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Mytilus edulis as a Bioindicator of Water Pollution: Potential Histopathology Biomarkers in Dublin Bay and Newquay, Co. Clare

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Declaration

I, Gillian McHugo, declare that this thesis is my own work except where stated through references or in the Acknowledgements, and that it is 5,306 words in length.

Signed: _____

Abstract

Mytilus edulis mussels are frequently used as bioindicators of water pollution. Sections of the mussels can be examined microscopically for abnormalities (histopathology) and compared between sites with known differences in pollution levels to validate them as biomarkers of pollution. This study examines mussels collected in Newquay, Co. Clare or transplanted from Newquay to Dublin Bay for 2 or 4 months.

When analysed individually none of the 19 histopathological variables examined were good biomarkers of pollution. 4 variables were absent in all mussels and 9 were present but not in significantly different proportions between the sites. 6 were present in significantly different proportions but also had a statistically significant difference between the initial samples from each site, which were collected from the same population and exposed to the same levels of pollution.

When the results for each variable were combined together into a histopathological score, indicating overall mussel health, a statistically significant result was found. However it was affected by the sex of the mussels, since there were differences in the ratio of male and female mussels in the samples. To correct this one variable that was clearly affected by sex (atresia; the degeneration of the female oocytes) was removed. This new histopathological score was found to be a potential biomarker of water pollution since it was significantly higher in mussels that spent the duration of the study in the more polluted site and was not affected by sex, however its use is questionable since the individual variables were not significant.

Acknowledgements

I would like to thank Professor Wilson and Dr Giltrap for all of their help and encouragement over the course of this project. Dr Giltrap was particularly helpful in the lab, providing me with all the information and equipment I needed and examining a few of the samples herself as a quality control for my observations. Professor Wilson was my supervisor and gave me some excellent advice regarding the data analysis and many much needed corrections on an earlier draft of this thesis. I would also like to thank Andrea Lenderink for collecting and preserving the all samples that were examined as part of this project.

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1. Introduction

1.1 Marine Pollution

Marine pollution has a significant global impact, not just on marine and littoral organisms but on life in general. It has been estimated that 50% of the world's human population live a few 100km away from the coast and the estimated total impact associated with marine pollution by sewage is 3 million disability-adjusted life years (DALY) per year, with an estimated economic loss of 12 billion dollars per year (Shuval, 2003). These figures can only be expected to rise, along with sea levels, as more and more people come into contact with polluted sea water as a result of climate change. At Rio+20 world leaders agreed to 'take action to… achieve significant reductions in marine debris to prevent harm to the coastal and marine environment' (United Nations, 2012). Ireland also has other legal obligations to tackle marine pollution. For example the Convention for the Protection of the Marine Environment of the North-East Atlantic (or OSPAR Convention), which Ireland signed in 1992, requires signatories to 'take all possible steps to prevent and eliminate pollution' of the Atlantic (OSPAR Commission, 1992). In order to fulfil these obligations accurate measures of water pollution are needed, as set out in the Water Framework Directive which requires monitoring programmes of water quality to be established (European Parliament and Council, 2000). An integrated approach to marine monitoring is recommended by OSPAR (Davies & Vethaak, 2012). This allows for the fullest picture of the situation possible to be viewed and accounts for the problems associated with relying on just one measure of pollution. This is particularly important since there are many different types of pollutant in the marine environment and it would be impossible to measure them all using just one variable.

1.2 Mussel Histopathology

The common or blue mussel, *Mytilus edulis* (Linnaeus, 1759), has been used as a bioindicator since 1975 when Edward D. Goldberg suggested a global 'Mussel Watch' involving samples of mussels from sites all over the world being analysed annually to monitor global levels of water pollutants (Goldberg, 1975). There are many reasons why mussels are suitable as an indicator species, including their widespread geographic distribution and large, stable populations. They are sedentary, making it easy to move samples from one area to another in manipulative caging studies. They are also

