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LEC301: Dissertation with work placement

Investigating the impact of ultrasonic algal control on *Daphnia* in a freshwater ecosystem

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Declaration

I declare that, except where explicitly stated, this work is entirely my own. I have not submitted it in substantially the same form towards the award of a degree or other qualification. It has not been written or composed by any other person, and all sources have been appropriately references or acknowledged.

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Abstract

Developing environmental methods to control algal growth in lakes is necessary as current technologies are expensive and have environmental consequences. Ultrasound has been developed as a control measure; it is known to kill and inhibit growth of algae through acoustic activation and induction of programmed cell death (PCD). There is currently limited research into the effects ultrasound may have on non-target aquatic organisms, an issue this study begins to address through a field study and a laboratory experiment.

A man-made lake with sectioning walls at Forest Hills golf club, Lancaster was utilised as a field site; two bays were treated with mid-frequency wavelength (~580kHz) ultrasound and the other two were controls. Weekly measurements were taken over 2.5 months of numbers of *Daphnia* present, numbers of individual organisms and numbers of species. No detrimental effect was observed in ultrasound treated bays with regards to any of the three variables.

The laboratory experiment comprised two *Daphnia* cultures grown in RT (*Daphnia* growth) medium, one of which was subjected to ultrasound. For five days daily measurements were recorded of *Daphnia* numbers at various distances from the ultrasound source. This study indicated that ultrasound did not increase mortality in *Daphnia* nor did the presence of an ultrasound device influence the dispersion of *Daphnia* within the tank.

This study found mid-frequency ultrasound wavelengths (~580kHz) to have no damaging effect on *Daphnia*.

The greatest benefit of ultrasonic algal control would be if it could be developed to be applied to bodies of water that are used as a drinking source, these are significantly larger than the 2.4km^2 lake investigated and so parameters such as ultrasound frequency may need to be increased. If ultrasonic algal control technologies develop to be commercially viable, the issues of non-target organism damage will need to be addressed in greater detail.

Introduction and Background

Owing to the combination of natural aquatic process, such as circulation, flow, upwelling and subsequent relaxation, with human activities, such as intensive farming and mass industry, unnaturally large quantities of reduced nitrogen and phosphorus are frequently leached into water systems (Dai *et al,* 2012; & Sellner *et al*, 2003). The increase in nutrients provides the ideal habitat for algal and cyanobacterial reproduction and growth resulting in algal blooms, which, directly cause

several biological problems. If a body of water is a drinking source, expensive methods of purification, such as chlorination, are required to allow it to be free from toxins, and therefore, safe to drink and if the water serves as a recreational site there is a loss in terms of profit or enjoyment (Ahn *et al*, 2003; Dai *et al,* 2012; Eberhart *et al*, 2012; & Himberg *et al*, 1989). In addition to the unpleasant attributes of algal blooms namely it is unsightly and often produces a bad taste or odour (Ahn *et al*, 2003), cyanobacteria can also produce harmful toxins such as neurotoxins and hepatotoxins (Ahn *et al*, 2003 & Dai *et al,* 2012). For example, the major bloom forming *Microcystis aeruginosa* is a cyanobacterial species commonly found in eutrophic bodies of water, such as lakes, ponds and reservoirs, and produces microcystins, potent hepatotoxins (Yoshida *et al*, 2008). Above threshold levels, both microcystins (above 10000 cells ml^{-1}) and associated hepatotoxins can be poisonous to humans, causing liver failure, and also animals both domestic and wild: effects have been most frequently monitored in cattle, dogs, pigs and waterfowl (Beasley *et al* 1989; Jochimsen *et al*, 1998; Tango *et* al, 2004; & Wu *et al,* 2012).

Current methods of controlling algal blooms can be categorised into engineering, chemical and biological methods (Wang *et al*, 2011). Engineering methods are not permanent and include dredging the sludge or mechanical removal of the algae (Wang *et al*, 2011). Chemical methods, such as copper algaecides, are harmful to the surrounding environment due to the adverse effect they have on non-target freshwater organisms e.g. *Daphnia*, algaecides are also an expensive option for developing countries (Saro *et* al, 2012; Wang *et al*, 2011 & Wu *et al*, 2011). There is a safer biological option; planting macrophytes (e.g. *Myriophyllum verticillatum*) to intercept blooms, absorb the leached nutrients and excrete polyphenols with negative allelopathic effects resulting in inhibited algal growth, however the survival rate of these macrophytes is relatively low and so this is not a reliable method of control (Chang *et al*¸2012; & Wang *et al*, 2011).

An alternative option is becoming more available, and has been shown to be effective if applied correctly at irregular intervals daily throughout the year (Wu *et al,* 2011). Ultrasound radiation provides a reliable way of inhibiting algal growth and killing cells without the secondary pollution effects of chemical methods (Wu *et al,* 2011).

Successful ultrasonic algal control devices operate at mid frequencies (see table 1) (Wu *et al*, 2011). Short exposures of high frequency ultrasound can to be used safety in medicine e.g. during an ultrasound examination of a developing embryo (Tang *et al*, 2004). However, the higher frequency, acoustic, waves of ultrasound have the ability to cause greater damage to cells; at frequencies of 580 kHz, maximum intensity exposure has been found to reduce algal mass by almost 50% in only 30 minutes (Joyce *et al*, 2010).

Table 1: The frequency of ultrasound wavelengths used to control algal blooms in context with low and high frequency uses. (Chandrapala *et al, 2012,* Joyce *et al*, 2010 & Wu *et al*, 2011)

Ultrasound frequency	Intensity	Use
20-100kHz	Low	E.g. Food processing
580 kHz	Medium	E.g. Control algal growth
<20MHz	High	E.g. Medical imaging

There are thought to be two main ways in which ultrasound radiation can lead to algal cell death: disruption of the gas vesicles and also production of free radicals. Gas vesicles are vital to the function of planktonic microorganisms; they provide buoyancy which allows them to migrate vertically through the water column (Walsby, 1994). Buoyancy is necessary as it allows planktonic microorganisms, including cyanobacteria, to obtain light energy from the sun that penetrates into the top of the water column, which is necessary for photosynthesis (Addy & Green, 1996). Disruption of the vesicles would disrupt buoyancy and therefore restrict the light available for photosynthesis and will lead to a reduction in function and even cell death (Addy & Green, 1996; and Wu *et al*, 2011). Free radicals (messenger molecules) initiate programmed cell death and disruption of photosynthetic activities (Tang *et al*, 2004; & Ahn *et al*, 2003).

Gas vacuoles in algae are comprised of groups of microscopic vesicles, individually with a diameter of approximately 75 millimicrons and a length ranging from 0.2 – 1.0 microns (Bowen & Jensen, 1965). Gas vesicles are found in several planktonic species of algae (Sharma *et al*, 2010). These vesicles provide buoyancy which allows migration of cyanobacterial species e.g. *Microcystis aeruginosa*, through the varying depths of the water column (Sharma *et al*, 2010). This migration allows the algae to obtain the essential nutrients and light necessary for survival and the disruption of such systems often proves fatal (Sharma *et al*, 2010). When applied to algae, the high frequency sound waves causes collapse of the gas vesicles within the cells, and corresponding collapse of the vacuole, through a process known as cavitation (Bowen & Jensen, 1965; & Zhang *et al*, 2009). Acoustic cavitation is the rapid collapse of bubbles in water triggered by the ultrasound, this results in high temperatures (>5000K) and pressures (>100 MPa), known as hot-spots, within the cells (Zhang *et al*, 2009). There is a school of thought that this vesicle collapse is the primary cause of algal cell death in response to ultrasound (Tang *et al,* 2004; Ahn *et al*, 2003; & Nakano *et al*, 2001). When the vesicles are damaged, buoyancy is lost and the algal cells can no longer remain at the top of the water column; they become 'sedimented' (Ahn *et al*, 2003). As light often fails to penetrate the water column to the sediment, photosynthesis cannot occur and so these species of vesicle

containing algae are susceptible to damage from ultrasound (Ahn *et al*, 2003). However, this is not a one-off method of control. Damaged gas vesicles have been seen to reform, restoring full function in a relatively short time period (24 hours) (Lee *et al*, 2001), and so the repeated application of ultrasonic is vital to its long term success in algal bloom control i.e. frequent exposure. Further research is necessary to determine the biological mechanisms that allow this apparent vesicle reformation.

