment as the hydrophone and the R-4, and the recordings compared 10 times to determine a single conversion value. This conversion value can be used to convert Raven values to sound pressure levels (SPL), (dB re: 1 $\mu Pa^2/Hz$).

3.2.6 Data evaluation and analysis

Three variables were used to evaluate sound characteristics within tanks, in the acoustic environment which consisted of broadband noise. All recordings were evaluated using an FFT with resolution of 46.9 Hz (Sampling frequency of 48 kHz, 1024 point FFT using Blackman window). The units used for narrowband SPL throughout this research are dB re 1 $\mu Pa^2/Hz$, relative to a 46.9 Hz FFT bin width, which can be converted to dB re 1 µPa by adding $10\log_{10}$(bandwidth) (McCauley, 1998). The first of these variables, maximum SPL, representative of the highest spectral peak of all frequency analysis bands (width 46.9 Hz), was identified in each individual recording (sample). The maximum SPL's from each days' sample were then used to calculate a mean maximum SPL per tank. Mean maximum SPLs (dB re: 1 $\mu Pa^2/Hz$, 46.9 Hz FFT bin width), the second variable, were considered values of importance as they indicated the corresponding values (to the FFT bins) of the dominant frequencies within the system. The third variable, the mean average SPL (dB re 1 μPa^2, broadband (2 Hz to 20 kHz)), was calculated through averaging the mean SPL (dB re 1 μPa^2, broadband (2 Hz to 20 kHz)) of each individual sample, and then averaging these means, creating a grand mean referred to as the mean average SPL. Mean maximum and mean average SPLs were calculated from the three

days sampled, either from entire files of 5 minutes in duration or, in the case of night recordings, from randomly selected 5 minute sub-samples of larger files. The value which corresponds to the FFT bin number associated with the peak of the FFT (maximum SPL occurrence location), is referred to as the dominant frequency, and was identified in all recordings.

Data was evaluated based on comparison of highest maximum SPLs, mean maximum SPLs, mean average SPLs and dominant frequencies. Statistical analysis was undertaken using SPSS version 14.0 for Windows. If data satisfied normality, a one-way ANOVA was used to test for significance, where statistical assumptions of normality were not satisfied a non-parametric Kruskal-Wallis tied ranks test was used, followed by a Mann-Whitney U test. The significance for all statistical tests was set at $p < 0.05$.

3.3. Results

3.3.1 Broodstock tanks

Mean maximum SPLs, mean average SPLs and dominant frequencies (Table 1.), were not significantly different between the four large broodstock tanks sampled (mean maximum ANOVA: $p < 0.522$: mean average Kruskal-Wallis: $p < 0.154$), which highlights that the sound characteristics between the tanks were similar among all tanks of that size. Sound samples for individual tanks were then combined and the sound characteristics at three different depths (shallow, mid, deep), were investigated. Sound within tanks was found to be vertically 'layered', with sound pressure levels being positively related to depth (Figure 3.1). Mean maximum SPLs differed significantly with depth (Kruskal-Wallis: $p > 0.004$), the highest SPL was consistently found at the base of the tank, a level significantly higher than the mean maximum SPLs occurring at either the mid (Mann-Whitney U: $p > 0.002$), or shallow (Mann-Whitney U: $p > 0.038$), depths (**Error! Reference source not found.**). Mean average SPLs also showed a similar trend of increasing sound pressure with depth. The highest maximum SPL recorded was 124 dB re 1 $\mu Pa^2/Hz$, at the base of the tank, while the lowest was 115

dB re 1 μPa^2/Hz at the shallowest depth sampled, which represents an approximate trebling of sound energy between the two sampling locations. The dominant frequency was consistent among all depths sampled, with the peak of the FFT lying in the 4^{th} FFT bin, with a corresponding value of 187.5 Hz (Figure 3.2). It can be summated that in general, SPLs consistently increased with increasing tank depth.

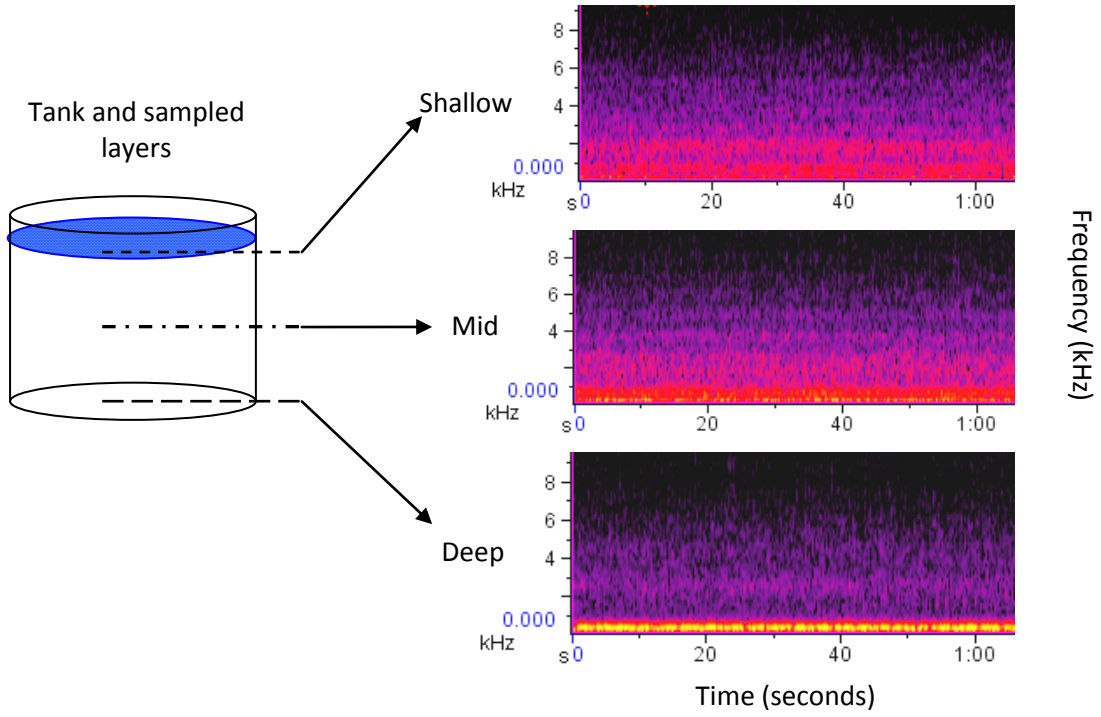


Figure 3.1. This figure depicts the multiple layers of a LB tank. These spectrogram images show the varying concentration of intensity according to frequency over time (determined by colour yellow = very intense, to black = very low intensity). The observed distribution of intensities between the top, middle and bottom sample show that highest intensities as well as the most concentrated distribution of the higher intensities is within the bottom recording. These images are from tank LB4 which demonstrates clear differences of all three layers. The rest of the LB tanks followed a similar pattern in depth.

Table 3.1 Summarization of tank type and samples statistics.

	Mean maximum dB re: 1 µPa²/Hz	*Mean average dB re: 1 µPa²*	*SD of the maximum*	*± SE of the*		*Highest maximums dB re: 1 µPa²/Hz*	*FP**
				Maximum	*Average*		
Rearing Tanks							Range
Mean of all R tanks	106	67	7.84	5.533	0.872	117	187.5-2625.4 Hz
Large Broodstock tank depths							
Deep	115	80	1.55	3.531	0.837	124	187.5 Hz
Middle	114	78	2.29	2.340	1.618	117	187.5 Hz
Shallow	112	77	4.65	3.825	1.177	115	187.5 Hz
Small Broodstock tank depths							
Deep	117	83	4.41	0.70	0.670	122	187.5 Hz
Middle	115	78	2.91	1.220	0.162	119	187.5 Hz
Shallow	110	73	4.68	1.553	0.293	120	187.5 Hz
Night vs. Day recordings							
Day LB1	111	74	7.84	2.61	1.928	124	187.5 Hz
Night LB1	105	74	1.67	1.671	0.364	109	187.5 Hz

*Frequency peak at highest recorded maximum

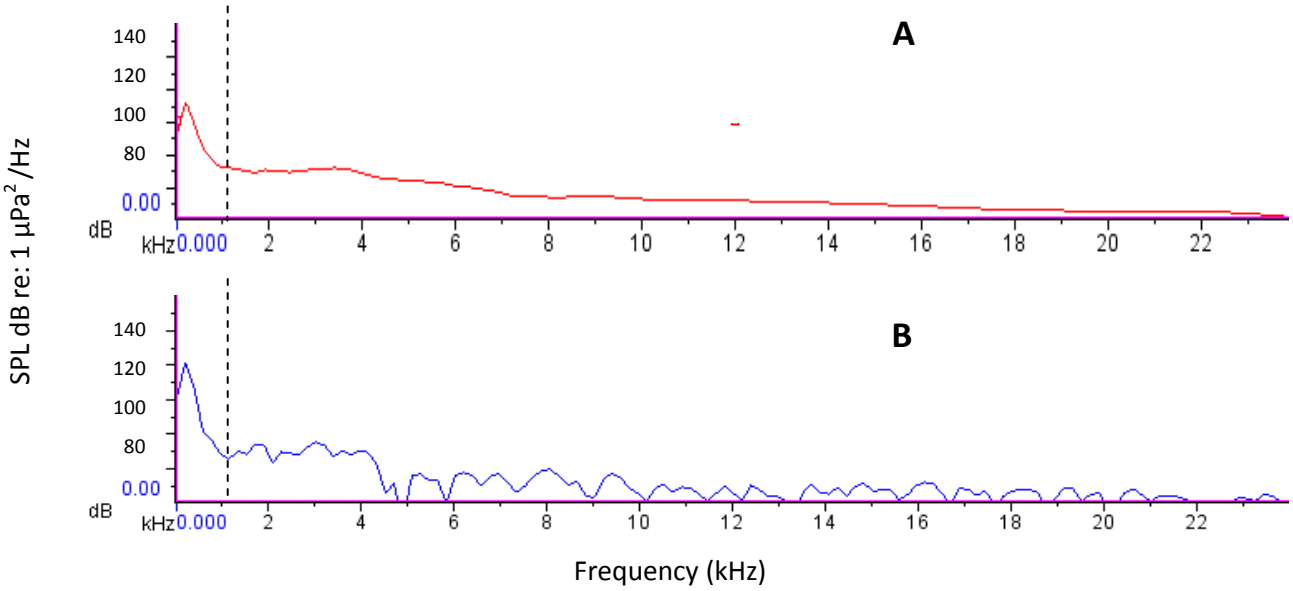


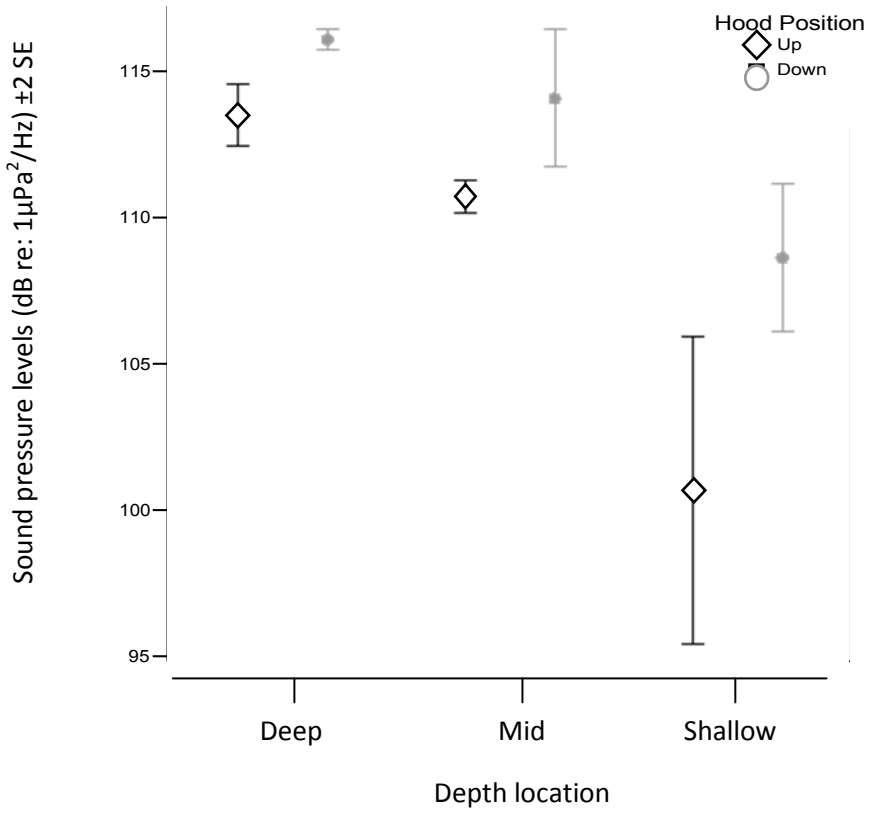
Figure 3.2. These spectrums display the relative intensity distribution across the sampled frequency range. A) Is a full 5 minute recording selected spectrum of the bottom recording from tank LB1 sample 1 using an FFT with the Blackman window, this FFT rounds frequency bandwidths over time to describe a generalized soundscape of that recording (318 Hz). The majority of higher intensity falls below 1000 Hz (designated by the dotted line). B) Is a spectrum slice at a specific point in time, and in this instance is where the highest recorded maximum was found. The dB values are displayed in original Raven output.

The sound characteristics of small broodstock tanks were similar to that observed for large broodstock tanks. Both mean maximum and mean average SPLs were significantly different between depths (mean maximum ANOVA: P>0.001: mean average Kruskal-Wallis: p>0.0001, Mann-Whitney U: deep/mid p> 0.047, deep/shallow p>0.0001, shallow/mid p>0.0001). The highest maximum recorded SPL of 122 dB re 1 µPa^2/Hz occurred at the deepest depth sampled: which was more than 2 dB re 1 µPa^2/Hz higher than the highest maximum SPL at the shallowest depth sampled. Similar to previous observations in the large broodstock tanks, the dominant frequency was consistently 187.5 Hz.