relatively pollution resistant and can survive in areas that are too polluted for other bioindicator species to tolerate. They concentrate pollutants by factors between 10^2 and 10^5 and have relatively low levels of enzymatic activity and so can increase the concentrations of pollutants to more easily detectable levels that have half-lives of several months (Goldberg, 1986). Additionally, they are also of commercial importance as seafood and therefore in relation to public health (Sunila, 1986). Early studies used chemical analysis to test the tissue of the mussels for the presence of a number of pollutants, including transuranic elements such as plutonium, halogenated hydrocarbons like polychlorinated biphenyls (PCBs), petroleum hydrocarbons including benzopyrene and heavy metals like lead and cadmium (Farrington *et al.,* 1983).

However mussels can be used as bioindicators of pollutants in other ways. Since exposure to pollutants and contaminants in the water can have a detrimental effect on the health of mussels we can also examine their tissue for biomarkers. These are measured characteristics that can be used as indicators of the biological state of the organism and can be seen with a microscope. By examining cross sections of mussel samples under a microscope we can observe the effect the pollutant has on the health of the mussel itself instead of testing for the presence of the pollutant using chemical tests. This is called histopathology and involves dissecting the mussels and examining cross sections of their tissue on slides (Calabrese *et al.,* 1984). For example stress caused by environmental conditions, such as high levels of hydrocarbon pollution in the water, can significantly increase levels of atresia (degeneration of the oocytes) in female mussels (Lowe & Pipe, 1987). Laboratory tests have shown that exposure to untreated municipal wastewater or sewage can negatively affect the health of the gills of mussels (Akaishi *et al.,* 2007) and the exposure of mussels to crude oil has been shown to negatively affect their digestive system, causing epithelial thinning (Lowe, Moore & Clarke, 1981). It has also been found that the incidence and size of granulocytomas (an inflammatory response involving aggregation of cells, mainly granulocytes) increases with increasing pollution levels (Lowe & Moore, 1979).

1.3 Sites Chosen

Two sites were chosen for *M. edulis* caging studies; Dublin Bay and Newquay, Co. Clare. The Dublin Bay site was found to be higher than Newquay in levels of 23 out of 30 contaminants tested, including a number of polychlorinated biphenyls (PCBs),

cadmium, mercury and lead (Giltrap *et al.,* 2014). This is as a result of its location. It is beside Dublin city and the associated suburbs which are home to more than 1 million people. Dublin Bay contains Dublin Port which is the largest port in the country, accounting for 2/3 of Irish port traffic. Dublin is also home to a large number of industries and the Ringsend Waste Water Treatment Works (Lenderink, 2010). In contrast Newquay has little anthropogenic input, the main sources of potential pollution are agricultural run-off and untreated waste from domestic sources (Giltrap *et al.,* 2014). The levels of the contaminants tested in Newquay are of a similar range to those found in other less populated areas of Ireland such as Kinvara Bay, Co. Galway and the Shannon estuary while Dublin Bay had levels similar to other highly populated, industrial areas like Cork Harbour (Giltrap *et al.,* 2014). In general, the human impact on marine ecosystems around the coast of Ireland is classed as ‘high’, however this impact is classed as ‘very high’ in the area around Dublin Bay. Globally, 41% of the world’s oceans were classed as ‘medium high’ to ‘very high’. There are very few areas where the impact is classed as ‘very low’, mostly occurring at the poles (Halpern *et al.,* 2008).

2. Methods

2.1 Sample Collection

The *M. edulis* samples examined were collected as part of a previous pilot study in 2009 (Lenderink, 2010). The study took place at two sites with different levels of water pollution; the control or un-polluted site was in Newquay, Co. Clare and the more polluted, experimental site was in Dublin Bay, at the North Bank Lighthouse. The locations and coordinates of the sites can be seen on the map below (Fig. 1).

Figure 1: Map of Ireland showing the study sites with coordinates; the Dublin Bay site is marked with a circle and the Newquay site is marked with a square. Coordinates from Lenderink, 2010 and map of Ireland from: http://www.irish-tv.com/irishgreats/images/ireland_outline_map2.GIF.