The action of messenger molecules formed from the sonolysis of water, e.g. OH radicals, has also been noted (Mason, 2007a). The production of these messenger molecules is thought to be due to the presence of gas (oxygen) (Hart and Henglein, 1985). In experiments involving the irradiation of solutions with ultrasound, solutions in the absence of oxygen failed to produce free radicals; the H atoms forms instead H_2 and the majority of OH forms hydrogen peroxide (Hart and Henglein, 1985). However, with oxygen present there is formation of HO_2 and OH radicals and O atoms (Hart and Henglein, 1985). With such free radical production is it easy to see how other, non-target, organisms may be jointly affected.

'Sonoxide' is a water purification system developed by Ashland in 2002 (Mason, 2007b). The system combines an air supply with high frequency ultrasound exposure; this combination harms previously healthy cyanobacterial cells through the production of free radicals (Mason, 2007a; & Mason, 2007b). The cells are then triggered into 'Programmed Cell Death' and in doing so produce, as of yet unidentified, "signalling protein molecules" which are transmitted to the other cells in the algal bloom (Mason, 2007b). Programmed cell death is then triggered by these signalling protein molecules in cells in the biofilm of the water, including algae (Mason, 2007b).

Ultrasound can also be utilised to control non-vesicle containing algae as long as it is a filamentous species such as the *Spirogyra* genus (Purcell, 2009). Although there have been far fewer studies conducted into these species of algae and the effect ultrasound has upon them, it has been shown that when silica is present in the cells, cavitating gas bubbles directly compromise the structure of the filament, leading to damage to the joints and cell lysis (Purcell, 2009).

Although there is limited understanding at present of the specific mechanisms that disrupt photosynthesis, studies have shown that, in addition to the collapse of gas vesicles, inhibition of photosynthetic systems of blue-green algal blooms is also apparent; studies have shown that ultrasound can reduce photosynthetic activity by 40.5% (Lee *et al*, 2001; Zhang *et al*, 2006a; & Wu *et al,* 2012). Ultrasound-induced free radical production also directly inhibits photosynthesis (Ahn *et al*,

2003 & Lee *et al*, 2001). It is thought to be a consequence of the free radicals produced by the cativation of water, thereby, damaging Chlorophyll a (Wu *et al,* 2012).

In studies examining the possible impact of increased UV-B exposure due to ozone depletion, it has been shown that excess light can result in production of free radicals (largely hydroxyl and carbon-centred) (for example: Hideg and Vass, 1996; & Kumagai *et al*, 1999). It is therefore possible that ultrasound induces stress in plants in a similar way to UVB, stimulating the production of free radicals (Kumagai *et al*, 1999; & Wu *et al,* 2012).

Further study revealed that when used at lower (but still dangerous) frequencies (20 kHz), damage caused by ultrasound is largely physical; and so vesicle damage is the cause of cell death. At higher frequencies (580 kHz) there are far greater numbers of free radicals produced from the cavitation of water causing faster cell death, and more complete control of growth of the algal bloom (Joyce *et al*, 2010). Thus the aim of purification systems is to function at the minimum frequency that produces enough free radicals to initiate sufficient programmed cell death within the bloom in order to provide a long term solution to algal growth. Most devices made to control algal bloom, therefore, operate at a frequency of at least 40kHz, although more commonly at 580kHz (Mason, 2007b).

In addition to the benefits of ultrasound radiation in controlling the growth of algal blooms it can also be used to remove toxins produced by algae in a body of water (Wu *et al*, 2011). Pilot studies have shown that ultrasound at frequencies of 400-650 kHz can effectively be used to degrade toxins which can otherwise have damaging effects on health (Ahn *et al,* 2003; Song *et al*, 2006; & Wu *et al,* 2011). The mechanisms leading to the degradation of these microcystin toxins result from attacks from hydroxyl radicals on benzene ring and Adda peptide residue causing a cleavage of the Mdha-Ala peptide bond (Song *et al*, 2006).

Studies have been conducted using high frequencies of ultrasound (19kHz) with the intention of killing bacteria, phytoplankton and zooplankton transported in ballasts of ships to reduce species invading non-native ecosystems (Holm *et al*, 2008). With relatively short exposure times (1-4 seconds) 90% reduction in live phytoplankton cells has been shown to occur (Holm *et al*, 2008). However in these studies, the test species (brine shrimp *Artemia* sp) and conditions (saline water) are unlike the freshwater lakes and ponds where the ultrasound equipment used to control algal blooms would be installed.

It is widely accepted that the complex relationships in ecosystems are difficult to understand and that it is almost impossible for us to predict the absolute outcome of addition or removal of a species. Invasive species can often pose a significant threat to the health of an ecosystem and its

ability to function (Nehring & Kolthoff, 2011). An example of such a species is Ludwigia grandiflora, the water primrose, which originates in South America is currently threatening aquatic ecosystems of Germany after being introduced in 2004 (Nehring & Kolthoff, 2011). Initially only a few individuals were noted in Germany, however since 2009 rapid growth and spread of the plant have led to it being placed on the German Black List for fears that the new species will greatly harm the existing native ecosystems (Nehring & Kolthoff, 2011). If ultrasound is found to harm a component of the aquatic ecosystem it will be difficult to predict the possible consequences.

In a UK freshwater ecosystem there are likely to be several different species of organisms, depending on trophic status; the poorer the quality of the lake the lower the levels of available nutrients and so species adapted to these conditions flourish where others cannot survive (Reynolds, 1998). In addition to the plant life within a freshwater ecosystem, there are likely to by several types of zooplankton; species of: *Daphnia, Cyclops, Rotifer,* for example and also macroinvertebrates e.g. species of *Crustacean, Oligochaeta, Nematoda,* amongst others (Reynolds, 1998).

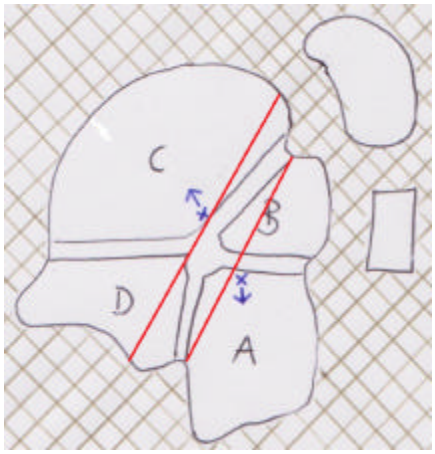
The aim of this investigation is to establish whether the use of ultrasound as a method of algal bloom control has any detrimental effects on an individual species (*Daphnia*) within an aquatic, lake ecosystem.

Materials and method

Field Experiment

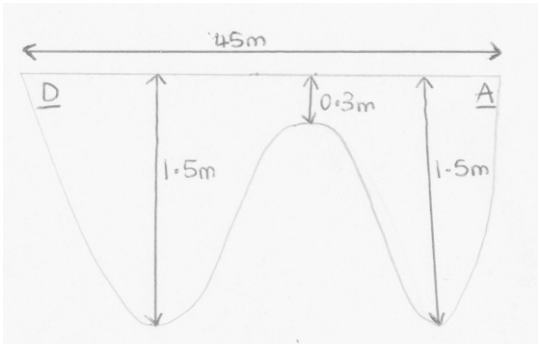
This phase of study was undertaken at Forest Hills golf club, Hazelrigg, Lancaster. An established man-made lake, with approximate area 2.4km^2, is situated there and in addition to the access to an electricity supply that the ultrasound demands, the lake has the unusual benefit of being partitioned into four sections. These barriers provide walls allowing a pathway for access to the fountain situated in the lake's centre, however the main benefit for the purpose of this study was the separation allowing for two sections of the lake to be subjected to ultrasound (A&B) while the other two may as the control (C&D) (see figures 1a, b, c & d). Ultrasound can penetrate solids with relatively small thicknesses, which explains its use for medical imaging (Chan & Perlas, 2011) however, with careful placement of the ultrasound unit, wavelengths into control areas can be eliminated. One ultrasound unit was placed per treatment section on edge of a perimeter wall (see figure 1b), the ultrasound unit faced into the section and was angled down slightly to avoid wavelengths passing over the wall into a control section. Some assumptions need to be made at this stage, for more information, see "Assumptions and limitations".