3.3.2 Hood position

The position of the tank hood influenced the pressure levels of the soundscape. The hood in the down position resulted in maximum SPLs being consistently higher at all depths (

Figure 3.3), although this



difference was not significant (ANOVA p< 0.28).

Figure 3.3. Mean (±SE) sound pressure levels at different depths and hood position. Although the result is not statistically significant the trend suggests that there is an influence on sound levels across depths are affected by the hood position.

3.3.3 Identification of sound components and influences and airstone evaluation

Samples of a tank with all system components deactivated revealed the strong influence of sound from neighbouring and adjacent tanks (Figure3.4 , A). This clearly demonstrates the process of sound transmission throughout the facility. Background noise associated with other operating systems influences all individual tanks soundscapes, regardless of what system components were operating. Analysis of the sound generated by the pump revealed that this system component was the dominant contributor of sound and the primary contributor of the 187.5 Hz frequency (Figure3.4, B.). The pump was identified as the source of the 187.5 Hz frequency peak as it was the component with the highest individual SPL increase at that specific frequency bin. The contribution of the heater system was insignificant as there were no observable differences in the sound characteristics of the tank regardless of the operational state of the heater. Aeration had a distinct effect on sound characteristics resulting in the energy extending into frequencies >7,600 Hz and as high as ~22 kHz

(Figure 3.4, C). Frequencies between ~2.5-10 kHz showed an appreciable increase in energy, increasing by 10-20 dB re 1 μPa^2/Hz.

Comparing the spectral characteristics when all system components are off, to all system components operating, reveals that the frequency range detected changed from ~20-7,300 Hz to ~20-19,000 Hz, while energy content predominantly increased across the ~20-7,300 Hz range. A 10 dB re 1 μPa^2/Hz increase in the dominant frequency of 187.5 Hz was also evident. The increase in SPLs when all system components are operational (Figure3.4, D), greater than that observed from any single component (Figure 3.4, B-C), suggests interaction of the sounds emitted by individual system components.

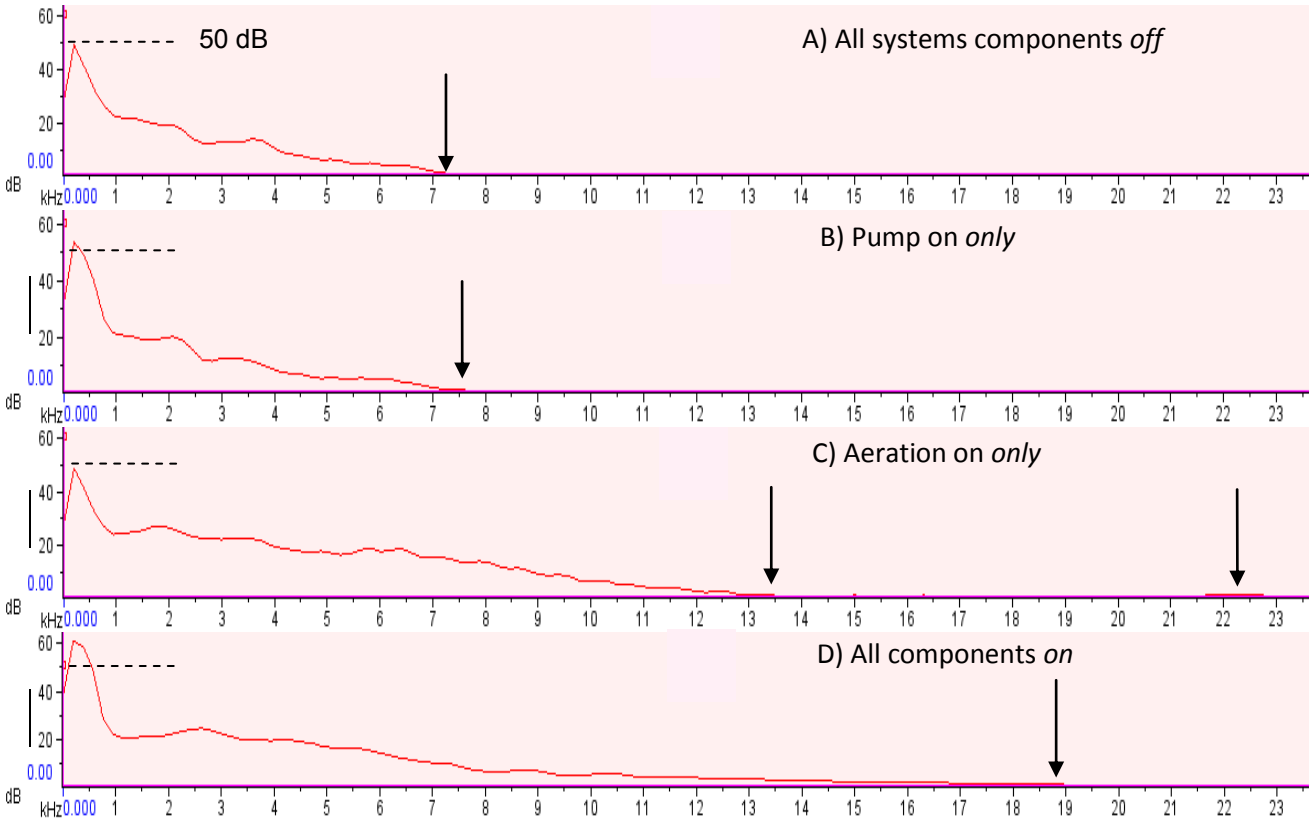


Figure 3.4. Together these spectra from LB 3, in relative scale, depict the individual system components and the contribution and effect for each: (A) all system components off, (B) pump on only, (C) aerator on only, and (D) all system components on. The arrows indicate where the sound dissipates and in (D) where it re-occurs at a higher frequency. In A. all machinery input of that tank has been shut off, however it is clear that the other tanks with in the facility contribute to the sounds within the tank. Displayed spectrums set at 318 Hz bandwidth for overall feature display.

3.3.4 Airstone evaluation

As a result of aeration being identified as a major contributor to the soundscape the sound properties of fine and course airstones were investigated. The highest maximum SPL of 119 dB re 1 $\mu Pa^2/Hz$ was recorded from the coarse airstone which produced larger bubbles and more intense in frequencies below 8 kHz. A significant difference existed between the two airstone types (ANOVA: $p > 0.039$), mean maximum SPLs were consistently ~9dB re 1 $\mu Pa^2/Hz$ higher across the ~20-8000Hz range when using the coarse stone. The variation among recordings was based on the change of the frequency point of highest SPL, which was at 187.5 Hz due to the surrounding facility noise.

3.3.5 Day vs. night recordings

Mean maximum SPLs were significantly higher during the day than during the night, this difference was significant (Kruskal-Wallis: p >0.019). Variability of mean maximum SPLs was also distinctly different between day and night periods, with the coefficient of variation for day and night samples being 7.08% and 1.59% respectively. Highest maximum SPLs recorded during the day were ~15 dB re 1 $\mu Pa^2/Hz$ higher than the observed maximum SPLs during the night with the mean maximums varying ~6 dB re 1 $\mu Pa^2/Hz$ (Table 1). The dominant frequency remained unchanged between night and day samples and was identical to the previous observation of 187.5 Hz. Mean average SPLs were not significantly different between day and night periods (Kruskal-Wallis: p <0.605).

3.3.6 Generation of transient sounds

The characteristics of transient sounds differed substantially between hard and soft objects. Impacts of the hard object on tanks were characterized by an initial SPL of ~6 dB re 1 $\mu Pa^2/Hz$, which attenuated rapidly but persisted for ~1.0 sec after the initial impact (Figure 3.5A). Impacts of soft objects were characterized by lower SPLs of ~1 dB re 1 $\mu Pa^2/Hz$, which rapidly attenuated and were indistinguishable from the tank background sound after ~0.25 sec (Figure 3.5B). Initial SPLs were also observed to be influenced by the location of the impact; both hard and soft objects had higher SPLs when impacts occurred at the top of the tank.

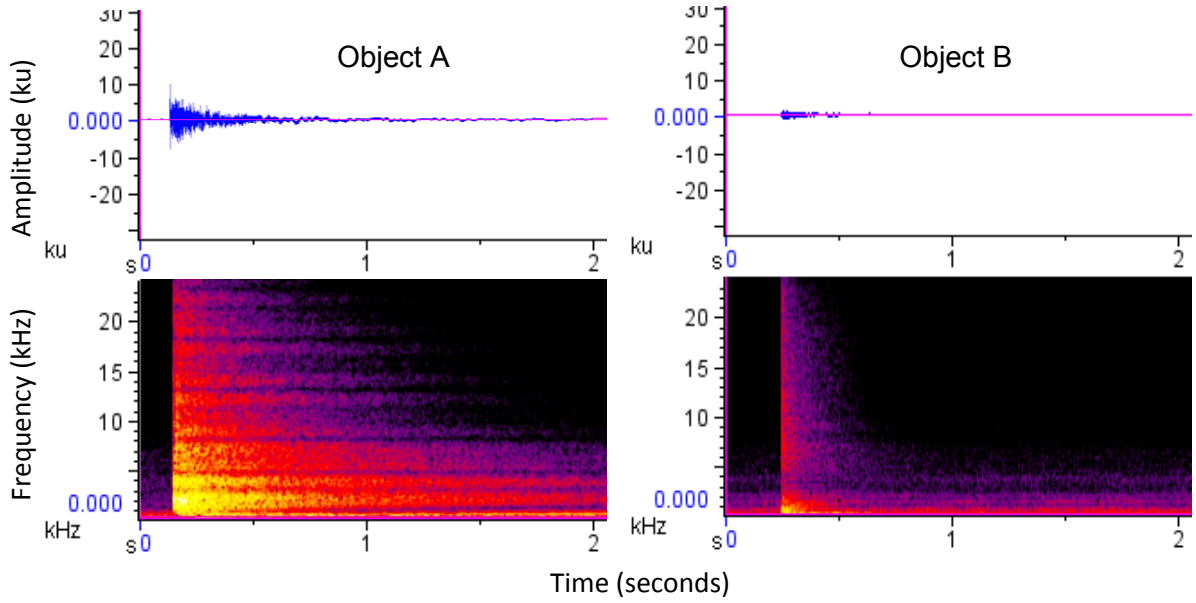


Figure 3.5. These images show the oscillograms and sonograms of two very distinct sound shapes. The shapes reflect the structure and densities of each object. The top image is a waveform of each with the coinciding spectrogram below. Yellow represents the highest intensity and black the lowest. SPLs and amplitude are represented in relative Raven values.

3.3.7 Rearing tanks

The mean maximum SPL among juvenile rearing tanks was 106 dB re 1 µPa^2/Hz. The mean maximum SPLs from individual rearing tanks were not significantly different (ANOVA: p <0.958). The highest maximum SPL recorded was 117 dB re 1 µPa^2/Hz, with a dominant frequency of 206 Hz. (Table 1).

3.4. Discussion

The results of this study clearly show that the equipment used in re-circulation facilities, such as aerators, pumps and filtration systems make a dominant contribution to the acoustic environment of aquaculture facilities. The study has also uncovered three important characteristics; firstly the sound within tanks has a vertically layered distribution, with SPLs, positively related to depth, secondly SPLs within tanks were consistently and significantly higher during the day in comparison to night, while also having a higher variability during the day, and thirdly each component of the recirculation system has a unique acoustic signature with regard to frequency and SPL.

Sound within broodstock tanks had a vertically layered distribution, with sound pressure increasing with depth. The unique acoustic characteristics existing in these tanks strongly suggests that the combination of tank design, structure and equipment components play a considerable role in determining the acoustic conditions within tanks. The overall soundwave interactions become unique and difficult to evaluate, as Hawkins (1993, p131-132) described that sounds within a restricted environment become considerably more complex with changes in the structural characteristics of these sounds. The tanks used within this facility are far from an ideal acoustic tank (Parvulescu, 1964).

In the case of this particular facility, the broodstock tanks are situated directly on concrete with the associated pump located adjacent to the bottom of the tank on the same concrete slab. This design exposes tanks to receive pump noise via three separate channels: 1) directly through the water, 2) across the air-water interface, and 3) via direct vibration through the adjacent concrete slab. Of these three sources, the transfer of vibrational energy directly from the pump to the tank base will have greatest single effect. This appears to be reflected in both the vertical layering of sound pressure and the evaluation of the acoustic signature of individual equipment components. For example the highest SPLs were constantly found at the base of the tank, with the base having SPLs ~8dB higher than the surface SPL, in addition SPLs measured at the base of the tank were centred at 187.5 Hz, a frequency that was later identified as a characteristic of the pump. The influence of the pump was considerably less in the middle and surface layers of the tank which was evident by the considerably lower mean SPLs at the 187.5Hz frequency. This suggests that sound intercepted possibly through shear effects or refraction of water flow as it rises through the water column. Another explanation for the vertical layering of sound is that sound transmission through the water is also influenced by rising air bubbles from aeration stones. This effect is similar to that of air bubble curtains used to reduce sounds transmitted from pile driving (Würsig *et al.*, 2000). There is also the influence of sound waves interacting in a constructive and destructive manner such as in multipath

losses, and specifically, Lloyds mirror effects (Malme *et al.*, 1995) and sound transmission influences associated with cutoff frequencies due to the restricted size of the tank environment. Together these may disrupt and alter the sound transmission properties of the water due attenuation, refraction, reflection and absorption.