The mussels were originally collected from Newquay, Co. Clare in August 2009. The experimental mussels were transplanted to the Dublin Bay site where they were placed in oyster bags with 14mm mesh and attached below the low tide mark to the

ladders of the North Bank Lighthouse. This ensured that the mussels would be kept submerged during low tides. The control mussels were placed in Newquay under similar conditions. The first samples of mussels were collected at t0 in August when the mussels were initially placed in the sites. At t0.5 in October mussels were sampled from the Dublin Bay site. The final samples were collected at t1 in December from both the Newquay and Dublin Bay sites.

As a result of this previous study the mussels had already been preserved and stained in cross sections on slides in accordance with the methods laid out in the International Council for the Exploration of the Seas (ICES) Techniques in Marine Sciences (TIMES) *Mytilus edulis* histopathology manual (Bignell *et al.,* Unpublished).

2.2 Data Collection

The histopathology of the mussels was examined by viewing the cross sections on slides using a light microscope (Olympus Bx41). The mussels were measured and their sex, gonadal stage and adipogranular (ADG) rate were assessed. The mussels were carefully examined using a scanning technique for parasites and other abnormalities. Keys from the ICES manual were used to identify these potential biomarkers (Bignell *et al.,* Unpublished). A summary of the variables examined and the methods and measurements used to record them can be found in the table below (Table 1).

Table 1: Table showing the name of the variables, methods used to identify them and the measurements recorded.

Variable	Method Used	Measurement Recorded
Length of the mussel	Maximum dimension measured with a ruler	Nearest 1 mm recorded
Sex of the mussel	Identified using keys from the ICES manual (Bignell *et al.,* Unpublished)	1 for male, 0 for female
Gonadal stage of the mussel	Identified using keys from the ICES manual (Bignell *et al.,* Unpublished)	Scored using the mussel development stages (Ellis *et al.,* 1998b) recommended in (Bignell *et al.,* Unpublished)

Variable	Method Used	Measurement Recorded
Adipogranular (ADG) rate of the mussel	Identified using keys from the ICES manual (Bignell *et al.,* Unpublished)	Scored using *Mytilus* spp. adipogranular (ADG) tissue scoring index from (Bignell *et al.,* 2008)
Level of digestive epithelial atrophy	Identified using keys from the ICES manual (Bignell *et al.,* Unpublished)	Scored using epithelial atrophy scale (Kim *et al.*, 2006), adapted from (Ellis *et al.,* 1998a) and recommended in the ICES manual (Bignell *et al.,* Unpublished); 0 for no atrophy, 1 for less than 50% atrophied, 2 for averaging 50% atrophied, 3 for greater than 50% atrophied, 4 for 100% atrophied
Presence of: - *Ancistrum mytili,* gregarine, *Marteilia* sp., and *Mytilicola* sp. parasites - apoptosis - atresia - brown cells in the gills, gonads, and vascular connective tissue or digestive diverticula - granulocytomas - inflammation - intersex characteristics - metacercarial stages - pearls - *Rickettsia*-like organisms or *Chlamydia*-like organisms in the digestive glands and gills	Identified using keys from the ICES manual (Bignell *et al.,* Unpublished)	1 for presence, 0 for absence

2.3 Data Analysis

Microsoft Excel was used to compile the results. The gonadal stage and ADG rate were converted into presence or absence type data by comparing the actual results with those predicted for mussels at that time of year according to a representative graph showing typical the reproductive cycle of *M. edulis* (Bignell *et al.,* Unpublished). A mussel was scored as 0 if the stage or rate was within the normal range predicted for the time of year and 1 if the stage or rate differed from the predicted result, meaning it was abnormal. The level of digestive epithelial atrophy was categorical since all the mussels scored either 0 or 1 on the scale. The categorical variables (normality of gonadal stage and ADG rate of the mussels, level of digestive epithelial atrophy, presence or absence of *A. mytili,* gregarine, *Marteilia* sp.*,* and *Mytilicola* sp. parasites, apoptosis, atresia, brown cells in the gills, gonads, and vascular connective tissue or digestive diverticula, granulocytomas, inflammation , intersex characteristics, metacercarial stages, pearls and *Rickettsia*-like organisms or *Chlamydia*-like organisms in the digestive glands and gills) were then summed for each mussel to produce a 'histopathological score'. A lower score means that the mussel is healthier since it has less parasites or abnormalities. A higher score indicates a less healthy mussel that has a greater number of parasites and abnormalities.