Figure 1a: Image from Google maps satellite view showing the Forest Hills golf club visitors centre and 2.4km^2 lake divided into the 4 sections: A and C were treated with ultrasound while B and D acted as the control group and were not. Red dots indicate the location of sampling.



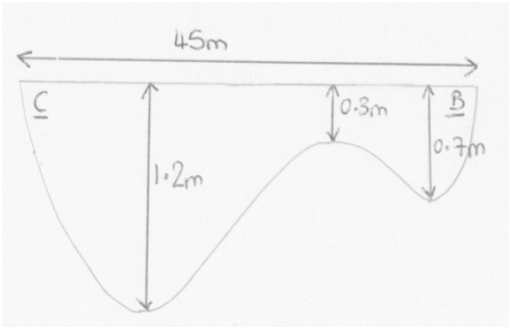
(Not drawn to scale)

Figure 1b: A hand-drawn schematic showing the Forest Hills golf club lake divided into the 4 sections by the manmade walls: A and C were treated with ultrasound while B and D acted as the control group and were not. The crosses mark placement of ultrasound unit and arrows show direction of wavelengths emitted. The red lines indicate the locations of cross sections in figures 1c and 1d.



(Not drawn to scale)

Figure 1c: A hand-drawn schematic showing approximate maximum depths of 2 of the 4 sections of the Forest Hills golf club lake: D & A. Location of cross section shown on figure 1b.



(Not drawn to scale)

Figure 1d: A hand-drawn schematic showing approximate maximum depths of 2 of the 4 sections of the Forest Hills golf club lake: C & B. Location of cross section shown on figure 1b.

The Ultrasound was installed late June (25th) and in addition to a zooplankton sample taken prior to installation, measurements were then taken weekly throughout July and August for a total of eight additional weeks.

Measurements were taken using a sampling tube designed by Dr Jackie Parry of the Lancaster Environment Centre which Dr Parry has found to be an easier and more effective way of examining zooplankton than a net (Personal Communication, 15th June 2012). The accessible sediment was disturbed, by kicking, and a plastic bottle was used to collect water just below the surface to ensure the same depth of water column was being sampled. For each data collection day one sample was taken from each lake section (A, B, C and D). Each sample was gathered by filtering the contents of 5

x 500ml bottles through the net at the three positions illustrated in figure 1a (therefore a total of 7.5L of lake water was filtered to obtain one sample).Once collected in the tube, specimens were carefully placed in containers filled with 70% ethanol and 30% pure water. The nets were examined for any caught individual organisms and these were carefully removed with tweezers and added to the sample container, finally the net flushed through with several sprays of pure water. The samples where then transported back to the laboratory in the containers of ethanol and pure water and identified (to species level where possible) using images and descriptions from a number of freshwater identification guide books (Macan, 1959; Olsen *et al,* 2001; & Greenhalgh & Ovenden, 2007). Before identification, each sample was randomly assigned (using a random number generator) a number and this replaced the label identifying lake section on the sampling tube. This task was carried out by an individual who had no role in the species identification, this removed bias from the identification process as the origin of the sample (treatment or control) was unknown.

As small freshwater zooplankton are shown to be sensitive to temperature; their growth can be limited due to temperature extremes (Vidal, 1980; & Napper, 2009), the water temperature of each bay was recorded at every sampling location on each visit.

Laboratory experiment

Two tanks, previously constructed for another experiment involving the company, were provided by Sustainable Soil and Water Ltd in order to conduct the laboratory experiment. The first tank (the control) was a simple 0.38m x 0.25m x 0.69m design, the second (the treatment) was the same dimensions but with an additional cylindrical protuberance which houses the ultrasound unit, both are made from a thick polymer (see figures 2a & 2b).

Figure 2a: The plastic 0.38m x 0.25m x 0.69m tank used as the control in the laboratory experiment. (Provided by Sustainable Soil & Water Ltd)

Figure 2b: The plastic 0.38m x 0.25m x 0.69m tank with ultrasound attachment, used as the treatment in the laboratory experiment. (Provided by Sustainable Soil & Water Ltd)

The in-built ultrasound unit is a "Pool Tec 10" obtained from hughes-sonic-systems.com, the device operates at 110-240V and emits ultrasound at a frequency range of between 45-60Hz. Although this is lower than the frequencies used in lakes, the structure of the tanks reflects wavelengths more due to the normal incidence with which the wave comes into contact with the walls (90°) (Fellah *et al*, 2003), therefore in order to replicate the conditions of the lake as closely as possible a lower frequency is used. The *Daphnia* were kept in RT media which was created following the procedure described by Tollrian (1993) (see table 2). This choice was influenced by the PhD research of Piers Napper (2009) which selected RT medium as a next best substitute to spring water, which would have been too expensive to purchase for two large tanks which require at least 65 L each in order to fully submerge the ultrasound unit.

Table 2: Elements (mg l^{-1}) in RT medium adapted from Tollrian 1993. Two elements were excluded from the stock solution (Li (LiCl) and Se ($Na_2SeO_3.5H_2O$)) due to lack of supply. As they were only trace elements and their benefit to daphnia could not be identified their absence was not thought to be significant. * TES = $C_6H_{15}NO_6S$ (*N*-Tris [hydroxymethyl] –methyl – 2 – aminoethane – sulphonic acid; Sigma T-1375)
8.5ml HCL (1N) per tank was used to lower the pH to 7.9 and a conductivity was found to be >220 µS and so no additional $Ca(OH)_2$ was required.

Trace elements **(stock solution)**	
EDTA (disodium salt)	500.0
B (H_3BO_3)	572.0
Fe ($FeCl_3$)	322.46
Mn ($MnCl_2.4H_2O$)	72.0
K (KBr)	7.5
Mo ($Na_2MoO_4.2H_2O$)	12.5
Cu ($CuCl_2.H_2O$)	6.5
Co ($CoCl_2.6H_20$)	20.0
I (KI)	0.6
10ml stock solution 1^{-1} medium	

Main elements	
TES*	85
$CaCl_2.2H_2O$	39
$NaNO_3$	50
$MgSO_4.7H_20$	20
$Na_2SiO_3.5H_2O$	10
KCl	10
$CaCO_3$	13
$Ca(OH)_2$	30

The *Daphnia* were fed on a supply of dried blue green algae (*Spirulina*) every 3 days, found to be an acceptable substitute to live algae if unavailable (Napper, 2009). They were kept by a window within their preferred temperature range (15-20°C) (Napper, 2009) and with equal access to sunlight.

The number of *Daphnia* present was measured daily, with sampling occurring at the same time each day. 360ml samples were collected from the same depth in the water column daily (in the morning) and were taken at 5 fixed distances from the ultrasound unit (1= closest to unit, 5= furthest away). The samples were then examined under a microscope and numbers of *Daphnia* were recorded, once counted *Daphnia* were returned to their original tank.

Statistical analysis

The field measurements contained data nested within categories; the data can be grouped into treatment and control but then sub-divided into the lake sections A, B, C and D. The data were analysed for differences in weekly measurement of *Daphnia* number, number of species and number of individuals. This analysis was carried out using a two-level nested-design hierarchical ANOVA (analysis of variance) in the statistics software package SPSS. The fixed factor for the ANOVA was the week of the observation and the nested factors were the treatment type (treated vs non-treated) followed by the lake section (A and C or B and D).

Further analysis involved calculating coefficient of variance (%) in SPSS, as the scales of the variables (number of *Daphnia*, number of species and number of individuals) were different, calculation of the coefficients of variance allowed for comparison of variation of the data between the three.

The water temperatures for each lake section were averaged and linear regression performed in SPSS to test for a relationship between each of the three variables and the temperature of the water. This was a precautionary measure to ensure that the slight, natural fluctuation in water temperature was not sufficient to cause increased mortality to the zooplankton, in particular *Daphnia*.

The laboratory data were analysed in two ways: firstly the number of *Daphnia were calculated* and secondly the data was tested to determine if there was a relationship between number of *Daphnia* and distance from the ultrasound unit.

To test the number an independent t-test was performed (also known as a Levene's test), in addition, data were plotted of number against time and a linear regression fitted to further determine if ultrasound effected reproduction or mortality.

The relationship of *Daphnia* number and distance from ultrasound was tested by performing a further linear regression.