At present there is a paucity of information regarding the effect(s) that sustained long term exposure to sound has on fish health. Consequently the vertical layering of SPLs revealed in this study may have important implications for the vertical distribution of fish within culturing tanks, considering they are restricted in space and unable to fully avoid the sounds, the fish may actively select the layer that causes the least acoustic irritation. However the lifestyle, such as benthic species, may override any potential discomforts. It has been demonstrated that domestic rainbow trout are capable of avoidance learning when presented with a frightening unpleasant stimulus (Yue *et al.*, 2004), while the vertical distribution of captive fish in tanks has been shown to be modified by environmental stimuli such as light (Sogard and Olla, 1993) and structural features (Noble *et al.*, 2007). Despite these effects, the active selection of depths may be overridden by lifestyle of the fish, for example benthic fish that have a strong association with the substrate may remain in the bottom region of the tank despite any such acoustic irritation.

This study has also revealed that structures that do not actively generate sound can influence the sound environment within tanks. Broodstock tanks are regularly fitted with 'hoods' that seal over the top in the tank, this enables tight control of the photothermal environment and is regularly used for the conditioning of broodstock. Our data has shown that these hoods cause sound intensification within the enclosed tank, with the hood in the down position sound levels were effectively increased at the mid-level of the tank. These hoods appear to have the effect of encapsulating sound and reflecting it back into the water, thereby intensifying the overall soundscape. This effect has the potential to further stress fish during photothermal manipulation, ultimately leading to poor

reproductive condition and/or performance. However, the extent of what characteristic of the hood is responsible of this effect is not known and it is recommended that this effect be further evaluated as our data was statistically limited.

The comparisons between day and night SPLs were designed to isolate the contribution of facility personnel to the sound environment. Day recordings differed from night recordings as far as day recordings included facility personal moving around and working in close proximity to tanks. Although, the dominant frequency (187.5 Hz) was consistently the highest SPL for day and night samples. Comparing day and night samples the highest maximum SPLs were found to occur during the day and differed by up to 15 dB above those measured at night. Although this effect cannot unequivocally be attributed to facility personnel, it is interesting to note that mean maximum SPLs were considerably more variable during the day than at night, such an effect would be consistent with variable nature of the activities of facility personnel. This finding underscores the effect that the activities carried out by personnel managing the facility can have a significant influence on the sound environment.

An event not addressed in this papers' evaluation, but one that maybe of concern, is daily fluctuation. For instance during times of rush hour along the road that is adjacent the facility, or hours where construction, heavy machinery is being used and how that may influence the day to day and hour by hour fluctuations of the soundscape.

The prevailing acoustic environment within tanks is a combined product of the acoustic contribution of each component. By measuring the acoustic attributes of individual components it was possible to isolate which components were dominant and quantify an acoustic identification of each. This is important as it enables a direct comparison between the acoustic properties of individual equipment components and the hearing capabilities of fish. Unsurprisingly the dominant acoustic source was

found to be the pump at low frequencies (~187.5 Hz) and aeration at higher frequencies (≥2,500 Hz). Sound levels within tanks reflected this being dominated by these frequency components. The frequency level of 187.5 Hz had the highest SPLs consistently re-occurring across tanks, suggesting that this frequency component is the dominating acoustic influence in the tanks. The importance of this finding is that this frequency falls within the hearing capabilities of a range hearing generalists and hearing specialists as well as being detectable by the lateral line (Popper *et al.*, 1988; Hastings *et al.*, 1996; Popper, 2003).

A variation in SPLs was also noted between two different airstone types. This variation appears to be a function of the relationship between the particle size of the air stones and bubble size. Finer particle size airstones create smaller radius bubbles, and consequently higher frequency sounds as well as higher SPLs. This is an important feature in minimizing the sound input within the tanks themselves at the level of the dominant occurring frequency. This characteristic could be included with other methods suggested by Davidson *et al.* (2007), such as modifications to inlet and effluent piping as well as substrate changes to drastically reduce SPLs within recirculating tanks.

With respect to transient sounds, these sounds could be considerably more stressful than sustained continuous sounds on fish. The continual nature of specific sounds allows the fish to generate a level of predictably and hence may result in a degree of adaptation to such sounds. Transient sounds, such as those generated by impacts on the tank walls, are stochastic consequently these types of sounds are unpredictable and the fish has no expectation of the sound. This may generate a fright or escape type response. The position at which an impact occurs is important with regard to the intensity of transient sounds. Sounds were considerably more intense when they occurred at the top of the tank, a result of the changing acoustic properties of tanks. The combination of shape change and connection with the concrete may change the resonance of sound by absorbing or even

reflecting sound waves (Tiwari *et al.*, 2004). The observed transient impacts were more intense at the top of the tank than at the base where the tank changes shape from a straight to a curved sided.

The facility surveyed is primarily designed to house tropical marine wild collected broodstock fish and/or to grow out juvenile hatchery fish. Juvenile rearing and quarantine tanks have a different system setup to broodstock tanks, such as the proximity of pumps, location and overall size. Consequently, the wild caught fish are initially exposed to a lower sound levels (approximately 10 dB re 1 $\mu Pa^2/Hz$ less), prior to being moved to the broodstock tanks that have louder environments. This graduated transition from the quieter juvenile rearing/quarantine tanks to the larger broodstock tanks may allow fish a period of acclimation to the facility's ambient sound environment.

There are substantial differences between the soundscapes of natural habitats and aquaculture facilities. This aquaculture soundscape was dominated by continuous high SPL at low frequency sounds particularly around 187.5 Hz. In comparison to natural habitats which are dominated by a range of lower and higher frequency distributions dependent upon conditions, location (shallow vs. deep water), and presence of biological noise (Urick, 1983; Greene, 1995; Cato, 2006a). The strength of these ambient sounds will also vary according to the prevailing time, conditions and locations. While an aquaculture recirculating facility soundscape is not subjected to these same fluctuations or the same sounds. Wild caught fish may therefore have adverse responses to the aquaculture soundscape. The responses may vary depending on the habitat from where they were captured as they would be accustomed a very different sound environment.

The effect that aquaculture related sounds has on fish health and welfare is just beginning to be investigated (Bart *et al.*, 2001; Hastings and Popper, 2005; Wysocki *et al.*, 2007). It is currently known that extreme sounds can cause inner ear damage, and that these sounds have recently been found to elicit endocrine stress responses in a range of species (Smith *et al.*, 2004; Wysocki *et al.*,

2006). There is evidence that some aquaculture related sounds do not adversely affect rainbow trout (Wysocki *et al.*, 2007). Yet the effect of aquaculture related sounds have on the auditory system and non-auditory responses across a range fish species remains to be comprehensively evaluated.

Broadening the understanding of the types of soundscapes that are associated with different aquaculture facilities, such as an enclosed recirculating system, will allow further investigation into the evaluation of the potential influences of these soundscape environments. The linkage between sound and stress has the possibility to adversely impact on the production of existing species and introduction of new species into aquaculture production. Therefore, establishing a baseline of information, as this work contributes to, in relation to facility types, may assist in bringing species that are more sensitive into culture production.

Chapter 4. Evaluation of short term continuous sound on the physiological stress responses in Barramundi (*Lates calcarifer*)

4.1. Introduction

When a stressor is directed at a sensory perception (*ie*. sight, hearing, smell) it increases the chance for a stress response in vertebrates. This is because fish can respond to stressors at levels of exposure considerably lower than levels of stimuli that induce stress responses in terrestrial species (Wendelaar Bogna, 1997). With consideration of the heightened sensitivity, it is important to establish the outcomes of poorly understood stimulus, and one such as sound.

Within the current literature few species have been assessed for their responses to sound stress, and only one species (rainbow trout, *Oncorhynchus mykiss*) has been evaluated for effects from aquaculture sounds (Wysocki *et al.*, 2007b; Davidson *et al.*, 2008). Rainbow trout demonstrated no physiological stress to the tested aquaculture sounds. Wysocki *et al.* (2007b) suggests that because this species originates from a loud habitat would predispose them to louder sounds and allow a higher tolerance to relatively higher ambient sounds. However, hearing in fish is highly variable across species (Popper and Fay, 2010) as are stress responses (Barton, 2002). Yet, this is only one species out of many aquaculture important species.

The few articles in regards to aquaculture soundscapes, completed by Bart *et al.* (2001), Davidson *et al.* (2007), Craven *et al.* (2009) have provided a general range and understanding of the contributions and characteristics of various aquaculture facilities. In addition, it is important to consider that each facility will use different equipment, with associated varied acoustic properties, causing each to be acoustically unique. For instance in Wysocki *et al.* (2007b), the sounds used were quiet facility noise, modified to include tonal sounds (25, 29, 58 Hz), which created tank ambient sounds simulating a commercial scale recirculating soundscape, with the SPL peaking typically >100 Hz. In contrast, Craven *et al.* (2009) witnessed peak SPL at approximately 187 Hz, and Bart *et*

al.(2001) surveyed a low frequency peak between 25-250Hz and a high frequency peak between 630-2000 Hz for a range of tank arrangements. Every study provided a range of sound pressure levels (SPL) corresponding to the frequency ranges. This presents an endless combination of characteristics; nevertheless, what is common between the combinations is the presence of high SPL, low frequency components throughout various recirculating facilities.

To further understand the aquaculture soundscapes in relation to stress, the intention of this research was to determine if there is an initial physiological stress response, a threshold of response, and potential for adaptation in juvenile barramundi (*L. calcarifer*) from the soundscape characteristics based on Craven *et al.* (2009). The fish will be evaluated for stages of responses to the exposure of three levels of aquaculture facility sounds across a time course of exposures over a 24 hour period.

4.2. Materials and Methods

For this experiment, the methodology of the sound room and control room acoustics follow that described in Chapter 2. This experiment required that one sound level be played at a time, and for each of the three levels a control treatment was used. The sound levels chosen and equipment restrictions were established by previous work (Craven *et al.*, 2009). A ten minute looped recording of a recirculating facility was used as the sound recording. The sound level was altered for each of the three trials, Level 1 (L1) 124 dB re: 1 µPa ^{2}/Hz which corresponds to the highest level recorded in Craven *et al.* (2009), Level 2 (L2) 130 dB re: 1 µPa ^{2}/Hz, Level 3 (L3) 139 dB re: 1 µPa ^{2}/Hz, with all measurements taken at a frequency of 187.5 Hz, which corresponds to the maximum SPL. The recordings used applied the peak energy at 187.5 Hz and replicated the shape of the surrounding frequencies to closely match the recordings collected from Craven *et al*. (2009). All replied recordings were measured to guarantee the appropriate sound was projected. This frequency was chosen based on previous evaluations of a known occurring frequency and the likelihood that this frequency is within the detectable near-field and far-field perception of barramundi.

Barramindi (*L. calcarifer)* were chosen as the test species based on specific reasons. These fish were easily accessible from a local fingerling grow out facility. The fish were hatchery reared, from the same cohort and were grown out to a manageable size for blood sampling (~100-150g). Barramundi are also an important aquaculture species, especially with regard to the commercial popularity in the Asian-pacific region. It is not known what the species hearing thresholds are, however based on their phylogeny barramundi would fall within the 'hearing generalist' category.

4.2.1 Exposure period and sampling procedure

The duration of exposure was based on work completed by Smith *et al.* (2004), where they found an initial response to sounds at 10 minutes of sound exposure and continued the experiment for a 24 hour period. For this experiment exposure times were established at 10 min, 6 hours and 24 hours. The time intervals were established to determine if there were initial responses, prolonged responses and possible initial signs of adaptation.

Prior to the acclimation of the fish to the experimental tanks the sound was pre-set to establish sound levels. Once the tanks were set, the fish were relocated to the room where a period of acclimation took place prior to the start of exposure. The fish were acclimated for approximately 24-48 hours dependent upon the return of normal feeding behaviour.

Three groups of tanks were spread across the room with each group consisting of three tanks that corresponded to a specific sound time exposure (i.e. 10 min, 6 hours and 24 hours). This allowed a spread of the tanks across the room and one from each group per exposure period to be sampled. This single tank selection was also applied to the control tanks. Each tank in both the sound and control rooms held five fish, and after the exposure period, all fish within the particular tanks were sampled. The tanks were drained gradually to approximately one-third of the tanks capacity, where 2-phenoxyethanol (at 0.1% volume) was injected into the tank water via an external tube to

minimise visual stress. Once the fish reached level-five anaesthesia, distinguishable by loss of equilibrium and cessation of opercula movements, they were removed and sampled. Following measurement of length and weight the fish was placed on a foam bed with the operculum and eyes covered with a damp towel. Blood samples were then collected using heparinised syringes via the caudal vein. Once the syringe was filled with blood (~1ml) the fish was placed into a recovery tank. Samples were collected for haematocrit and placed on ice until all samples were completed. Once all samples for that period were collected, the blood samples were immediately centrifuged (5000rpm for 5 minutes). Plasma was collected and all samples placed in -80°C storage until samples could be further evaluated. The sampling procedure was replicated across each sound level for the same exposure durations.

4.2.2 Whole blood and plasma sample analysis

4.2.2.1. *pH and Haematocrit*

Whole blood was sampled for haematocrit using heparinised capillary tubes that were spun (3000rpm for 5 minutes) and measured immediately. Haematocrit calculation used was:

$$Heamatocrit = \frac{(length\ of\ Erythrocytes\ x\ 0.985)}{Total\ length\ of fluid}\ x\ 100$$

The pH was recorded using a Shindegen pH meter Mini, which was calibrated using their specific commercial provided calibration solution (7.0 pH). After meter was calibrated with calibration fluid, ~50ul of plasma was placed on the meter tip and the pH determined after approximately 3 seconds.