The computer programme R (version Rx64 3.1.1) was used to test for a statistically significant difference between the results from the different sites (R Core Team, 2014). The numerical variables (length and histopathological score) were plotted and checked for normality using visual analysis of the histograms and boxplots were drawn to help visualise the data. Then ANOVAs were used to test for significant differences between the data sets (Dublin Bay t0, t0.5 and t1, Newquay t0 and t1). If the results were significant Tukey's HSD post-hoc tests were used to identify the significant differences. For the categorical variables Chi-square tests were used to test for differences between the data sets and the Marascuilo procedure was used to identify the significant differences (Prins *et al.,* 2003). The categorical variables were plotted using bar plots showing the proportion of mussels in each category. A general linear model was run using the 'lm' function in R to test the relationship between the histopathological score and the length of the mussels and a two-way ANOVA was run to test the effects of site and sex on the histopathological score of the mussels. The results collected for atresia (degeneration of the oocytes) were removed from the calculations

for histopathological score since it was clearly affected by the sex of the mussels and the tests were repeated using this new histopathological score.

3. Results

3.1 Variables Absent from All Mussels

The following variables were not found in any of the mussels examined:

- Gregarine parasites
- Intersex characteristics
- *Marteilia* parasites
- Pearls - None of the mussels examined had any pearls present in the tissue preserved on slides but this could be a result of the difficulty of slicing pearls into sections as part of the mussels (Bignell *et al.,* Unpublished). This was kept in mind as the mussels were examined under the microscope however no obvious holes that could have potentially contained pearls were found.

3.2 Insignificant Variables

When tested with Chi-square tests there was no significant difference in the proportions of the following variables found between the sites:

- Level of digestive epithelial atrophy (X-squared = 7.2519, df = 4, p = 0.1232)
- Presence of apoptosis (X-squared = 9.3364, df = 4, p = 0.05322)
- Presence of brown cells in the gills (X-squared = 5.8191, df = 4, p = 0.2131)
- Presence of brown cells in the gonads (X-squared = 2.2431, df = 4, p = 0.6911), only 4 mussels had visible brown cells in their gonads.
- Presence of inflammation (X-squared = 3.1129, df = 4, p = 0.5391), only one mussel showed evidence of inflammation.
- Presence of metacercarial stages (X-squared = 6.8978, df = 4, p = 0.1414)
- Presence of *Mytilicola* parasites (X-squared = 7.4024, df = 4, p = 0.1161)
- Presence of *Rickettsia*-Like Organisms or *Chlamydia*-Like Organisms in the digestive glands (X-squared = 6.5255, df = 4, p = 0.1632), only 4 mussels had RLO/CLO in their digestive glands.
- Presence of *Rickettsia*-Like Organisms or *Chlamydia*-Like Organisms in the gills (X-squared = 4.6511, df = 4, p = 0.325)

3.3 Variables with Significant Differences between Dublin t0 and Newquay t0

Although these variables had significant results according to Chi-square tests, when examined using the Marascuilo procedure they had significant differences between Dublin t0 and Newquay t0. Since these came from the same original population of mussels in Newquay before the study started, and had been exposed to the same conditions, any differences between them cannot be due to pollution. An example of the results found and a list of variables affected can be found below.

3.3.1 Presence of Atresia

Since atresia is the degeneration of the oocytes it is only found in female mussels. To correct for this only the presence or absence of atresia in female mussels was taken into account. A paired bar chart showing the differences in proportion of female mussels with atresia was plotted and examined (Fig. 4).