Assumptions and limitations

Due to uncontrollable nature of some aspects of a field study there are a few limitations of the experiment and assumptions that must be made.

Though the lake is split into four sections these sections are not of equal surface area and in addition they are not of equal depth. From observation, sections A and D are deeper than A and B with due to lack of access it is not possible to assess the volume of each section (see figure 1c). It is also difficult to judge where the perimeter boundaries for each section lie are there are large numbers of reeds present surrounding the lake. Due to this limitation, this study assumes that volume of the bays is not a factor in the effect of ultrasound or on the number or composition of species present.

Each bay varied in terms on plant composition and density. This potentially could impact results as certain organisms may prefer the habitat of one bay over other, regardless of ultrasound. Again, due to access, there is no data in this study on plant composition in bays and as such is a limitation. For the duration of this study it will be assumed that the lake habitat is uniform enough not to cause a significant organism preference for bay that will impact the effects of ultrasound.

While the bays have barriers that prevent large-scale transfer of water, organism and, in the case of this study, ultrasound exchange they are not fully independent. Due to the placement of ultrasound units it is very unlikely that ultrasound wavelengths were reaching the control sections of the lake. However, it is undeniable that water and organisms would be able to move between bays. As this study was investigating the presence of organisms and species compositions within the bays and not mortality, this is not a major limiting factor. Even if the zooplankton did not appreciate the ultrasound and had a behavioural reaction to it which involved moving from one bay to another, this would be shown in the results as ultrasound bays would have lower number. However, during the study we will assume that each bay is independent in terms of ultrasound treatment.

Results

Field experiment

The results of water temperature, displayed in table 3, show the limited fluctuation in temperature. By calculating linear regression in SPSS (see figures 3a, 3b and 3c) it was shown that in each lake section (A, B, C and D) there was no relationship between temperature and numbers of Daphnia, numbers of species or number of organisms sampled, see table .

Table 3: The weekly average water temperature (°C) for each lake section. The water temperatures were measured at the same water collection points as shown in figure 1a. Calculations were performed in SPSS.

Average recorded water temperature (°C)	Week water temperature recorded								
	1	2	3	4	5	6	7	8	9
A	15.5	15.4	15.4	15.4	14.5	15.6	15.2	14.3	15.9
B	15.6	14.4	15.7	14.2	14.3	14.1	14.6	14.8	14.6
C	14.4	14.7	15.3	15.3	15.3	15.8	15.0	14.4	15.3
D	15.3	15.2	14.7	15.2	14.4	15.3	15.5	14.4	14.8

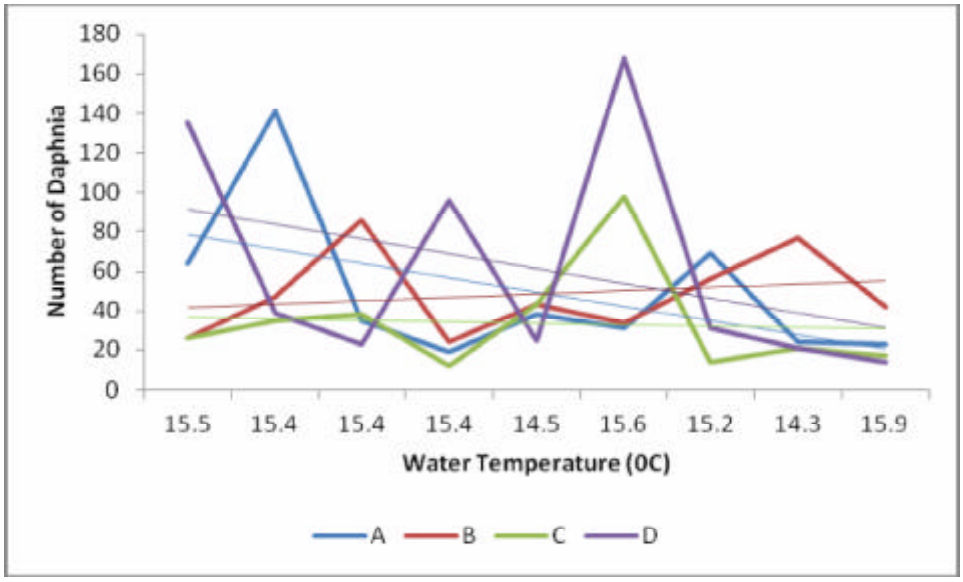


Figure 3a: Regression lines fitted to data from each lake section (A, B, C, D) to test the relationship between the number of *Daphnia* observed and the average water temperature of each lake section ^{0}C. Linear regression in SPSS found no relationship between temperature and *Daphnia* numbers for any of the lake sections (see table 4).

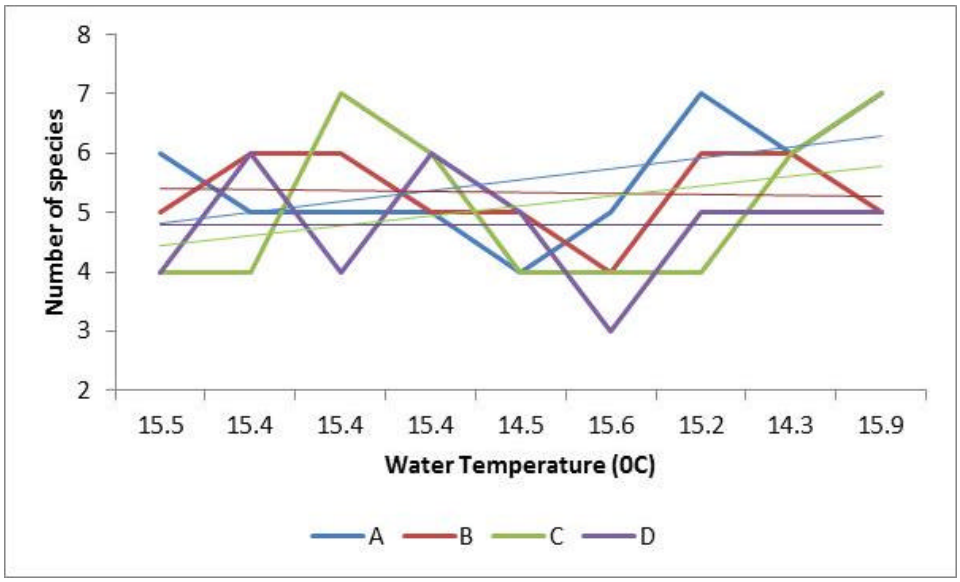


Figure 3b: Regression lines fitted to data from each lake section (A, B, C, D) to test the relationship between the number of species observed and the average water temperature of each lake section ^{0}C. Linear regression in SPSS found no relationship between temperature and species numbers for any of the lake sections (see table 4).

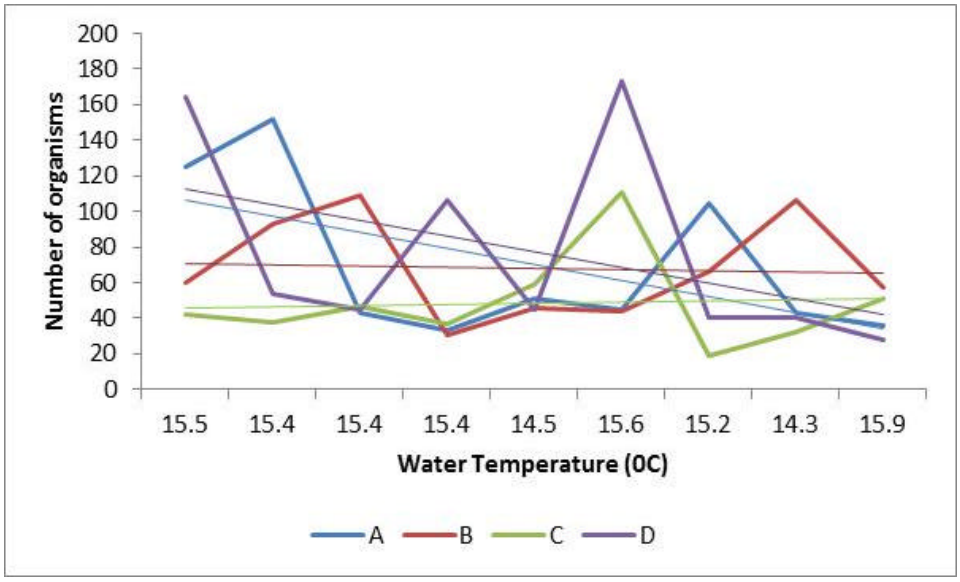


Figure 3b: Regression lines fitted to data from each lake section (A, B, C, D) to test the relationship between the number of individual organisms observed and the average water temperature of each lake section ^{0}C. Linear regression in SPSS found no relationship between temperature and the number of individual organisms for any of the lake sections (see table 4).