4.2.2.2. *Osmotic content of plasma*

Osmotic content was determined by the evaluation of Na^+ and K^+ ion concentrations and plasma osmolarity. The ion concentrations were determined using a flame photometer (Sherwood 410) where the plasma samples were diluted at a 1/400 for K^+ and 1/4000 for Na^+ and later corrected to 1/4000 dilutions for standardisation. The concentration was determined from the calculation based around the standard curves of incremental dilutions of standards K^+ 0.25mM/L and Na^+ 0.5 mM/L . The osmolarity was determined using an osmometer (Osmomat 030, Cryosopic osmometer,

Gonotec) with plasma samples diluted to 1/3 and determining Osmol/kg. Calibration solution used was commercial stock standard of 300 mosmol/kg.

4.2.2.2.1. Plasma glucose and cortisol determination

Glucose concentrations were determined using a Glucose Assay Kit by Caymen Chemical (USA) and plate reading spectrometer (Bio-tek microplate reader ELx800), the procedure used followed kit specifications. The absorption wavelength used was 515nm, which is within the suggested range of detection. The standards were plotted, and evaluation of the standard curve equations determined glucose concentrations per sample, the concentrations were calculated to correct for the weight of the fish (mg/dl/g).

Cortisol concentrations were determined using an EIA Cortisol Assay Kit (Cayman Chemical, USA), the methods used followed the specific kit instructions. Samples were diluted to a 1/10 dilution and were measured using an absorption wavelength of 405 nm (Bio-tek microplate reader ELx800). Plates were best when developed for the full 120 minute development period. Cortisol concentrations were calculated as described by kit and later corrected for fish weight, therefore all cortisol results are referred to as pg/ml/g.

4.2.3 Statistical analysis

All data was evaluated for statistical parametric assumptions (ie. normality, homogeneity and independence) and post-hoc tests were only applied when results were significant. Where parametric assumptions were not met related non parametric analysis was completed(ie. Mann-Whitney U). Controls of the same sampling period (i.e. 10 min, 6 hours and 24 hours) were pooled if no significant difference occurred between the days sampled, for instance if the control 10 minute exposure tanks did not significantly differ between runs of the experiment then samples for that 10 min exposure were pooled. Data pooling was used due to the restriction of the size of the experiment room limited the total number of fish sampled per day of exposure treatments. Where appropriate, the data was analysed SPSS 16 for Windows, and results were determined significant by

MANOVA, ANOVA tests, Confidence Intervals of 95% (CI) and all statistical significance was determined at $p<0.05$. All results are presented and later discussed within the following sections.

4.3. **Results**

Initially the analysis of pH and haematocrit values demonstrated no significance at Level 1, where Level 2 and Level 3 showed significance at 6 hours of exposure. There were also significant differences displayed at Level 2 and Level 3 at 24 hours of exposure Table 4.1). Data trends displayed an increase in pH from 10 minutes through to 24 hours, a trend suggesting a change in acid-base balance. Haematocrit values were only significant at Level 2 and the 6 hour exposure point where the control expressed larger haematocrit values (Table 4.1 4.1). The results for the osmotic content were not significant across levels, exposure period and or treatment and control (Table 4.1).

The glucose results displayed a different trend, with the highest means occurring with the controls, and significantly different concentrations between the control (6 hrs: 1.126 mg/dl/g, ±CI 0.237) and treatment occurring within the sixth hour of exposure and occurring at Level 1(0.494 mg/dl/g , ±CI 0.175), Level 2 (0.396 mg/dl/g, ±CI 0.241) and Level 3 (0.234 mg/dl/g, ±CI 0.067) as well as at t 24 hours of exposure in Level 3 (control 24hrs: 0.648 mg/dl/g, ±CI 0.189; Level 3 24hrs: 0.506 mg/dl/g, ±CI 0.209).

With the cortisol a clear trend was presented across Level 3 and corresponding exposures. The results demonstrated that among the 3 SPLs evaluated and the control at 10 minutes there were no significant differences (MANOVA; $p> 0.05$, $f=0.252$, $df=3$). Following into the sixth hour of exposure Level 3 significantly increased in cortisol concentration (L3 $\bar{x}$ = 1.025 ±CI 0.253) from the control and the other sound levels (MANOVA; $p<0.0001$, $f=10.571$, $df=3$, *post hoc* Tukey Level 3: from L1 $p<0.01$, L2 $p<0.0001$, control $p>0.001$). At the 24 hour exposure the same trend continued with a further increase in cortisol concentration (L3 $\bar{x}$ =1.173 ±CI 0.362) where the other levels and the control

stayed relatively constant compared to the 6 hour exposure results. The trend resulting is the gradual increase of cortisol over a 24 hour period (Figure 4.1).

Table 4.1. This table provides all the significance values for the labelled analysis results

		pH ANOVA *df= 1*	Haematocrit value ANOVA *df= 1*	Osmotic content MANOVA per level			Glucose mg/dl/g ANOVA *df= 1*
				Osmol/kg *df=2*	Na^- 0.5mM/L *df=2*	K^+ 0.25mM/L *df=2*	
Level 1 124 dB	10 minutes	*p*< 0.131 *f*= 2.433 $\bar{x}$ =7.99 CI ±0.0697	*p*< 0.103 *f*= 2.892 $\bar{x}$= 35.09 CI ± 3.261	*p*> 0.050 *f*= 1.080	*p*> 0.050 *f*= 1.079	*p*> 0.050 *f*= 3.064	*p*> 0.050 *f*= 0. 341 $\bar{x}$= 0.595 CI ± 0.202
	6 hours	*p*< 0.196 *f*= 1.763 $\bar{x}$ =8.06 CI ±0.072	*p*< 0.886 *f*= 0.021 $\bar{x}$= 37.12 CI ± 3.66	$\bar{x}$= 0.1727 CI ± 0.017	$\bar{x}$= 83203.46 CI ± 16769.44	$\bar{x}$= 228.19 CI ±32.23	*p*> 0.005* *f*= 14.649 $\bar{x}$= 0.496 CI ±0.175
	24 hours	*p*< 0.139 *f*= 2.333 $\bar{x}$= 7.85 CI ±0.055	*p*< 0.829 *f*= 0.019 $\bar{x}$= 33.53 CI ± 4.91	$\bar{x}$=0.149 CI ± .007 $\bar{x}$= 0.1739 CI ±.0197	$\bar{x}$= 61894.37 CI ± 17919.09 $\bar{x}$= 60321.174 CI ± 11820.71	$\bar{x}$=233.44 CI ±51.64 $\bar{x}$=225.79 CI ±22.76	*p*> 0.050 *f*= 0.072 $\bar{x}$= 0.691 CI ± 0.210
Level 2 130 dB	10 minutes	*p*< 0.517 *f*= 0.432 $\bar{x}$= 7.85 CI ±0.066	*p*< 0.856 *f*= 0.084 $\bar{x}$= 34.20 CI ± 2.82	*p*> 0.050 *f*= 1.435	*p*> 0.050 *f*= 0.759	*p*> 0.050 *f*= 0.273	*p*> 0.050 *f*= 0.070 $\bar{x}$= 0.554 CI ± 0.228
	6 hours	*p*> 0.019* *f*= 6.417 $\bar{x}$ =8.03 CI ± 0.043	*p*> 0.001* *f*= 16.096 $\bar{x}$= 38.23 CI ± 2.70	$\bar{x}$= 0.151 CI ± 0.012 $\bar{x}$= 0.142 CI ± 0.016	$\bar{x}$= 57417.59 CI ± 13521.88 $\bar{x}$= 39327.617 CI ± 12584.88	$\bar{x}$= 212.99 CI ± 20.97 $\bar{x}$= 204.722 CI ±27.57	*p*> 0.001* *f*= 24.829 $\bar{x}$= 0.395 CI ± 0.241
	24 hours	*p*< 0.700 *f*= 0.153 $\bar{x}$=8.05 CI ±0.047	*p*< 0.973 *f*= 0.006 $\bar{x}$= 26.38 CI ± 3.24	$\bar{x}$= 0.156 CI ± 0.014	$\bar{x}$= 64065.45 CI ± 16007.53	$\bar{x}$= 228.02 CI ± 30.42	*p*> 0.050 *f*= 1.407 $\bar{x}$=0.854 CI ± 0.139
Level 3 139 dB	10 minutes	*p*< 0.295 *f*= 1.148 $\bar{x}$ =7.83 CI ±0.0641	*p*< 0.932 *f*= 0.007 $\bar{x}$= 39.82 CI ± 2.57	*p*> 0.050 *f*= 1.251	*p*> 0.050 *f*= 0.666	*p*> 0.050 *f*= 0.725	*p*> 0.050 *f*= 1.372 $\bar{x}$= 0.3943 CI ± 0.107
	6 hours	*p*> 0.013* *f*= 7.258 $\bar{x}$=8.042 CI ± 0.051	*p*< 0.202 *f*= 1.721 $\bar{x}$= 32.58 CI ± 1.66	$\bar{x}$= 0.213 CI ± 0.0137 $\bar{x}$= 0.159 CI ± 0.0141	$\bar{x}$= 58348.68 CI ± 9772.63 $\bar{x}$= 61377.99 CI ± 15859.21	$\bar{x}$= 225.99 CI ± 17.15 $\bar{x}$= 205.70 CI ± 41.83	*p*> 0.006* *f*= 12.559 $\bar{x}$= 0.234 CI ± 0.067
	24 hours	*p*< 0.773 *f*= 0.085 $\bar{x}$ =8.05 CI ± 0.050	*p*< 0.440 *f*= 0.615 $\bar{x}$=33.21 CI ±4.60	$\bar{x}$= 0.147 CI ± 0.007	$\bar{x}$= 43811.725 CI ± 11072.83	$\bar{x}$= 190.18 CI ± 24.77	*p*> 0.005* *f*= 13.412 $\bar{x}$= 0.509 CI ±0.209
Control	10 minutes	$\bar{x}$= 7.87 CI ± 0.167	$\bar{x}$= 37.46 CI ± 5.71	$\bar{x}$= 0.185 CI ± 0.012	$\bar{x}$=61621.34 CI ± 5866.77	$\bar{x}$= 265.79 CI ±57.135	$\bar{x}$= 0.513 CI ± 0.162
	6 hours	$\bar{x}$= 7.95 CI±0 .0514	$\bar{x}$= 37.63 CI ± 3.99	$\bar{x}$= 0.147 CI ± .0055	$\bar{x}$= 56887.93 CI ± 13399.56	$\bar{x}$= 201.885 CI ±30.05	$\bar{x}$= 1.126 CI ± 0.237
	24 hours	$\bar{x}$= 8.03 CI ±0.099	$\bar{x}$= 33.09 CI ± 2.33	$\bar{x}$= 0.213 CI ± 0.0973	$\bar{x}$= 59003.09 CI ± 18939.05	$\bar{x}$= 256.22 CI ± 74.88	$\bar{x}$= 0.648 CI ± 0.189

* Levels of significance

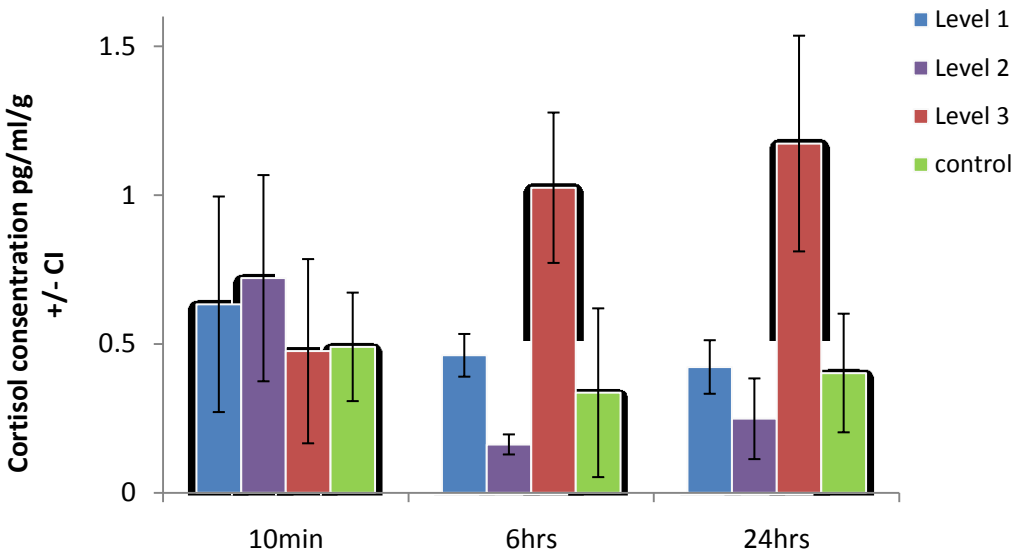


Figure 4.1. This depicts the trend observed of the Level 3 cortisol concentrations. Level 3 displays the only level with a significant increase of cortisol from the control at 6 hours and at 24 hours.

4.4. Discussion

The cortisol results showed an effect in the stress responses in juvenile barramundi, with an increase of concentration across the 24-hour period of exposure. The result varies from what would be more expected, which would be a greater response at 10 minutes than at 24 hours. This would be more similar to Smith *et al*. (2004), where goldfish showed initial responses in the first 10 minutes thereafter there was no stress response detected.

Throughout the other results, trends were inconsistent among the various indices of stress. The effect of inconsistencies is mainly due to the high variability between individual fish, as well as the varied results of the controls. Similar to the sound treatment fish, the control fish were exposed to uncontrolled external sounds, although a quieter environment may have heightened the fish's awareness to changes in the in the room environment. Therefore the inclusion of sound (stronger background sounds) within an aquaculture facility may mask sounds that occur at fluctuating levels

throughout the day, and thus, benefit the fish by reducing the level of the response to changes in the background soundscape.

One factor that may have affected the fish's responses to these sounds is that these fish are hatchery reared; therefore, they developed from eggs to larvae through growout in an aquaculture setting. Development within this type of soundscape may have allowed the fish to adapt previously to a range of sounds, which may reduce the strength of response that may have otherwise been present. An example of a culture practice that has an effect on fish in development is the use of cryopreservation of fertilised eggs that has displayed effects on the weight and length of steelhead fish milt (Hayes *et al.*, 2005). The process of cryopreservation as well as other various culture practices could also interfere with the developmental processes of the sensory system.