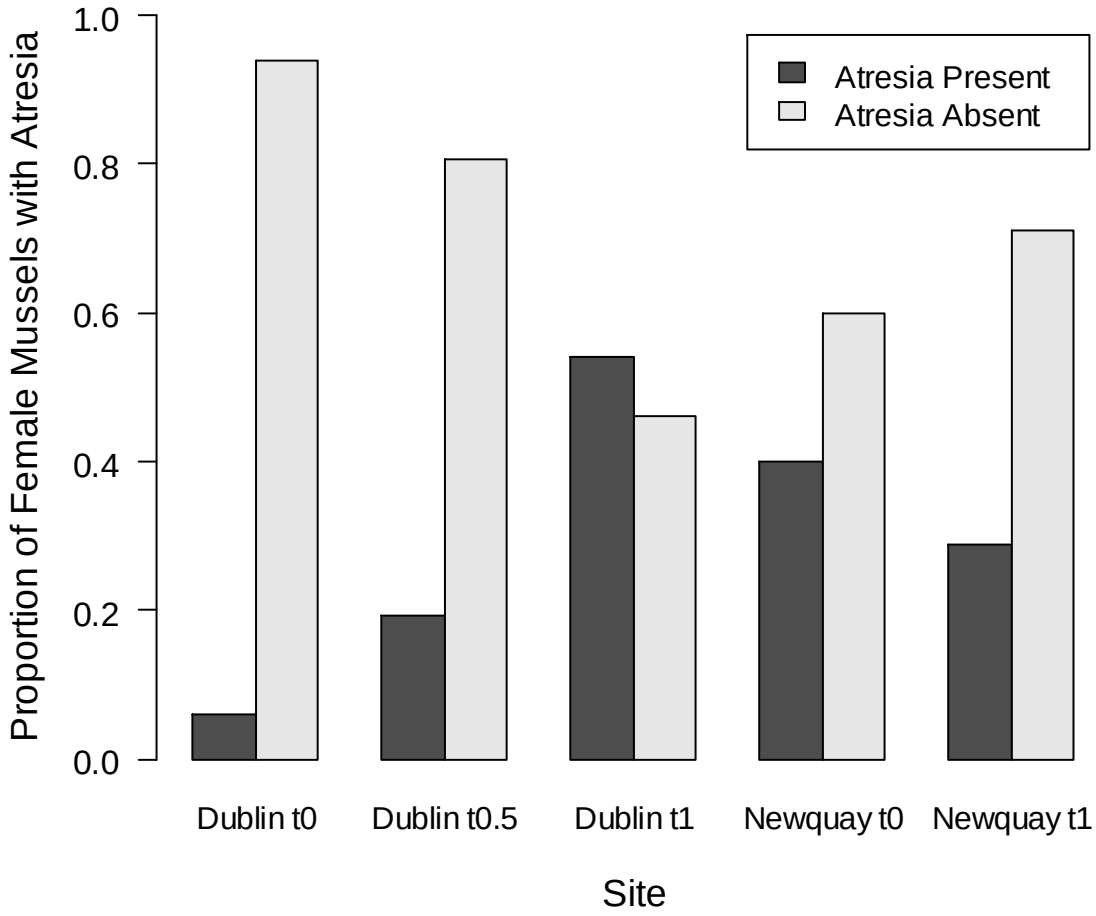

Figure 2: Paired bar chart showing the proportions of female mussels with atresia present and absent in the different sites. The dark bars show proportion of mussels with atresia and the lighter bars show the proportion of normal mussels with atresia absent.

A Chi-square test found there to be a significant difference in the proportion of female mussels with atresia from each site (X-squared = 15.1645, df = 4, p = 0.004372). When tested using the Marascuilo procedure, there was a significant difference in the proportions of female mussels with atresia from Dublin t0 and Newquay t0 (difference = -0.394, absolute difference = 0.394, critical range = 0.115). Since these samples were taken from the same population of mussels exposed to the same conditions of pollution before the study began any differences in proportion of mussels with atresia can't be due to their exposure to pollution.