Table 4: Results from linear regressions performed in SPSS to test the relationships between each of the three variables (number of *Daphnia*, number of individuals and number of species) and the average weekly water temperature (^{0}C) in each lake section (A, B, C and D). All adjusted R^2 values are low, the highest only 0.385, showing poor fits of the data points to the trend line. All p-values were greater than 0.05 proving no observed significance between water temperature and each variable.

Section tested	Variable tested for relationship with water temperature	Adjusted R^2	P-value	Significant? (<0.05)
A	Number of Daphnia	0.127	0.313	No
	Number of individuals	0.124	0.343	No
	Number of species	0.037	0.346	No
B	Number of Daphnia	0.077	0.238	No
	Number of individuals	0.212	0.119	No
	Number of species	0.036	0.292	No
C	Number of Daphnia	0.214	0.118	No
	Number of individuals	0.385	0.044	No
	Number of species	0.125	0.748	No
D	Number of Daphnia	0.237	0.104	No
	Number of individuals	0.180	0.141	No
	Number of species	0.136	0.845	No

The numbers of Daphnia present in both control and ultrasound-treated sections of the lake fluctuated greatly (figure 4a). In the control sections (B and D) the highest average recorded number was in week 6 with a mean of 101 *Daphnia* detected in each section per 7.5L sample and the lowest in week 9 where a mean of only 29.5 *Daphnia* were recorded. In ultrasound treated sections the largest average number of *Daphnia* found per 7.5L sample per week was during week 2 where a mean of 87.5 *Daphnia* were collected and the lowest was week 4 where the mean was only 19

Daphnia per section. This fluctuation in results can be shown in figure 4a, where whilst the numbers decrease from week 1 to week 9, due to the data in the interim weeks there is no clear trend in the data. The lack of obvious trend is shown by performing a hierarchical (nested) ANOVA (results in table 5) where the p-value calculated when comparing number of *Daphnia* between treated and non-treated groups (p=0.875) illustrates the lack of significant relationship; ultrasound had no effect on *Daphnia* numbers in this field study.

The number of individual organisms present in control sections of the lake and ultrasound treated sections of the lake again varied greatly (figure 4b). In the control sections (B and D) the highest average recorded number of individuals was in week 1 with a mean of 112 individual organisms detected in each section and the lowest in week 5 where a mean of only 50.5 individual organisms were collected. In ultrasound treated sections the largest average number of individual organisms found per week was during week 2 where a mean of 95 individual organisms were collected and the lowest was week 9 where the mean was only 32 individual organisms per section. This fluctuation in results can be shown in figure 4b, where whilst the numbers decrease from week 1 to week 9 there is again no clear trend in the data. The lack of obvious trend was again shown by performing a hierarchical (nested) ANOVA (results in table 5) where the p-value calculated when comparing number of individual organisms between treated and non-treated groups was shown to be 0.229 therefore there is no significance and it can be said ultrasound had no effect on individual organisms numbers in this study.

The number of species present in control sections of the lake and ultrasound treated sections of the lake varied once more (figure 4c). In the control sections (B and D) the highest average recorded number of species was in weeks 2,7 and 9 where a mean of 6 species were detected in each section and the lowest in weeks 6 and 8 where a mean of only 3.5 species were collected. In ultrasound treated sections the largest average number of species present per week was during weeks 3, 8 and 9 where a mean of 6 species were collected and the lowest was week 5 where the mean number of species per section was found to be 4. These smaller fluctuations in results can be seen in figure 4c, where whilst the numbers decrease from week 1 to week 9 there is again no clear trend in the data. The lack of obvious trend was again shown by performing a hierarchical (nested) ANOVA (results in table 5) where the p-value calculated when comparing number of species between treated and non-treated groups was shown to be 0.288 therefore there is no significance and it can be said ultrasound had no effect on the number of species present in this study, a full list of species found in this study can be seen in appendix I & II.

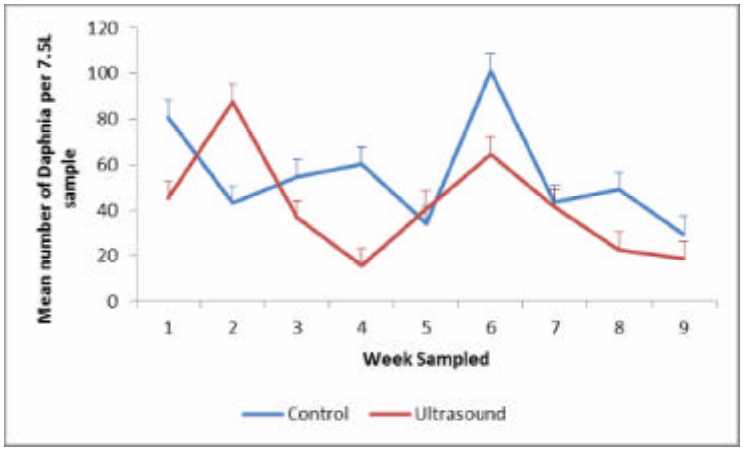


Figure 4a: Line graph illustrating how number of *Daphnia* fluctuated over the 9 week study, both in control (blue) and ultrasound treated (red) sections of the lake. Each data point represents the mean number of *Daphnia* observed per visit in a 7.5L sample from either a control or treatment bay as labelled. A hierarchical ANOVA shows no significant trend in the data collected (see table 3). Error bars represent +1SE of the mean. Calculations were performed in SPSS.

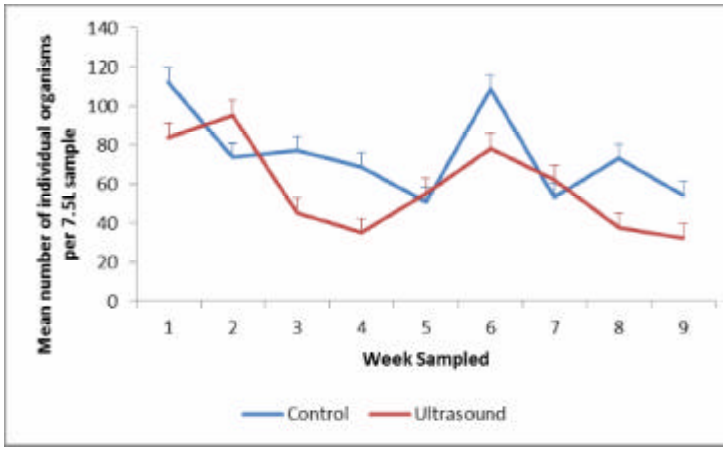


Figure 4b: Line graph illustrating how numbers of individual organism fluctuated over the 9 week study, both in control (blue) and ultrasoun treated (red) sections of the lake. Each data point represents the mea number of individual organisms (see appendix I & II for full species lis observed per visit in a 7.5L sample from either a control or treatment ba as labelled. A hierarchical ANOVA shows no significant trend in the dat collected (see table 3). Error bars represent +1SE of the mean. Calculation were performed in SPSS.

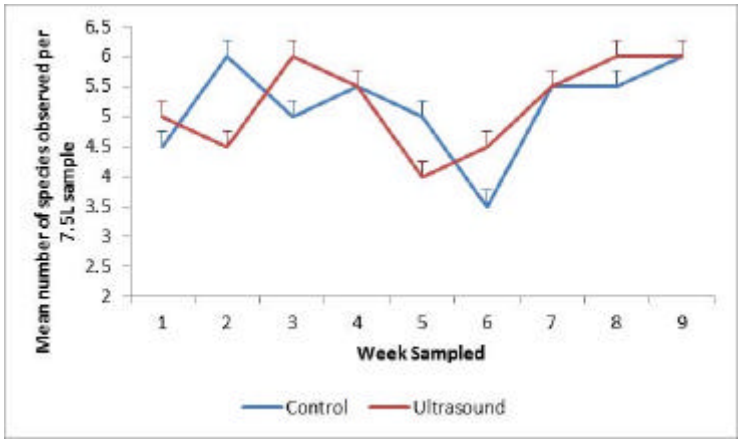


Figure 4a: Line graph illustrating how numbers of different species present in samples fluctuated over the 9 week study, both in control (blue) and ultrasound treated (red) sections of the lake. Each data point represents the mean number of species (see appendix I & II for full species list) observed per visit in a 7.5L sample from either a control or treatment bay as labelled. A hierarchical ANOVA shows no significant trend in the data collected (see table 3). Error bars represent +1SE of the mean. Calculations were performed in SPSS.