The barramundi juveniles used in this experiment were hatchery reared, transported from the hatchery facility to the fingerling growout location and subjected to variations in temperature. The events of their rearing history could affect have their development (Wysocki *et al.*, 2007b). The development within the hatchery environment with the addition of transportation stress, may have potentially have Increased stress threshold, disabled the hearing sense even increased hearing thresholds, and hence their varied stress response. In agreement with Wysocki *et al.* (2007b), further investigation into this effect is required.

The conclusions of this experiment are the presence of a stress response within the initial and secondary stages of stress, and the increase of this response over time suggests that the stress in the fish is increasing. However, as the other stress analyses resulted in a range of inconsistencies, further testing should be carried out. It would also be beneficial to continue these exposures over a longer period and determine if the trend of increasing cortisol carries over into the tertiary levels of response. Also this experiment would benefit from a larger sample pool which may correct for the

range of responses and together with the change in the duration would further determine the effects of these sound environments on fish.

Chapter 5. Identifying if aquaculture sounds have an effect on the growth of Barramundi

An integral part of aquaculture species development and production improvement is associated with maximising the potential for growth. Producing hardy, well developed, fast growing fish ensures a faster turnover of product and maximises plate size within minimal time. These factors are important in maintaining a cost effective and economical industry, and therefore it is important to create the ideal environment for fish to grow in.

The creation of ideal conditions through the elimination of negative stress causes is an important factor to support growth. In some cases the addition of a stimulus may reduce stress levels under specific conditions, such as extended light periods (Papoutsoglou *et al.*, 2007). However, this has to be done carefully, as over stimulation may have the opposite outcome, reducing feeding and causing developmental issues even reduction in disease resistance (Barton, 2002). Due to the normally intensive culture setup in aquaculture, a small change can impact the whole stock, therefore identifying potential stressors is beneficial for fish culture productions.

Many intensive culture systems are made possible by being designed on recirculating systems, as they allow for greater control of culture conditions (*ie.* water temperature, water quality, photoperiod, nutrition). A factor often overlooked within recirculating facilities is the unique sounds generated per facility, which are controllable through system layout, setup, and the types of equipment used (Davidson *et al.*, 2007; Craven *et al.*, 2009). Known related facility soundscapes cover a range of frequencies, but predominantly the dominant sound levels are due to frequencies typically below ~200Hz (Bart *et al.*, 2001; Craven *et al.*, 2009).

Limited studies have been completed on the effects of these soundscapes on the growth and development of fish. The studies available focused on the same species (rainbow trout) under similar

sound conditions (Wysocki *et al.*, 2007b; Davidson *et al.*, 2008). The evaluation of one species is not enough as stress responses and hearing are species specific, fish culture conditions are also species specific.

This report will evaluate the effects of three sound levels on the growth of juvenile barramundi for growth trends and changes. This will be achieved by the application of baseline information and recordings provided from the work completed by Craven *et al.* (2009), which is shown in Chapter 3, as well as applying the same sound conditions used in Chapter 4.

5.1. Methods and materials

The experiment took place within the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University. Details regarding room lay out and acoustic set up are presented in Chapter 2, as well as the acoustic baseline information provide in Chapter 3.

5.2. Sound exposure and sampling techniques

Three sound groups were run simultaneously, with each group set at a designated sound level, 124, 130, 139 dB re 1µPa ^{2}/Hz, with all measurements taken at a frequency of 187.5 Hz, this corresponds to the maximum SPL. These sound levels were verified through sampling prior to the fish entering the room. Fish weights and lengths were measured 48 hours prior to the beginning of sound treatments to establish initial values, and to ensure correct lengths sizes in each tank. Specifically each group of tanks had a tank with fish a small size (16.5-18cm), medium size (18-19.5 cm), and large size (19.5-22cm). The aim of this was to allow the fish to grow at similar rates within each tank, because barramundi express dominance that often results in cannibalism (Parazo *et al.*, 1991) with the largest fish inhibiting the growth and development of the other fish present. Therefore starting the experiment with fish within the same size group would reduce a proportion of this effect as grading was not an option during the duration of the experiment.

For this experiment the evaluation of the effect on the fish was viewed from a tank level perspective, therefore for the feeding regime, the food consumed refers to total tank consumption. Measurement of this was achieved through the following steps. Initially a 500ml beaker was filled with dry pellet feed, Riddley Aqua feed Barramundi feed floating pellet, size 4-6mm, and the total weight of the beaker and pellet was measured. The fish were then fed to satiation once a day, where uneaten pellets were collected, dry pellets were replaced back in the beaker, and the beaker reweighed for the total pellets eaten. The total feed consumed for that day was calculated by subtracting the beaker weight prior to feeding from the post feeding beaker weight.

All fish were sampled every 14 days (sampling scheme; initial- day 0; S1- day 14; S2- day 28 mid sample; S3 - day 46, Final - day 56). To reduce handling stress the fish were lightly anaesthetised. The tanks were drained slowly to approximately 1/3 of the tanks' capacity, where 2-phenoxyethonal (at 0.1% volume) was injected into the tank water via an external tube to minimise visual stress. Once the fish reached level-five anaesthesia, distinguishable by loss of equilibrium and cessation of opercula movements, they were ready to be sampled. This technique was effective as it allowed the fish to be sampled quickly with fast recovery (within ~1 minute of placement in recovery tank). Once all fish were removed and sampled from a tank, any required maintenance occurred prior returning the fish to the tank as to not further disrupt the fish during the experiment.

The RNA/DNA sample collection occurred during three of the five sample periods. An initial sub sample of 36 fish of the cohort was collected just after the other fish were relocated to the treatment tanks. The other two samples occurred at the mid sample (4 weeks) and at the final sample (8 weeks) again with 36 fish sample per sample period. During the weight and length sampling, at random 3 fish from each tank were fully euthanized after general anaesthetic in a high concentration of 2-phenoxyethonal (+1.1ml/l based on Velisek *et al.* (2007),. White muscle tissue

was collected immediately from the posterior end of trunk, beginning of caudal region, and weighed. The tissue sample was then sealed in a cryo tube and snap frozen in liquid nitrogen. Once final samples were collected all samples were moved into -80 freezer for storage and analysed within 6 months.

5.2.1 RNA/DNA analysis

The analysis of the white muscle tissue was adapted from combination of (Humphrey *et al.*, 2007; McGinty *et al.*, 2008). Frozen tissue was shaved removing 50-100mg, tissue was finely sliced and put into 1ml of TE buffer (Tris-EDTA with 1% sarcosyl). Sample in buffer was then homogenised for 60 seconds and placed on ice, before being probe sonicated at 50% amplitude for 60 seconds. The samples were then centrifuged for 8 minutes at 1200g and chilled to 4 °C, after which aliquots of supernatant was removed and snap frozen in liquid nitrogen until all samples had been prepped. For the RNA DNA quantification (Quant-it Kits by invitrogen, RNA broad range assays and DNA broad range both 20-1000 ng) standards were prepped by serial diluting a 100ng concentration solution of both RNA and DNA standard. The remainder of processes followed kit specified methods however all samples were diluted to a 1/100 dilution with TE buffer. The samples were measured for fluorescence using methods and equipment specified in McGinty *et al*. (2008). This dilution was the most effective among the trial standards and samples with the total concentration of sarcosyl 0.0384%. RNA and DNA concentrations were calculated based on kit recommendation and correcting for the dilution factor. Ratio was calculated by dividing the RNA value by the corresponding DNA value, which gives the total RNA per DNA.

5.2.2 Growth analysis

Weight (g) and length (total length in cm) data was complied and compared. Data of individual fish weight and length was then grouped per tank, as well as the pellet weight consumed per day, and compiled into weekly totals. The growth indices used to determine variations in growth among the three sound levels are:

1. Specific growth rate (SGR) calculated the percentage of growth grown per day or set time frame. Since the fish were sampled every 14 days the time frame is 14 days. For the purposes of this experiment, total weight includes the combined weight of the tank opposed to individual fish. The SGR is calculated by knowing total wet weight gained subtracted by the total weight started divided by the period. For this equation Bw_f is body weight final and Bw_i refers to body weight initial.

$$\%\ growth\ per\ day(s) = \left[\frac{(lnBw_f - lnBw_i)}{(number\ of\ days)}\right] \times 100$$

2. Condition factor (Barlow *et al.*, 1995) (K) is a calculation of weight (g) to length (cm) ratio per sampling point. This calculation is calculated per individual as it is an instantaneous measure but the data is then pooled in relation to tank, where W is the weight and l is length.

$$K = \left(\frac{W}{l^3}\right) \times 100$$

3. To determine the rate of food consumed in relation to the total weight gained, food conversion rates were calculated (FCR) every sampling period. Where W_f is equal to the weight final and W_i is equal to the initial weight total together, $W_f - W_i$ is equal to the total weight gained over the period of interest.

$$FCR = \frac{Dry\ food\ consumed\ (g)}{W_f - W_i}$$

5.2.3 Statistical analysis

For all analysis methods, the results were tested for statistical normality and homogeneity. Growth results were evaluated by methods of repeated measures, where a group or sample is repeatedly measured across a period of interest, measures were compared by applying a MANOVA (Quinn &

Keough, 2002) tests and any significance found was established by post hoc (Tukey's test) or further comparison tests were completed. The fixed factors were the periods of sampling (Initial, sample 1, sample2, sample 3, Final) and the sound levels tested (Level 1,2,3 and control)where each level the tanks were pooled to increase the strength of the tests as the number of fish with in the tanks was limited. MANOVA test were applied as of the multiple categories within the fixed factors. The MANOVA allows for a more time efficient test, which is actually many ANOVAs that are run against each of the designated fixed factors. All data was evaluated in Excel (Microsoft Windows 2007) and SPSS 16.0 (for Windows) and where appropriate, Tukey HSD post hoc evaluation was used, Confidence intervals (CI, 95%) were applied and all significance was determined at $p< 0.05$.

5.3. Results

5.3.1 Growth analysis

The results for the condition factor generally showed Level 3 consistently demonstrating the lowest K value compared to the other levels and the control. Level 3 trends away from the other groups at the second to last sample of the experiment duration, however following the drop in condition, at the last sampling point the condition level appears to recover in line with the other treatment groups where there is no significance across treatments (

Table 5.1).

Table 5.1. This table provides all the significance information for the MANOVA test completed for the condition factor analysis.

Sampling period	Significance ANVOA *df=3*	Specific significance Tukey's post hoc test	Mean +/- SD
Initial	P=0.0001, *f=7.328*	Form L3 L2 p>0.007 control p>0.0001	L1=1.256 ± 0.0654 L2=1.286 ± 0.0882 L3=1.206 ± 0.0793 C =1.303 ± 0.1005
S1	P<0.01, *f=3.546*	From L3 L3 p>0.021	L1=1.287 ± 0.0589 L2=1.316 ± 0.0653 L3=1.254 ± 0.0738 C =1.312 ± 0.0541
S2	P=0.01, *f=3.898*	From control L3 p>0.005	L1=1.212 ± 0.1723 L2=1.225 ± 0.0769 L3=1.180 ± 0.0635 C =1.268 ± 0.09004
S3	P=0.0001, *f=8.374*	From L3 L1 p>0.022 L2 p>0.001 control p>0.0001	L1=1.256 ± 0.0540 L2=1.278 ± 0.1223 L3=1.189 ± 0.0774 C =1.296 ± 0.05702
Final	p>0.05, *f=1.345*	None	L1=1.247 ± 0.0942 L2=1.262 ± 0.0775 L3=1.260 ± 0.0601 C =1.286 ± 0.0758

A similar trend for Level 3 is reflected within the SGR and FCR. For the SGR within the sampling periods of S2 and S3, Level 3 again shows a greater decline (mean FCR ±CI from 2.102 ± 0.380 to 1.509 ± 0.310 and then up to) in growth rate for that period. It then recovers for the following time period, with an increase in growth between the S3 and final sample periods (increase to mean FCR ±CI 1.729 ±0.507) (Figure 5.1). Despite this change, across treatment level and sampling period there was no significant differences (MANOVA; df=9, p>0.05, f=1.061). The results of the FCR displays at first an increase between S2-S3 and a large decrease in FRC for the final sampling periods, which suggests that the fish are not converting the total feed eaten to body mass. The fish were also inconsistent across each sampling periods, providing no significant differences (MANOVA; df=9, p>0.220, f=1.326). The other levels 1, 2 and the control maintain a more consistent rate of food conversion across the experiment duration (Figure 5.2). Comparing all growth indices, fluctuations

appear to occur at Level 3 only, while Level 1 and 2 are relatively consistent with the control with no significant differences.