3.3.2 Other Variables with Significant Differences between Dublin t0 and Newquay t0

Other variables that had a significant difference between Dublin t0 and Newquay t0, and what the difference was, can be found in the table below (Table 2).

Table 2: Table showing variables which had a significant difference in the proportions of mussels with and without the condition between Dublin t0 and Newquay t0. In order for the difference to be significant according to the Marascuilo procedure the absolute difference must be greater than the critical range.

Variable	Dublin t0 - Newquay t0 Difference	Absolute Difference	Critical Range
ADG Rate Normality	-0.167	0.167	0.120
Presence of *A. mytili*	-0.2	0.2	0.097
Presence of Atresia	-0.394	0.394	0.115
Presence of Brown Cells in the Vascular Connective Tissue or Digestive Diverticula	0.327	0.327	0.107
Presence of Granulocytomas	0.153	0.153	0.119
Gonadal Stage Normality	-0.773	0.773	0.079

In addition, there was no significant difference in the proportion of mussels with *A. mytili* parasites between Dublin t0 and Dublin t1 (difference = -0.06, absolute difference = 0.06, critical range = 0.084), so the group of mussels that had spent the duration of the study in Dublin Bay had a similar proportion of mussels infected with *A. mytili* parasites as the group from the start of the study. This means that the pollution in Dublin Bay had no impact on the proportion of mussels with *A. mytili* parasites visible in their tissue. There were similar results for granulocytomas, which also had no significant difference in the proportion of mussels with granulocytomas between mussels from

Dublin t0 and t1 (difference = -0.1, absolute difference = 0.1, critical range = 0.105). There was actually a decrease in the proportion of mussels with brown cells in their vascular connective tissue or digestive diverticula in Dublin between t0 and t1 (difference = 0.26, critical range = 0.114) so it is clear that exposure to the pollution in Dublin Bay didn't increase the proportion of mussels with brown cells visible in their vascular connective tissue or digestive diverticula.

Similarly, there was no significant difference in the proportion of mussels with an abnormal ADG rate between Dublin t1 and Newquay t1 (difference = 0.096, critical range = 0.114) meaning that there was no significant difference in the proportion of mussels with an abnormal ADG rate between the mussels that had been exposed to the higher pollution levels in Dublin for the duration of the study and those that were in Newquay for the same length of time. There was also no significant difference in the proportion of mussels with an abnormal gonadal stage between Newquay t0 and Dublin t1 (difference = -0.007, absolute difference = 0.007, critical range=0.061). This means that mussels from the start of the study in Newquay and the end of the study in Dublin Bay had no significant difference in the proportion with abnormal gonadal stages for the time of year.

3.4 Histopathological Score

The histopathological score for each mussel was calculated by adding together the results for the categorical variables examined. This means that a mussel with a histopathological score of 0 has no parasites, evidence of degeneration or inflammation etc. and the gonadal stage and ADG rate are in the expected range for the time of year. The maximum possible histopathological score is 19; a mussel with this score would have every abnormality and parasite examined for. After calculating the histopathological score for each mussel a histogram was plotted and visually examined for normality. A series of boxplots were drawn showing the histopathological scores for each site (Fig. 3).