Table 5: Results from the hierarchical ANOVA showing no significance (all p values>0.05) between treatment (ultrasound) and control groups for number of *Daphnia,* number of individuals and number of species observed over a 9 week field study. Calculations were performed in SPSS.

	P value	Significant (<0.05)?
Number of *Daphnia*	0.875	No
Number of individual organisms	0.229	No
Number of species	0.288	No

Due to the large differences in data values and therefore mean averages of the data sets displayed in figures 4a, 4b and 4c coefficient of variance percentages (table 6) were calculated in excel in order to provide a comparison between the three. There was higher variability in the data sets containing sample numbers of Daphnia than both number of individuals and number of species data sets for control and treatment lake sections. The data sets containing number of individual organisms found contained more variability in both control and ultrasound treated bays than number of species sampled.

Table 6: The coefficient of variance (%) for the three test variables (number of species, number of individuals and number of *Daphnia*) in both control and treatment (ultrasound) groups. Percentage of variance is highest for the number of *Daphnia* observed and lowest for number of species. Calculations were performed in SPSS.

	Coefficient of variance (%)		
	Number of Species	**Number of individuals**	**Number of *Daphnia***
Control	20	30	42
Ultrasound	15	41	54

Laboratory experiment

In the control tank the number of *Daphnia* did not significantly alter throughout the course of the week (see figure 5a). The linear regression in SPSS calculated an adjusted R^2 of 0.101 (see table 7); this shows that the data points correspond poorly to the line of best fit. This lack of trend is confirmed with the P-value of 0.485 which shows no significant relationship between the number of *Daphnia* in the tank and the duration of the 5 day experiment. Similar results were found in the control tank as can be seen in figure 5b. The adjusted R^2 value of 0.095 shows the data points fit even less well to the trend line than the data provided through observations of the *Daphnia* number in the control tank. The linear regression provides a P-value of 0.478 (table 7) which again shows no significant relationship.

The lack in differences of number of *Daphnia* in each tank was confirmed with an independent t-test comparing the daily numbers of *Daphnia* observed. The t-test, performed in SPSS, calculated the mean daily *Daphnia* number to be for the 1.72 control and 1.40 in the treated tank (see table 8). The P-value calculated was 0.321 and so there is no significant difference between the *Daphnia* numbers in both tanks.

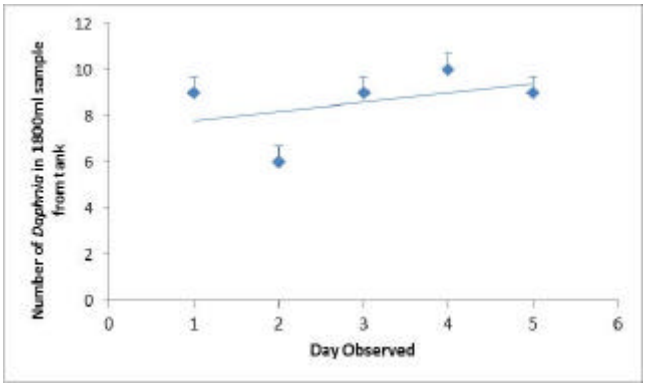


Figure 5a: A scatter plot displaying the relationship between the total numbers of *Daphnia* observed in the 1800ml of water sampled from the control tank (no ultrasound) daily over the course of the experiment. Each data point represents the total number of *Daphnia* sampled per day. A linear regression was fitted to the data series in SPSS and a significance of fit to the data points of 0.174 was calculated, indicating poor fit. Calculations were performed in SPSS.

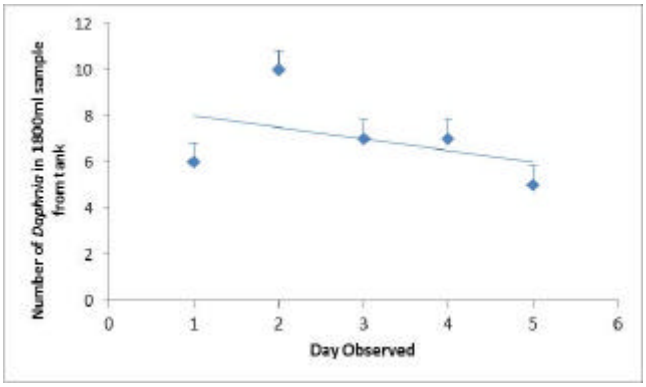


Figure 5b: A scatter plot displaying the relationship between the total numbers of *Daphnia* observed in the 1800ml of water sampled from the experimental tank (with ultrasonic device present) daily over the course of the experiment. Each data point represents the total number of *Daphnia* sampled per day. A linear regression was fitted to the data series in SPSS and a significance of fit to the data points of 0.179 was calculated, indicating an almost equally poor fit of data to a trend line as in the control tank. Calculations were performed in SPSS.

Table 7: Linear regression data analysing relationships between the number of *Daphnia* over time and the placement of *Daphnia* in relation to the placement of the ultrasound emitting unit in both a controlled environment (no ultrasound) and a test environment (with ultrasonic device present). As all p-values were >0.05 none are classed as a significant. Calculations were performed in SPSS.

Tank tested	Variables tested	Adjusted R^2	P-value	Significant? (<0.05)
Control	Number of *Daphnia* and Day	0.101	0.485	No
	Average daily *Daphnia* number and distance from ultrasound unit	0.169	0.562	No
Ultrasound	Number of *Daphnia* and Day	0.095	0.478	No
	Average daily *Daphnia* number and distance from ultrasound unit	0.0258	0.699	No

Table 8: T-test data analysing the difference in means between *Daphnia* numbers in a control (no ultrasound) and a test environment (with ultrasonic device present). As the p-value is >0.05 there is no significant difference between the environments. Calculations were performed in SPSS.

Comparison	Mean average number of *Daphnia* per section per day	P-value	Significant? (<0.05)
Control	1.72	0.321	No
Ultrasound tank	1.40		

The placement of the *Daphnia* within the tank was also investigated. In the control tank there was no ultrasound unit present and so the measurements of distance from ultrasound (1-5) mirror the distances from the unit in the test tank. In both cases; control and test, adjusted R^2 values were low: 0.169 and 0.0258 respectively, indicating a lack of trend in the data sets (see table 7). Linear regression provided P-Values of 0.562 for the control data and 0.699 for the test data; therefore there is also no significant relationship between number of *Daphnia* and placement in the either tank.

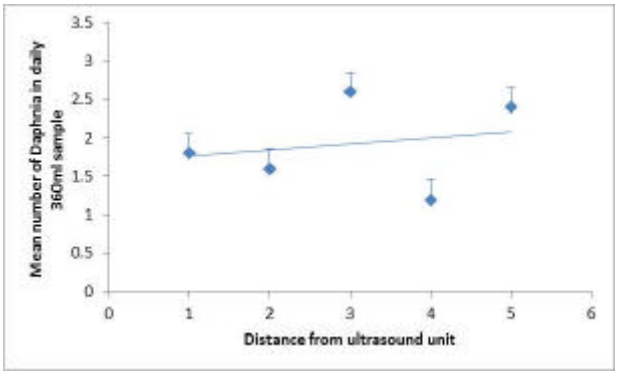


Figure 6a: A scatter plot showing the relationship between the average numbers of *Daphnia* observed in the 360ml of water sampled at each distance from the ultrasound unit in the control tank (no ultrasound). Each data point represents the average number of *Daphnia* observed over the duration of the experiment. A linear regression was fitted to the data series in SPSS and a significance of fit to the data points of 0.123 was calculated indicating poor fit. Calculations were performed in SPSS.