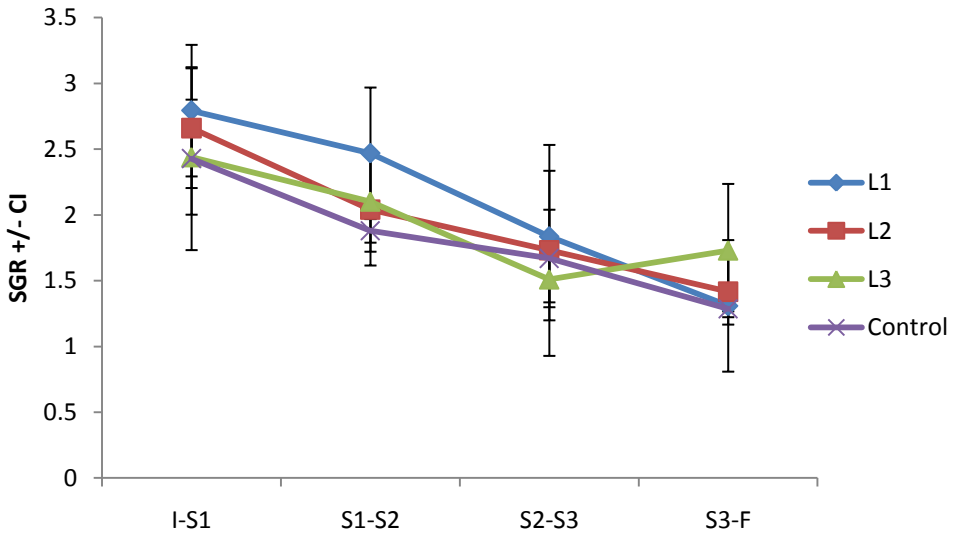


Figure 5.1. SGR for barramundi overall displayed a decline in the rate of growth over the duration of the experiment. For Level 3 for the last period between S3-F the growth rate increases where as the other treatments remain within the same slope/rate of growth for that period.

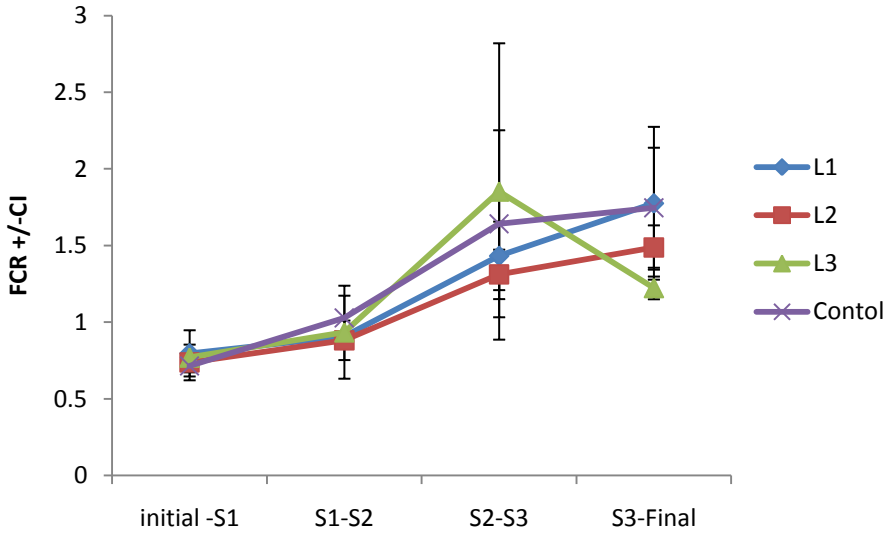


Figure 5.2. The FCR across the full duration of this experiment L1,L2 and the control are consistent across the sampling periods where L3 displays high variation from the other treatments after the S1-S2 sampling period.

5.3.2 RNA DNA ratios

The results of the RNA/DNA analysis show no significant differences (MANOVA; p<0.481, *f*=0.832, df=3) across treatments or controls. Although there is no significant data there are some minor

trends displayed. The control is quite consistent across all sampling period, along with Level 2 that only displays a small incline of RNA to DNA. Level 1 displays a decrease from the initial sample to the mid sample but the ratio increases more than any other level. Evaluating Level 3 it seems to be the inverse result of what was displayed in Level 1. Level 3 increases in RNA from the initial sample but then considerably declines from the mid sample to the final sample (Figure 5.3).

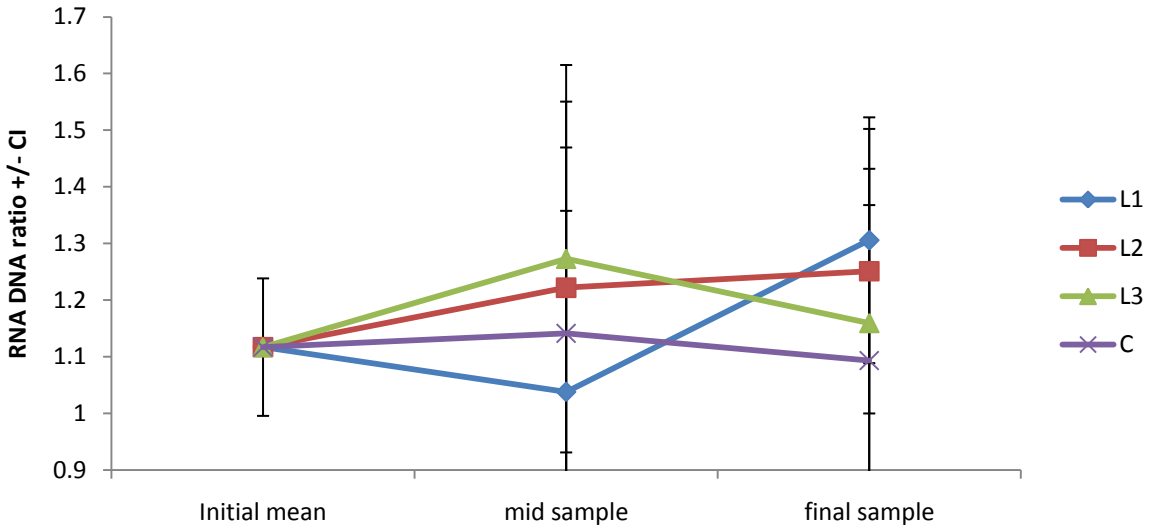


Figure 5.3. The RNA DNA results depict a varied range of RNA production for L1 and L3 where L1 increases in RNA production more than any other treatment and L3 expresses the inverse of L1. There is also a high variability across individuals, illustrated by the confidence interval (± CI), explaining the lack of significant differences among the treatments.

5.4. Discussion

Evaluation of the results across the growth indices and the RNA DNA ratio allows only trends to be suggested. The results and their analysis do not show a definable effect from the exposure of the sounds in this experiment. The responses of the fish under the Level 3 treatment displayed the highest range of variability across the duration of the experiment then the other treatment levels. There are some trends among the results for Level 1 and Level 2, which showed some signs of increased growth, which could possibly suggest that some level of sound may provide advantageous stimulation. This experiment may have benefited from continuing; however, this was not possible within the time frame of the study.

Based on the range of variability, particularly that expressed in Level 3, the information provided within this work is consistent with that of Wysocki *et al.* (2007b), Davidson *et al*.(2007; 2009), who found no effect on the growth of rainbow trout. Again, it is possible that hatchery reared animals have been exposed to various conditions that may have caused developmental sensory effects which would influence a fish's level of response. Both rainbow trout and barramundi evaluated for effects to soundscapes are of domesticated lines. It is possible that their domestication influences their susceptibility to being stressed. This implies, that not only are there possible effects of developmental changes due to various culture conditions during early life stages (Hayes *et al.*, 2005), but that it is also possible that these fish may have been bred for resistance and maximum potential within a cultured environment, the process of which may have indirectly adjusted their inherited sensory perception and stress response.

With consideration that selective breeding is currently both the focus of aquaculture research, and is being incorporated in regular culture practices, the idea of effects form domestication seems plausible. It is important to remember that during the selective breeding process some characteristics can be unexpectedly bred out or enhanced, such as disease resistance (Tave, 2003). It is possible that fish selected for the highest rate of growth and disease resistance may inconsequently also display the highest tolerances to sound stress. There are also developments for breeding programs that have focused on reducing the heritability of stress responses (Pottinger and Carrick, 1999), so specifically breeding a less stress responsive line of rainbow trout, where these fish express a moderate to high heritability of cortisol response (Fevolden *et al.*, 1999).

In combination with possible developmental and domestication influences, these sounds may not be perceived as severe enough to induce a stress response. This means that sound would possibly need to be at much higher intensities for a consistent response to occur. The levels used in this work, and

their dominant frequency, would not be a concern for a facility operating with similar soundscape conditions with a similar species.

Chapter 6. Stress Adaptation: Can fish adapt to random and predicted sounds

6.1. Introduction

In the world of acoustics there are two main contributions to a soundscape. The first, noise, an example of which is ambient noise, consists of various combinations of continuous and transient sound sources, which for the ocean can include wind, rain, and wave action, these are all sounds with no single point source (Greene, 1995). The second contribution is from signals, acoustic events that stand out from the background noise (Fay *et al.*, 1978) and have a point source. Examples of signals can include a single tones or impulsive events, such as marine mammal vocalisations.

Continuous sounds have been identified as possible causes of stress in many animals, and in more recent times, fish (Hastings and Popper, 2005). However it has been proposed that transient sounds might be a more prominent source of stress (Davidson *et al.*, 2009; Popper and Hastings, 2009). A fundamental aspect of aquaculture is that fish are restricted and completely surrounded by the facility soundscape, which presents unique a situation. It can be asked, how will a fish perceive sudden changes within an environment that is filled with continuous sounds, but not of natural origin or of biological significance? In the aquaculture environment, these sounds could include those associated with construction and maintenance activities, such as loud banging from hammers to impacts on tank walls.

This raises the question, that after a period of exposure, do fish adapt to the occurrences of the transient sounds and selectively disregard the sound by no longer responding to it? Fish are known to use adaptive filtering in the lateral line system (Webb *et al.*, 2008), specifically that the fish are able to cancel hydrodynamic stimuli associated with its own movements (Montgomery and Bodznick, 1994). It is also considered probable that fish, similar to most vertebrates, are able to differentiate between conspecific, heterospecific and background signals, also known as auditory

segregation (Bergman, 1990), to differentiate between important and non-important biological information within a natural habitat. There are also possibilities of auditory masking, where signals maybe masked or cancelled-out by another signal (Fay *et al.*, 1978). Therefore if fish respond to changes in the environment, at what point does a fish successfully apply sensory adaptations to an exposure regime?

The purpose of this work is to examine the physiological stress responses to periods of exposure to random and constant interval sounds over a period of 24 hours to establish initial responses through to possible occurrences of adaptation.

6.2. Materials and methods

For the evaluation of transient sounds the rooms are the same as described in Chapter 2. For this experiment sounds of impacts on tanks were previously collected and described in Chapter 3.

6.2.1 Sounds, exposure periods and sampling procedure

The sounds used for the response determination were comprised of two components. The first is the background sound, formed from a recording of an aquaculture facility soundscape, with a peak SPL at 187Hz of 124 dB re: 1 $\mu Pa^2/Hz$, coinciding with the lowest level from the continuous sound experiment (Chapter 4). The background noise was added to create a realistic environment within the room, and its addition standardises the background noise that all tanks are exposed to, removing any possible effect due to small room location variations. The second part of the sound of exposure was the overlaid impact sounds.

There are two impact sounds, the first was representative of facility personnel impacting the tank wall (low frequency impact at 200 Hz), and the second representative of a tool knocking a tank wall (higher frequency pulse with 2 peaks at 1500 and 3609Hz). The sounds chosen were selected based

upon the likelihood of occurrence within a functioning facility. The sounds were set to occur at 2 interval types, one random and the other constant (Figure 6.1)).

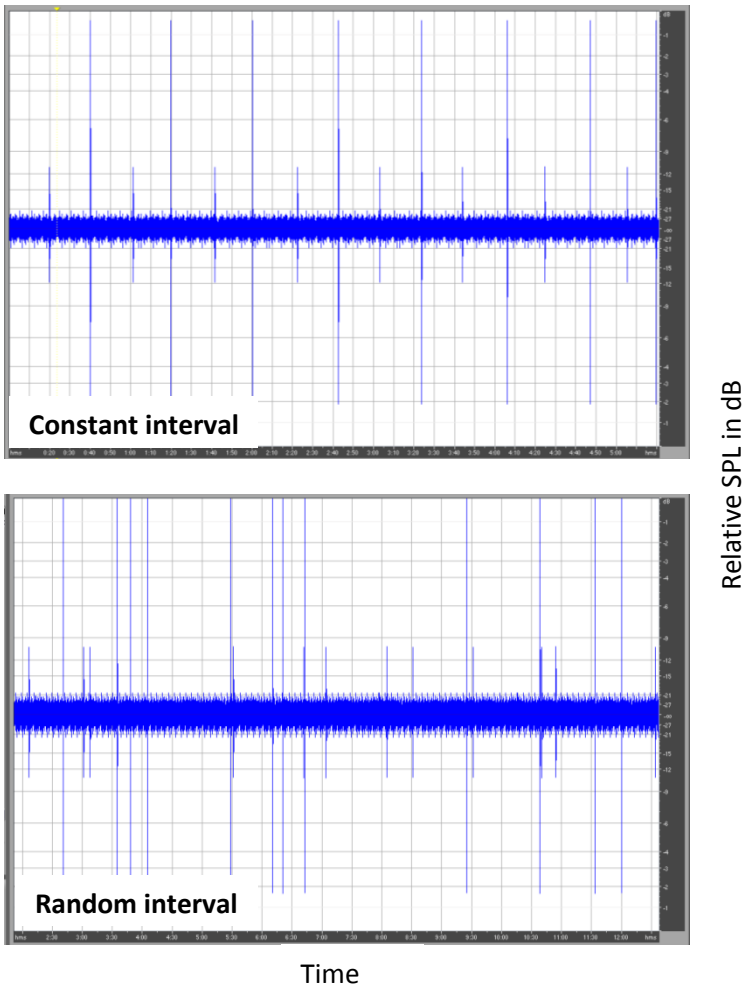


Figure 6.1. This figure depicts samples of the two interval types used. The top one is the constant interval sound with events evenly distributed between the two types of sounds used. The bottom is the random interval recording, in which the two sound events are irregularly inter-dispersed across the recording sample

The random sounds were chosen based upon the time interval of the recording (60min), allowing for 120 instances within that time frame. A random number table based upon the possible occurrence time in minutes and seconds was generated, and the location of the two impact sounds was taken from alternating numbers in the table. The 60 minute recording was then repeated for longer exposures. The constant interval sounds were set at an occurrence interval of 20 seconds across a 10 minute recording that was looped and the two impact sounds were incorporated into the recording in an alternating fashion.