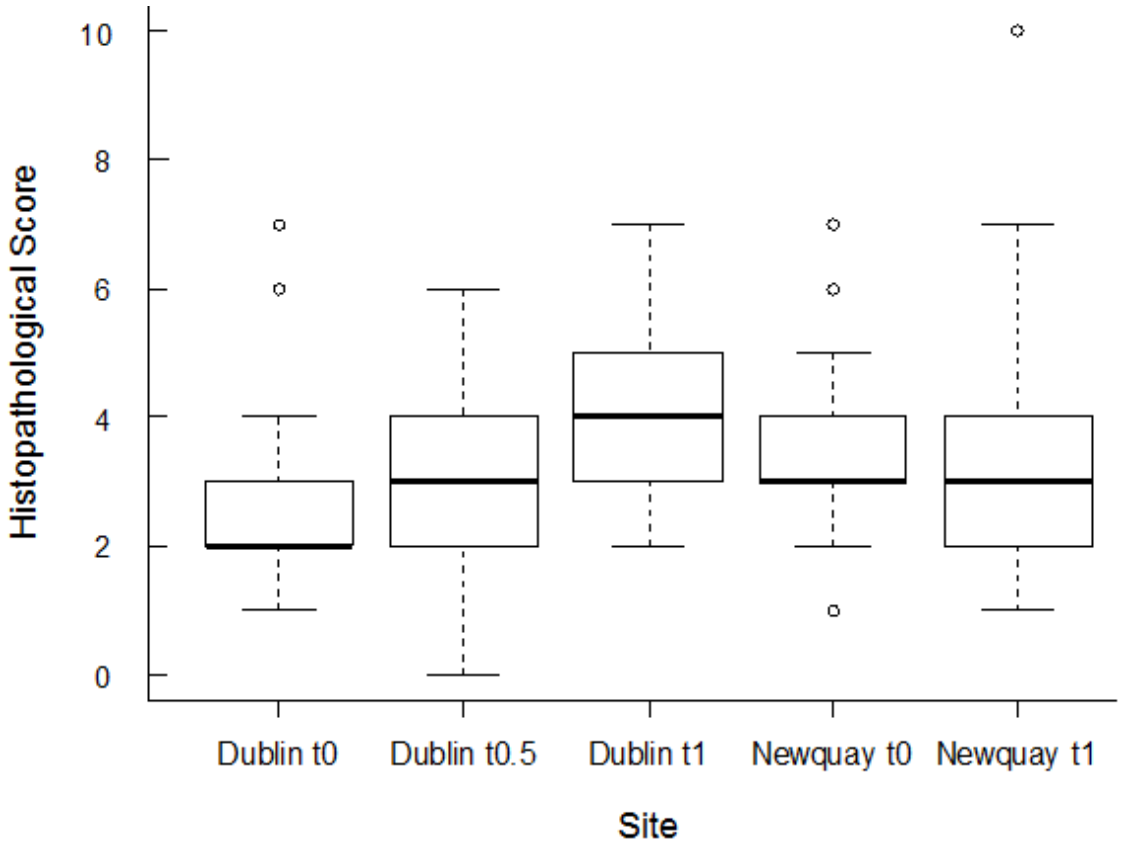

Figure 3: Boxplots showing the histopathological scores calculated for mussels from the different sites. A lower score indicates healthier mussels, the maximum score for any mussel is 19.

An ANOVA was used to test for statistically significant differences between the results from the different sites. A significant difference was found ($p = 9.06e^{-05}$, df = 4, sum of squares = 59.7) so Tukey's HSD post-hoc test was carried out. The mussels in Dublin Bay for the duration of the study had significantly higher scores than those that were in Newquay for the same period of time (Dublin t1 - Newquay t1, difference = 0.872, p = 0.038311). There were also significant differences between mussels that were in Dublin Bay for longer periods of time; Dublin t1 - Dublin t0 (difference = 1.46, p = 0.0000413) and Dublin t1 - Dublin t0.5 (difference = 1.275, p = 0.006786). This means that although the time spent in Dublin Bay did eventually affect the health of the mussels it took until the end of the study at t1 for these effects to be significant. This shows that while pollution levels of water do affect the health of the mussels living in them there is a minimum time period that the mussels must be exposed to the pollution for before the impact on their health is significant. However the histopathological score was calculated using variables that, when taken individually, were found to be insignificant or had significant differences between the two original groups of mussels from Dublin t0 and Newquay t0 so the results are questionable. Possible effects of the length or sex of the mussels on the histopathological score were then tested for.

3.5 Effect of Length

The lengths of the mussels were plotted as a series of boxplots (Fig. 4). The length of the mussels was plotted as a histogram and visually assessed for normality, it was found to be skewed and log transformed to correct this.