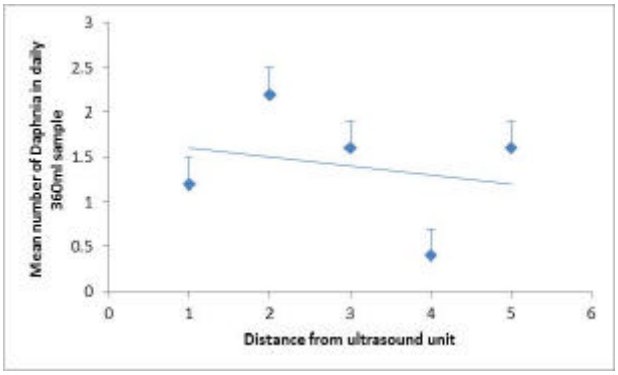


Figure 6b: A scatter plot showing the relationship between the average numbers of *Daphnia* observed in the 360ml of water sampled at each distance from the ultrasound unit in the experimental tank (with ultrasonic device present). Each data point represents the average number of *Daphnia* observed over the duration of the experiment. A linear regression was fitted to the data series in SPSS and a significance of fit to the data points of 0.057 was calculated indicating an even poorer fit than in figure 6a. Calculations were performed in SPSS.

Discussion

The analysis of the average water temperatures throughout the course of the experiment reveals that despite the natural fluctuations in temperature, there was no relationship between water temperature and any of the three variables (*Daphnia* number, number of individuals or number of species). It was necessary to measure temperature as is it possible a sudden change in temperature could have had a detrimental effect on zooplankton numbers and provided a false relationship between ultrasound and mortality, however this has not been the case. In addition to the lack of relationship between temperature and *Daphnia* in the study, this field experiment shows that commercial ultrasound use also does not appear to have a detrimental effect on the number of species within a freshwater ecosystem nor the number of these organisms. This indicates that the mid-frequency ultrasound wavelengths emitted do not reduce reproduction, increase mortality rates or negatively alter the environment in a way that decreases its suitability for zooplankton. The

laboratory experiment supports the above findings, by indicating that commercial ultrasound may not have a detrimental impact on Daphnia in this setting. In addition they show that Daphnia do not appear to migrate away from the source of the ultrasound. However, due to Daphnia being sensitive to laboratory conditions and the lack of repetition within the study this cannot be said with confidence. There were no signs however that ultrasound caused immediate mortality in Daphnia however as at least some specimens survived for the full 5 days being subjected to ultrasound wavelengths.

Field experiments are vital in testing ecological consequences of a non-natural influence as they involve the full ecosystem which is impossible to accurately recreate in a more sterilised environment (for example: Tipping *et al*, 1999 and Smith *et al*, 1999) however for a more controlled setting laboratory experiments provide data which is easier to interpret as there is a more obvious relationship between cause and effect (For example: Hartgers *et al*, 1998) and so conducting both often allows the deepest possible understanding (for example: Kestrup & Ricciardi, 2009). With this study the field experiment found ultrasound to have no short-term effect on the aquatic ecosystem of the freshwater lake and this was supported by the investigation conducted in the laboratory.

Although zooplankton are known as sensitive organisms, they are frequently used to assess good water quality (Gannon and Stemberger, 1978; & Lal *et al*, 1984). It may be possible that they are more resilient than algae to ultrasound stress. The sensitivity of *Daphnia* to certain abiotic stresses largely relates to their moulting cycle which is closely related to their reproductive cycle (see figure 6); if *Daphnia* are exposed to a pollutant at a particularly sensitive stage, this can be detrimental to the success of reproduction (Lal *et al*, 1984). This study shows that *Daphnia* are do not appear to be sensitive to mid-frequency ultrasound unlike other stresses such as copper, zinc and insecticides which are known to have detrimental effects on *Daphnia* (Hoang and Klaine 2007; & Hayasaka *et al*, 2012) however it does not say why this might be.

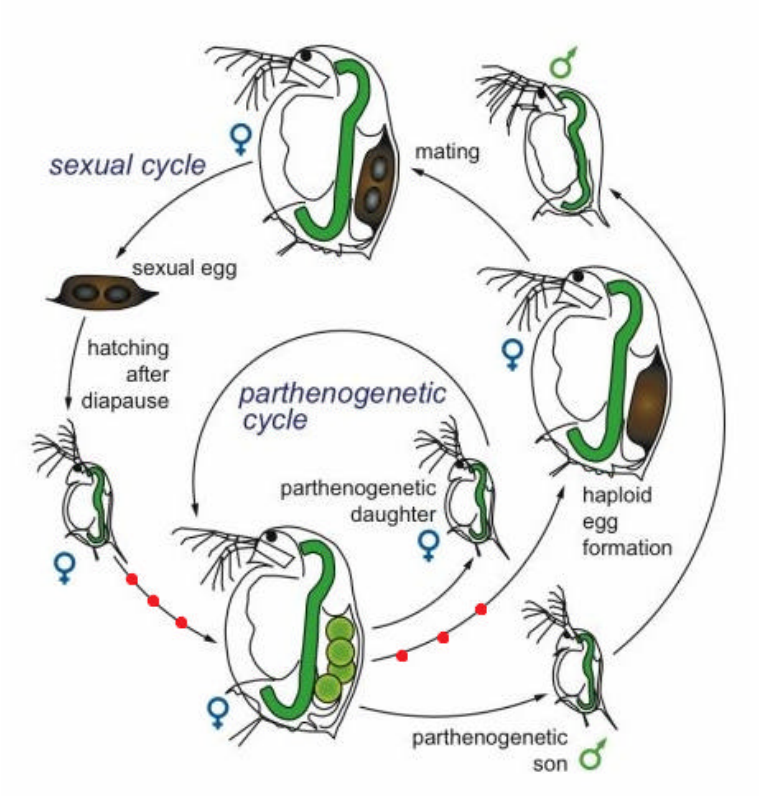


Figure 7: Diagram depicting the various morphological stages of *Daphnia*. The red dots represent the various moulting stages throughout the lifecycle which are undergone prior to maturity. During these moulting phases, *Daphnia* are particularly sensitive. (Adapted from: Ebert, 2005)

Daphnia, like other zooplankton, have the ability to swim because of their morphology; their 2nd antennae are used to propel themselves through the water (Ringelberg, 1999). This allows them to migrate through the water column allowing them to access nutrients, light, to locate suitable mates and, if conditions are unfavourable, to sink to the floor of the water system to lay their "resting eggs" (Destasio *et* al, 1995; & Ringelberg, 1999). Gas vesicles provide buoyancy which are needed by non-motile organisms to stay at the top of the water column (Walsby, 1994). However, as non-photosynthetic organsisms *Daohnia* are not required to remain solely at the top of the water column and due to their propulsion ability *Daphnia* do not require a gas vesicle (Walsby, 1994). As so much of the research isolated the disruption of the gas vesicle by acoustic cavitation (Tang *et al,* 2004; Ahn *et al*, 2003; & Nakano *et al*, 2001), and the zooplankton lack these air pockets this may explain the lack of effect ultrasound has on them. However, other studies suggested that the production of free radicals was the cause of algal reduction in non-vacuole containing algae (Mason, 2007a), the production of these messenger molecules could explain why high-frequency ultrasound has been proven to be fatal to zooplankton in ballasts of ships (Holm *et al*, 2008) but does not seem to explain the lack of fatality in the field portion of this study.

As ultrasound has been shown to be detrimental to some species of zooplankton (*Artemia* sp., *Ceriodaphnia dubia*, *Brachionus plicatilis*, *Brachionus calyciflorus*, and *Philodina* sp.) found in the ballast of ships (Holm *et al*, 2008) it is logical to conclude that under some conditions zooplankton will be affected by ultrasound. Even if zooplankton are not susceptible to endogenous free radicals being produced in algae as a result of ultrasonic wavelengths (which hasn't been investigated specifically in this study) and are more resilient to lower frequencies of ultrasound due to their lack of gas vesicles it is possible that at some, currently unknown, higher frequency of ultrasound wavelength, zooplankton will become sensitive to its effects.