The exposure periods were set at 10 minutes, 6 hours and 24 hours. The periods were chosen as mentioned previously, with 10 minutes looking for the initial response, 6 hours to evaluate stress level change, and 24 hours to determine if the fish were capable of stabilising their internal system and responses allowing possible adaption to occur.

For each exposure (10 minutes, 6hours, 24 hours) random and constant interval treatment experiments the time intervals were run separate. This eliminated the potential problem of the impact sounds over lapping and complicating the predetermined sound recordings. Therefore, only one group was used at a time. Each of the 3 tanks was filled with 4 fish, a total of 12 fish for treatment exposure and 12 fish in total for the control group. Once the period of exposure finished all fish from the tanks were sampled.

The tanks were drained gradually to approximately one-third of the tanks capacity, where 2-phenoxyethonal (at 0.1% volume) was injected into the tank water via an external tube to minimise visual stress. Once the fish reached level-five anaesthesia, distinguishable by loss of equilibrium and cessation of opercula movements, they were removed and sampled. Length and weight was collected, then each fish was placed on a foam bed with the operculum and eyes covered with a damp towel. Blood samples were then collected using heparinised syringes via the caudal vein. Once the syringe was filled with blood (~1ml), the fish was placed into a recovery tank. Samples were collected for haematocrit and placed on ice until all samples of that exposure were completed. Once all samples were collected, the blood samples were immediately centrifuged (5000rpm for 5 minutes). Plasma was collected and all samples were placed in -80°C storage until samples could be further evaluated. The sampling procedure was replicated across each sound level for the same exposure durations.

6.3. Whole blood and plasma sample analysis

6.3.1 pH and Haematocrit

Whole blood was sampled for haematocrit using heparinised capillary tubes that were spun (3000rpm for 5 minutes) and measured immediately. Haematocrit calculation used was:

$$Heamatocrit = \frac{(length\ of\ Erythrocytes\ x\ 0.985)}{Total\ length\ of fluid}\ x\ 100$$

The pH was recorded using a (Shindegen pH meter Mini) while plasma was defrosted for analysis separation. After the meter was calibrated with the provided commercial calibration fluid (pH 7.0), approximately 50ul of plasma was placed on meter tip and after a few seconds, the pH value was recorded.

6.3.2 Osmotic content of plasma

Osmotic content was determined by the evaluation of Na^- , K^+ ion concentrations and plasma osmolarity. The ion concentrations were determined using a flame photometer (Sherwood 410) where the plasma samples were diluted at a 1/400 for K^+ and 1/4000 for Na^- and later corrected to 1/4000 dilutions for standardisation. The concentration was determined from the calculation based around the standard curves of incremental dilutions of standards K^+ 0.25mM/L and Na^- 0.5 mM/L . The osmolarity was determined using an osmometer (Osmomat 030, Cryosopic osmometer, Gonotec) with plasma samples diluted to 1/3 and determining Osmol/kg.

6.3.3 Plasma glucose and cortisol determination

Glucose concentrations were determined using a Glucose Assay Kit by Caymen Chemical (USA) and plate reading spectrometer (Bio-tek microplate reader ELx800), and the procedure used followed kit specifications. The absorption wavelength used was 515nm, which is within the suggested range of detection. The standards were plotted, and evaluation of the standard curve equations used to determine glucose concentrations per sample, the concentrations were calculated to correct for the weight of the fish (mg/dl/g).

Cortisol concentrations were determined using an EIA Cortisol Assay Kit (Cayman Chemical, USA), and the methods used followed the specific kit instructions. Samples were diluted to a 1/10 dilution and were measured using light of absorption wavelength 405 nm (Bio-tek microplate reader ELx800). Plates were best when developed for the full 120 minute development period. Cortisol concentrations were calculated as described by kit and later corrected for fish weight, therefore all cortisol results are referred to as pg/ml/g.

Results are compared between the time exposure of the treatments and the controls for corresponding time exposures. This method was chosen because the interactions of interest are the variations between the exposure and control. The exposures and associated control were carried out simultaneously. The different exposures were not run at the same time as well as small variations within the cortisol analysis created additional variations among individual exposures. Visual comparisons are made of the data presented but not significantly tested due to the reasons stated.

6.4. Statistical analysis

All data were evaluated for the basic assumptions of parametric characteristics, which include testes for normality and homogeneity. Where appropriate results were determined significant by ANOVA (one way) and MANOVA (multivariate). All statistical significance was determined at $p<0.05$ and using SPSS version 16 (for Windows). Results were graphed and provided with confidence intervals (CI of 95%) and mean values. All results are presented and later discussed within the following sections.

6.5. Results

6.5.1 Random interval

The results for the random interval data present no effect on the stress responses of the barramundi to this interval sound (Figure 6.2**Error! Reference source not found.**). There was no significant

differences between the cortisol concentrations between treatment and the control at 10 minutes and 24 hours (ANOVA *df=1*, p>0.050, *f=3.260*; p>0.050, *f=3.742*). However, at 6 hours there is a significant difference (ANOVA: *df=1*, p<0.05, *f=4.857*) where the control expresses a higher concentration of cortisol then the treatment (mean (pg/ml/g) ±CI; 6hrs=0.00849±0.00224, C=0.0147±0.00552). The glucose results displayed no significant difference across exposures from the controls along with a range of varied results among the other stress indices (Table6.1).

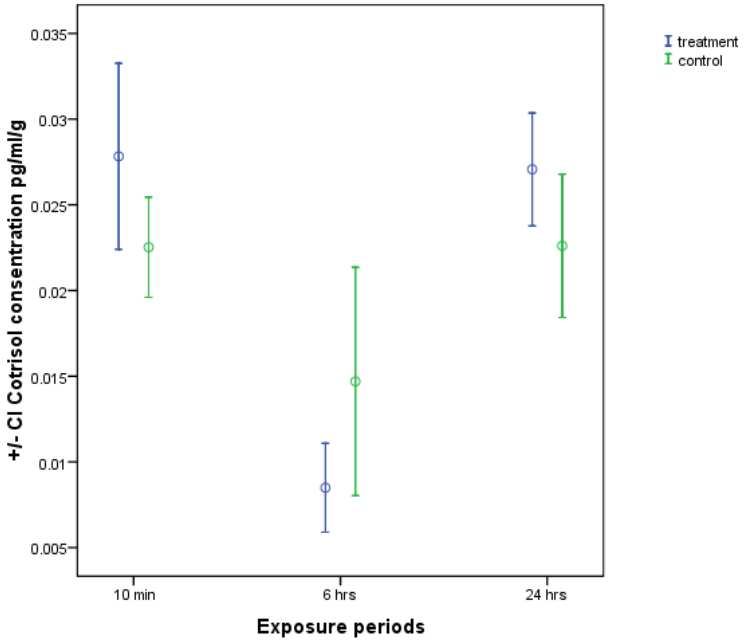


Figure 6.2. At 10 min and 24 hrs of exposure fish displayed the highest concentrations of cortisol, but as the control also displays higher levels of cortisol, the results are not significant. At 6hrs the cortisol concentrations decrease in the treatment and the control, however from the similarity of concentrations of the control across each sampling period, no effect is determined.

Table 6.1. Information is provided for results for the specific stress indices. Significance values are provided first, followed by the mean of each treatment at exposure in relation to the control mean, which is reported in reference to the exposure period.

Random		pH ±SE	Haematocrit value ±SE	*Osmotic content ANOVA* Osmol/kg ±SE *df=1*	Na^- 0.5mM/L±SE *df=1*	K^+ 0.25mM/L±SE *df=1*
10 min	Sig	p>0.016 *f=6.76**	p>0.05 *f=3.694*	p>0.05 *f=4.488*	p>0.05 *f=2.343*	p<0.01 *f=9.531**
	Mean ±SE	7.808±0.0259	43.531±2.887	0.219±0.134	744.47±23.329	19.734±1.817
	Control Mean ±SE	7.7±0.032567	35.986±2.658	0.191±0.173	866.957±16.473	17.173±1.218
6 hours	Sig	p>0.05 *f=0.01*	p>0.05 *f=2.041*	p>0.05 *f=1.115*	p>0.05 *f=0.0844*	p>0.05 *=0.764*
	Mean ±SE	7.736±0.0243	38.510±1.078	0.214±0.174	1119.308±16.864	18.227±1.429
	Control Mean ±SE	7.784±0.0188	36.624±0.789	0.220±0.178	946.495±16.089	19.192±1.425
24 hours	Sig	p>0.05 *f=0.051*	p<0.05 *f=6.774**	p>0.05 *f=1.478*	p>0.05 *f=0.923*	p>0.05 *f=1.992*
	Mean ±SE	7.85±0.0261	34.776±1.129	0.211±0.133	1169.2±22.903	18.415±1.831
	Control Mean ±SE	7.858±0.0259	39.364±1.369	0.197±0.136	992.626±23.269	16.784±1.824

*Significant difference present

6.5.2 Constant interval

The constant or predicted interval sounds displayed an initial response at 10 minutes, and the response declines over the 24 hour period, a trend strongly represented in the cortisol results (Figure 6.2). At 10 minutes exposure the treatment is significantly different from the control (ANOVA; *df=1*, p<0.05, *f=5.042*) and displays the highest concentration of cortisol among the 3 exposures (mean (pg/ml/g) ±CI; 10 min 0.0427±0.0183, 6 hours 0.0187±0.00626, 24 hours 0.0170±0.00353). After 6 hours of exposure there is still a significant difference between the treatment and the control (ANOVA; df=1, p<0.05, f=5.849), but by 24 hours this has reduced (ANOVA; df=1, p>0.05, f=3.017)(Figure 6.3). A similar trend is visible for glucose, however there are no significant differences (MANOVA: df=2, p>0.05, f=0.871) between the treatments and the control. Again, like the random interval results, osmotic content pH, haematocrit resulted in varying results (Table 6.2).

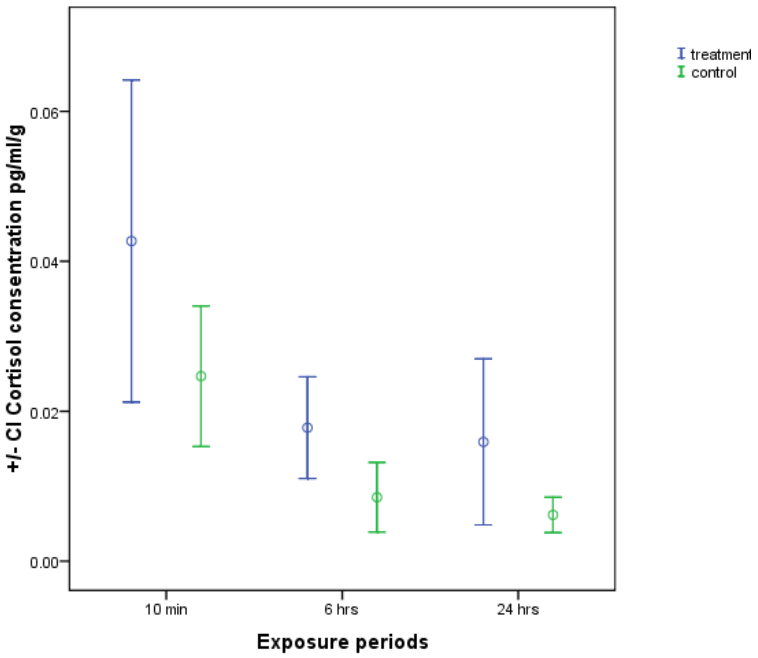


Figure 6.3. The cortisol results of the treatment group display the suggested trend described in the body text of the higher presence of cortisol in the first period of exposure followed by a decrease in cortisol level in the later durations. The control also decreases in cortisol concentration but the magnitude of the concentration of the treatment exposure are still much higher, solidifying the trend observed.

Table 6.2. Information is provided for results for the specific stress indices. Significance values are provided first, followed by the mean of each treatment at exposure in relation to the control mean which is reported in reference to the exposure period.

Constant		pH *df=1*	Haematocrit value *df=1*	*Osmotic content ANOVA* Osmol/kg ±SE *df=1*	Na^- 0.5mM/L±SE *df=1*	K^+ 0.25mM/L±SE *df=1*
10 min	Sig	P<0.01* *f=8.81*	p>0.05 *f=0.19*	p>0.05 *f=2.239*	p>0.05 *f=0.053*	p>0.050 *f=0.304*
	Mean ±SE	7.867±0.026	37.273±3.514	0.208±0.181	859.004±15.517	17.338±1.379
	Control Mean ±SE	7.7583±0.026	35.515±2.129	0.214±0.179	839.915±15.517	16.257±1.362
6 hours	Sig	P=0.001**f=14.57*	p>0.05 *f=0.429*	p>0.05 *f=0.06*	p>0.05 *f=0.698*	P<0.01* *f=13.161*
	Mean ±SE	7.875±0.032856	35.834±3.590	0.221±0.179	811.282±16.143	17.951±1.304
	Control Mean ±SE	7.709±0.0315	33.277±1.532	0.223±0.178	720.175±16.1429	13.808±1.279
24 hours	Sig	p<0.01* *f=12.21*	p>0.05 *f=2.295*	p<0.01* *f=10.233*	p>0.05 *f=0.228*	p<0.05* *f=5.701*
	Mean ±SE	7.867±0.0449	37.272±0.962	0.209±0.179	882.865±16.0893	17.925±1.406
	Control Mean ±SE	7.758±0.027061	35.515±4.179	0.218±0.177	922.634±15.676	15.944±1.384

*Significant difference present

6.6. Discussion

An anthropogenic environment completely devoid of natural conditions creates a new soundscape with its own contributions, fluctuations and unique signal events (Craven *et al.*, 2009). This experiment is the first (known) to evaluate effects of transient sound occurrences within an aquaculture environment and its influence on the stress response in fish. The research uncovered an unexpected combination of potential influences that led to further questions requiring answers.

The results for the constant internal sounds displayed a clear trend of an initial response in the cortisol that was also reflected for glucose, but not with the same level of significance. For cortisol, the concentration decreases over time, suggesting that the fish were physiologically adapting to the sound conditions. However, within the random interval data, the results are not significant nor do they give a clear demonstration of any trends or across any measure of stress response. For the random interval responses it would be expected that the responses would be similar to the constant interval sounds. Based on these inconsistencies as well as the varied results across the multiple measured indices of stress response, it is difficult to resolve whether these sounds are of more concern than continuous sounds.

It is still reasonable to expect that transient sounds would induce stress responses, but the sounds may need to be presented under different conditions. For example using underwater speakers and or physically creating impacts on the tank walls. This experiment intended to create a more realistic occurrence of sounds, so the transient sounds were in the presence of low-level SPL aquaculture soundscape sounds. Also with previous work completed (Chapter 4) the presence of sound within the confined type environment is most likely beneficial as it does appear to 'mask' some of the sounds that might otherwise be heard, in turn reducing significant response events.

In relation to determining if sounds affect fish, measuring stress responses is much more accessible than measuring audiograms. In saying this, it would also be worthwhile measuring audiogram responses to the presence of these sounds to determine what type of adaption in sound filtering or masking takes place. This may help reveal how the fish truly perceive this type of acoustic event. As sounds have been shown to mask the effects of other sound events, the hearing potential of each species will restrict the sounds heard or that will induce a response (Amoser and Ladich, 2005).

In conclusion this experiment highlights a few aspects of this approach that need to be improved upon. Much of the non-significant results were affected greatly by the range of responses across individual fish, which demonstrated the variable level of individual response to an acoustic event. Also these fish may be more adapted to unplanned acoustic disturbance based on their cultured life history than fish from a natural habitat. Overall this affect should be further investigated.

Chapter 7. General discussion and conclusions

7.1. Discussion

Over the period of this research, only a handful of peer reviewed articles have been released pertinent to the topic covered within this work (Davidson *et al.*, 2007; Wysocki *et al.*, 2007b; Craven *et al.*, 2009; Popper and Hastings, 2009). Craven *et al.*(2009) was the first full survey of a recirculating aquaculture facility soundscape since Bart *et al.*'s (2001) work that acoustically surveyed a range of different aquaculture setups. Davidson *et al.*(2007) did evaluate a recirculating facility but focused on methods of improvement rather than a full acoustic survey. The information provided by Craven *et al.* (2009) quantified a different range of acoustical characteristics, including the dominant contributions and day-to-night fluctuations. This work also described other influences that are common within many recirculating facilities of this type, such as hoods, and the contribution of facility background noise comprised of multiples of individual tank setups (each tank had its own pump, aeration and heater/chiller). The acoustics evaluation was the first step into evaluating these types of sounds on the stress responses of fish.

The three data chapters, Chapter 4,Chapter 5 and Chapter 6, used the baseline information gathered from Craven *et al.* (2009), such as the range of SPL as well as the dominant frequency. The frequency used (187 Hz) was ideal as it detectable within the near and far field sensory perception of fish (Weeg and Bass, 2002; Webb *et al.*, 2008) and that it is a realistic known event. The experimental designs focused on replicating the real soundscape rather than creating one.

The results of the three experiments detailed in Chapter 4, Chapter 5 and Chapter 6 suggest that there might be possible effects in terms of stress responses particularly with the cortisol initial response, but neither the initial responses through to the tertiary response resulted in consistencies between the stress indices within each experiment. The growth evaluation did not result in definite significances across the growth parameters, but the highest level of exposure (139 dB re: 1 $\mu Pa^2/Hz$)

did result in the largest deviation from the other treatments and control trends. This suggests that possible effects could arise, but as recommended within the chapters the experiment would need to continue for a longer period. The transient sound experiment is the first to evaluate these types of sounds (recorded or otherwise) in an aquaculture type of environment, and as such it would be wise to determine the various hearing sensory thresholds, and masking events, as well as accounting for the very high individual variations which can be a common occurrence (Barton, 2002).

Within the growth results there were possible trends of growth increase above the control in levels 1 and 2, so some levels of sound may stimulate the fish to eat and grow. The idea of benefits in this area is often overlooked as these soundscapes are commonly viewed as negative environments. However, as suggested among the previous chapters, the sound may mask facility activities, allowing the reduction in fright responses. It has been shown that for some fish (common carp, *Cyprinus carpio* L.) the effect of including sound such as music can reduce the stress response to another environmental condition (Papoutsoglou *et al.*, 2007).

A common discussion point throughout Chapter 4, Chapter 5 and Chapter 6 is the effect hatchery reared fish's life history might have on the animal's ability to perceive and sense its environment. In the literature there is not a complete understanding of the full influences of various culture conditions or the affects domestication might have on the fish's sensory system. For instance with the effect of cryopreservation and its influence on growth in steelhead trout (Hayes *et al.*, 2005), and water condition effects on skeletal development in flathead minnows (Blanksma *et al.*, 2009), both examples although very different are examples of condition changes. Therefore, the period of the eggs in cryopreservation and the concentration of calcium in water access both can greatly affect the development of a fish species. With these examples in mind, the affects of changes in temperatures, water condition, nutrition, and even other culture practices like transport and processing are

unknown in terms of the development of the sensory systems. This thought is agreed upon by Wysocki, Davidson *et al.* (2007b) and Popper and Hastings (2009).

Along with developmental influence, there is also the influence of domestication. The reasons behind this thought are that domestication has an influence on the productive capabilities and behavioural changes (Price, 1999). As there are breeding programs associated with reducing the stress responses in fish (Pottinger and Carrick, 1999) disease resistance and maximising growth, it is likely that the breeding or changes in genetic lines (Popper and Hastings, 2009) and life history has affected the stress responsiveness of the barramundi used for these experiments and as well as the other species evaluated by Wysocki *et al.* (2007b). Therefore together domestication and development may create fish less prone to experiencing a stress response to aquaculture sounds. In recommendation of the overall outcomes of this work and the work completed by Wysocki *et al.* (2007b) and by Davidson(2009), there is the potential that effects of sound stresses are of minimal concern for common cultured species.

7.1.1 Future directions

This work has prompted many ideas for future research, including further developing the understanding of developmental and domestication effects, and investigating potential causes to sensory perception changes. A reason for examining these areas is that it may benefit production to have fish less concerned with their acoustical environment. Another approach to evaluating these soundscapes is introducing wild caught species to the aquaculture soundscape and using the methods within this work to evaluate the process of adaptation. This approach would likely result in a more conclusive effect and adaptive response, as wild fish would never have been exposed to this environment before, unlike aquaculture produced fish. This approach would apply to broodstock, and it may create insight to some species that experience a lack of willingness or disinterest in reproducing in captivity.

Further work might also include more acoustic surveys of natural habitats, specifically habits of cultured species of interest. There is also limited information on Australian acoustic characteristics in freshwater locations, which is important for understanding natural habitats of fish such as barramundi. Fish hearing threshold studies have been predominantly focused on more temperate species, and it would be of benefit to evaluate more tropical species due to their vastly different living conditions.

7.1.2 Conclusion

In light of the limited stress effects the aquaculture soundscape has had on barramundi, the need for further experimentation and evaluation exists. However this body of work has provided a good baseline for future studies. The shortcomings and the insights this work has uncovered will build on the physiological understanding of acoustics on fish. The effects that have been poorly studied are now one-step closer to understanding an unknown effect.

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Chapter 8. Appendix

RNA /DNA ratio trials and step by step Procedure

Samples when not in liquid nitrogen they were maintain on ice. All equipment was cleaned and RNAzap treated, where appropriate DPEC water was used.

TE buffer = Tris-EDTA buffer solution

TES buffer = Tris EDIT Buffer

Tissue post harvest procedure: Step 1

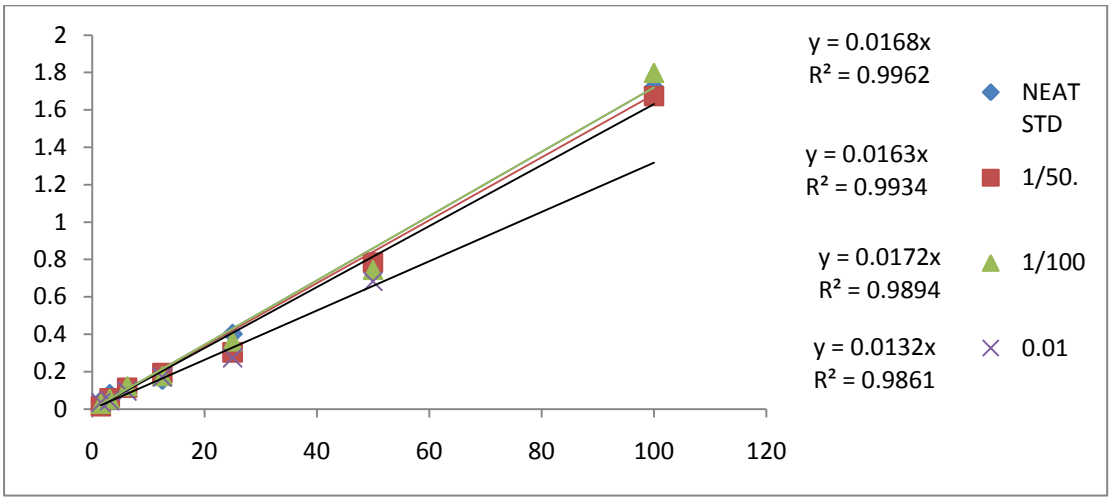
4. Remove single sample tube from Liquid nitrogen
5. With tweezers pullout frozen sample and shaving tissue: With use of sterile scalpel 'shave pieces off of frozen sample on to inside foil of scapple. Take ~50-100 mg of each. Continue to chop sample on scapple foil of until well broken.
6. The add sample to 1-2ml of TES* solution
7. Homogenise sample in glass tubes . Approximate time 1 minute (homogeniser blades were cleaned between sample use.
8. Sonicate (1 minute) Probe sonicator 50% amplitude 30 sec pulses
9. Sip down 8 min @1200g at 4 °C (remove middle fluid spin again)
10. Remove 2 aliquots of sample and snap freeze that this point

Plate and sample preparation: Step 2

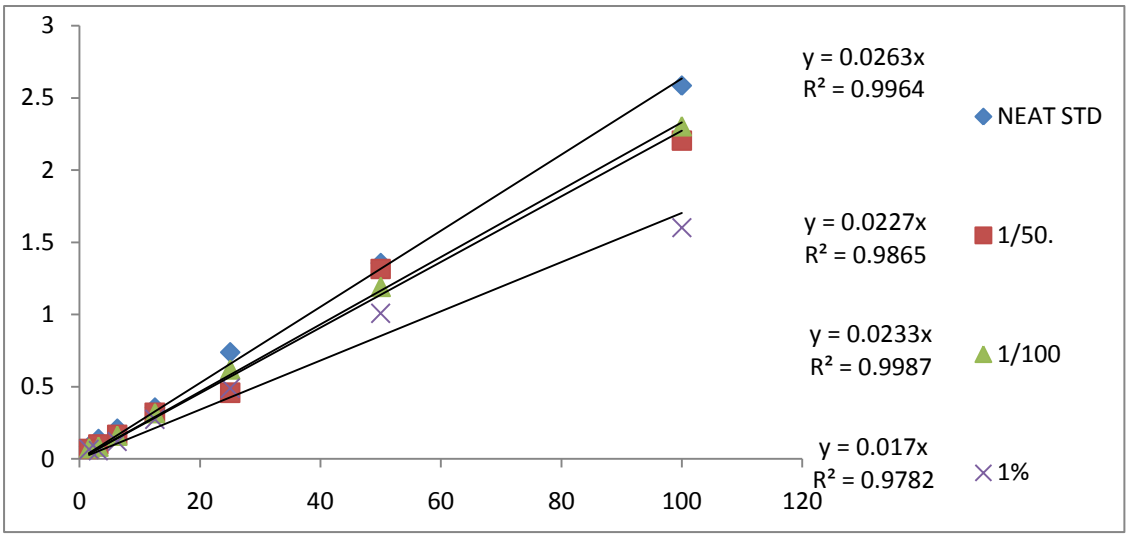
1. Made stock solutions of TES buffer and associated dilutions
2. TES (stock at 1% sarcosyl)
3. 1/100 TES = 1ml at 1/100 = 10 µl TES + 990 µl TE for standard spiking

4. Standards
    a. Concentrations in ng/µl (100, 50, 25, 12.5, 6.25, 3.125, 1.5625, BLK)
5. Total volume 52ul 50 µl Buffer + reagent and 2µl sample or standard (not including spike for STD)
6. 1/100 is 1µl of sample + 99ul of TE buffer (suggest doubling the volume it will be more consistent.
7. Sample and standard well total volume 52µl, 2µl of sample or standard pre diluted 50µl of reagent and buffer.
8. Reagent and buffer aliquot made per plate, procedure outlined by kit methods. Sample dilutions were used for both RNA and DNA kits as the standards dispelled the least effect from the TES spike on the fluorescence (standard results displayed in Appendix Figure 1).

RNA standard results



DNA standard results



Appendix Figure 8.1. The RNA standard at 1/50 had the best R2 value (0.99340) But 1/100 was very close DNA standards showed 1/100 with the best R2 value (0.9987). Also the Samples when tested for best dilutions were more consistent at a 1/100 dilution. Therefore to stay consistent across treatments as well as ease of plate method procedure the 1/100 dilution was used.