Due, again, to their ability to move (Ringelberg, 1999), it could simply be that the zooplankton are eliciting a behavioural response that is allowing them to escape the harmful wavelengths of ultrasound. Laboratory study indicated that this may not be the case as there didn't seem to be any relationship between numbers of *Daphnia* and location within the tank. This is perhaps not surprising as it is difficult to comprehend that this response would already exist as ultrasound is a new stress for zooplankton and behavioural responses are either taught through mimicry, innate or learned over a period of trial and error (Zentall, 2006), it is however possible that zooplankton may evolve to partake in a behavioural response in the future.

This research reveals that ultrasonic control appears to be a method of algal control that is safe to the *Daphnia,* and potentially other zooplankton, present in aquatic ecosystems such as the one studied. By using ultrasound as an alternative to previous control methods such as mechanical, chemical or biological means it is possible that the negative attributes associated with reliability and environmental damage can be avoided (Wu *et al*, 2011). Ultrasound units are smaller and easier to install and operate than mechanical machinery and technologies currently available as algal bloom control methods and therefore more accessible (Wang *et al*, 2011). This could be particularly important to small businesses or homeowners who wish to control algal blooms or if there is an unsightly algal bloom in a domestic, relatively small water system. And while chemical methods of control such as the copper algaecides mentioned previously have been known to harm non-target organisms (Saro *et* al, 2012; Wang *et al*, 2011; & Wu *et al*, 2011), this study has shown that Daphnia are not harmed when subjected to wavelengths of ultrasound and so suggests that ultrasonic algal control could be an ecosystem-friendly option. Easily the most environmentally friendly option are methods of biological control (Chang *et al*, 2012; &Wang *et al*, 2011), however due to their low rate of reliability this is not considered a feasible control method of large-scale algal control (Chang *et al*, 2012). While ultrasonic control requires a power supply in order to operate, making it less environmentally friendly than biological control, it is certainly more dependable (Zhang *et al*, 2006b).

Therefore using ultrasonic control instead of previously established methods of algal control will be easier and more readily available, have fewer environmental consequences and be a more reliable option.

Further study considerations

The field experiment conducted for this study examined the effect of ultrasound over a 9 week period due to time restrictions involved. A more thorough investigation may compare the effect of ultrasound treatment to control over a much longer time period as it common place in aquatic ecosystem monitoring studies. It is possible that while the wavelengths of ultrasound are not directly harming the zooplankton, perhaps due to their lack of gas vesicles, the loss of vesicle-containing organisms such as aquatic cyanobacteria and planktonic bacteria (Walsby *et al*, 1992), could pose a long term threat to the ecosystem. Either by the reduction of a species of unknown importance in the ecosystem or by the build-up of a substance released during acoustic cavitation, there are potentially unknown consequences that a nine week study would not pick up on.

Algal growth causes aesthetic problems in smaller bodies of water such as ornamental lakes and ponds (Ahn *et al*, 2003). Larger concerns arise when toxins contaminate drinking water (Ahn *et al*, 2003; & Dai *et al,* 2012) or when the presence of algae causes disruption of leisure activities such as when the Great North Swim had to be cancelled in 2010 (BBC, 2010). Contamination of larger bodies of water can therefore have large, detrimental economic consequences, which are becoming increasingly harmful to local societies due to the struggling world economy (Ahn *et al*, 2003; & BBC, 2010). Larger-scale studies need to be conducted firstly to determine the feasibility of the use of ultrasonic algae control for such big volumes of water and also to determine the practicalities of it e.g. how many units would be required, would power be available, would frequencies need to increase etc. The current expense of carrying out such investigations could prove to be beneficial to future generations if it provides a less expensive algal growth control method.

Previous study has shown that free radical production can lead to algal cell death when exposed to ultrasound (Mason, 2007a), however, the specific free radicals thought to be triggering programmed cell death are yet to be fully understood (Ahn *et al,* 2003; & Broekman *et al*, 2010). It is thought the difficulty in identifying these substances is due to their ability to be active at low quantities for very short periods of time, and therefore linking the presence of trace molecules to cell mortality is incredibly difficult (Broekman *et al*, 2010). While this study did not find a negative effect of ultrasound on zooplankton, it cannot be concluded that this indicates a resistance to these messenger molecules; only if free radicals are tested for, found to be present and still no detrimental

effects are observed could this conclusion be drawn and this will be incredibly difficult to achieve with today's technologies (Broekman *et al*, 2010).

Daphnia are known to be a sensitive zooplankton (Gannon and Stemberger, 1978; & Lal *et al*, 1984) however it is also know that other organisms, such as *Ceriodaphnia*, can be more sensitive to different stresses for example insecticides (Hayasaka *et al*, 2012). It may therefore be of interest to conduct further laboratory trials with freshwater species of zooplankton with a lower stress threshold than *Daphnia* to ensure that they do not suffer with the use of ultrasonic algal control.

Previous study involving saltwater ecosystems have shown that ultrasound at high frequencies can be used to remove non-native zooplankton species (Holm *et al*, 2008). It would be logical to conclude, therefore, that there is a limit to how much ultrasound zooplankton can receive before levels become harmful. As ultrasonic control has the potential to become a widely used technology, it would be sensible to conduct further study to determine the safe frequencies of ultrasound in various different conditions.

Conclusion

This study aimed to determine if new methods of ultrasonic algal control would cause detrimental effects on freshwater ecosystems, as some predecessor methods are prone to do. Studies in both the field and in the laboratory indicated that there are no such negative consequences of ultrasound use for *Daphnia* and ultrasound could develop into the best current method of algal control in terms of the lack of known environmental damage caused to non-target organisms.

Ultrasound still has its flaws: it cannot be a completely "green" technology, as it has been referred to in some literature (for example: Hutchinson, 2008) as it requires a power supply, it isn't a permanent, one-off solution (Wu *et al,* 2011) and there is no present way to determine the amount of money that large scale algal control by ultrasound may cost, of if it is even possible. However, previous studies show it is more reliable on a smaller scale than truly green biological methods of control and this study indicates that ultrasound may not have the same damaging effect on non-target organisms seen in other studies that used chemical methods of control (Wang *et al*, 2011; & Wu *et al*, 2011)

This study is a starting point for this area of research. It shows how on a small scale there is no apparent impact of ultrasound on *Daphnia,* and therefore may not be one for freshwater ecosystems as a whole but there are still many more points to address. Further research must be performed to develop the technology from its current application in small ponds, lakes and pools to operate at a more industrial scale. In order to control algal growth and prevent toxins in large bodies

of drinking water, particularly in developing countries where chemicals are currently often used out of necessity, larger, more powerful ultrasound devices will doubtless be needed. It is imperative that such advances in the equipment involved do not come at a cost to the non-target plants and organisms living as part of freshwater ecosystems and so studies such as this must be conducted at each stage of production.

Ultrasound technologies are presently used to great effect in the UK for algal control largely for aesthetic reasons, but their real social and environmental benefit will be further afield in economically less developed countries where they could replace currently used chemical alternatives. The potential is vast but these technologies are still at an early stage of development.

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Appendices

Appendix I: Raw data showing the sum species and corresponding number each week for lake sections B and D (the control sections).

	1	2	3	4	5	6	7	8	9
Capnia bifrons								1	3
Cyclops sp.		28	19	6	21	8	3	9	11
Cyclops strenuus	23	6	15		3	3	2		9
Daphnia	161	86	109	120	68	202	87	98	59
Diaptomus sp.	4								
Fly larvae		2					2		
Gammarus spp.	33	12	4	6	6	3	3	9	4
Hydropsyche sp.				1					
Hydrozetes lacustris		9	5	1					
Hydrozetes lacustris				2	3	1	3	7	10
Orthocladius sp.			2				5	22	12
Tanytarsus sp.	2	4							
Thaumalea testacea	1								

Appendix II: Raw data showing the sum species and corresponding number each week for lake sections A and C (the ultrasound treated sections).

	1	2	3	4	5	6	7	8	9
Caenis horaria	1		1						
Capnia bifrons							2	6	1
Cyclops sp.		5	5	12	17	11	6	7	7
Cyclops strenuus	26	7	2	10	5	7	12	7	6
Daphnia	86	175	73	38	81	129	83	45	37
Diaptomus sp	25								
Gammarous spp.	17	4	2	5	7	4	4	1	6
Hydropsyche sp.			1	1					
Hydrozetes lacustris	2		4	4		3	5	6	3
Nemurella picteti	25								
Orthocladius sp.			1			2	12	2	3
Scarodytes halensis	7								