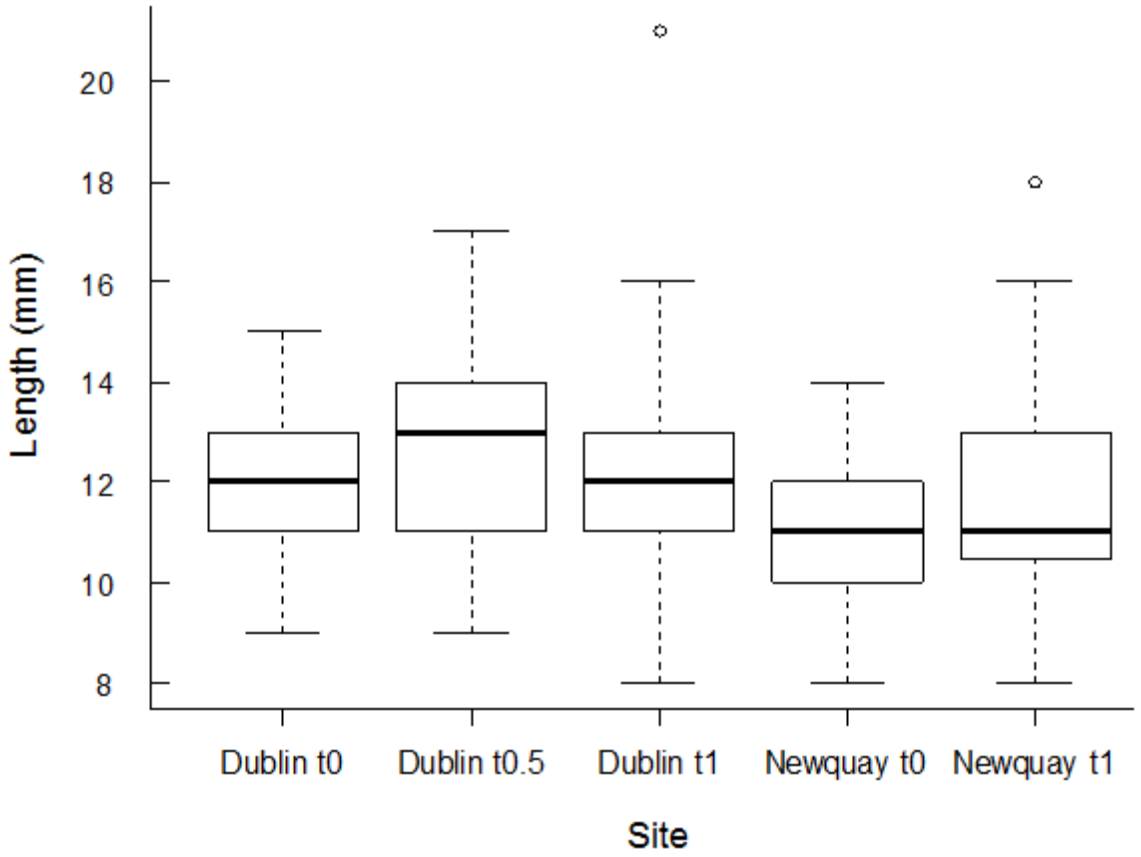

Figure 4: Boxplots showing mean length of the mussels from the different sites in millimetres.

An ANOVA was used to test for statistically significant differences between the logged results from the different sites. A significant difference was found ($p = 0.00699$, $df = 4$, sum of squares = 0.0746) so Tukey's HSD post-hoc test was carried out. There was only a significant difference found between the lengths of the mussels from t0 in Newquay and t0.5 in Dublin Bay (Newquay t0 - Dublin t0.5, difference: -0.070, $p = 0.0033974$). The reason for this is unknown since when collecting the mussels care was taken to ensure that they were all of a similar size (Lenderink, 2010). A general linear model was run using the 'lm' function in R to test the relationship between the histopathological score and the length of the mussels. It found that there was no significant relationship between them ($p = 0.985$).

3.6 Effect of Sex

A paired bar chart was plotted showing the proportions of male and female mussels from each site (Fig. 3) and the differences were examined using a Chi-squared test and the Marascuilo procedure.

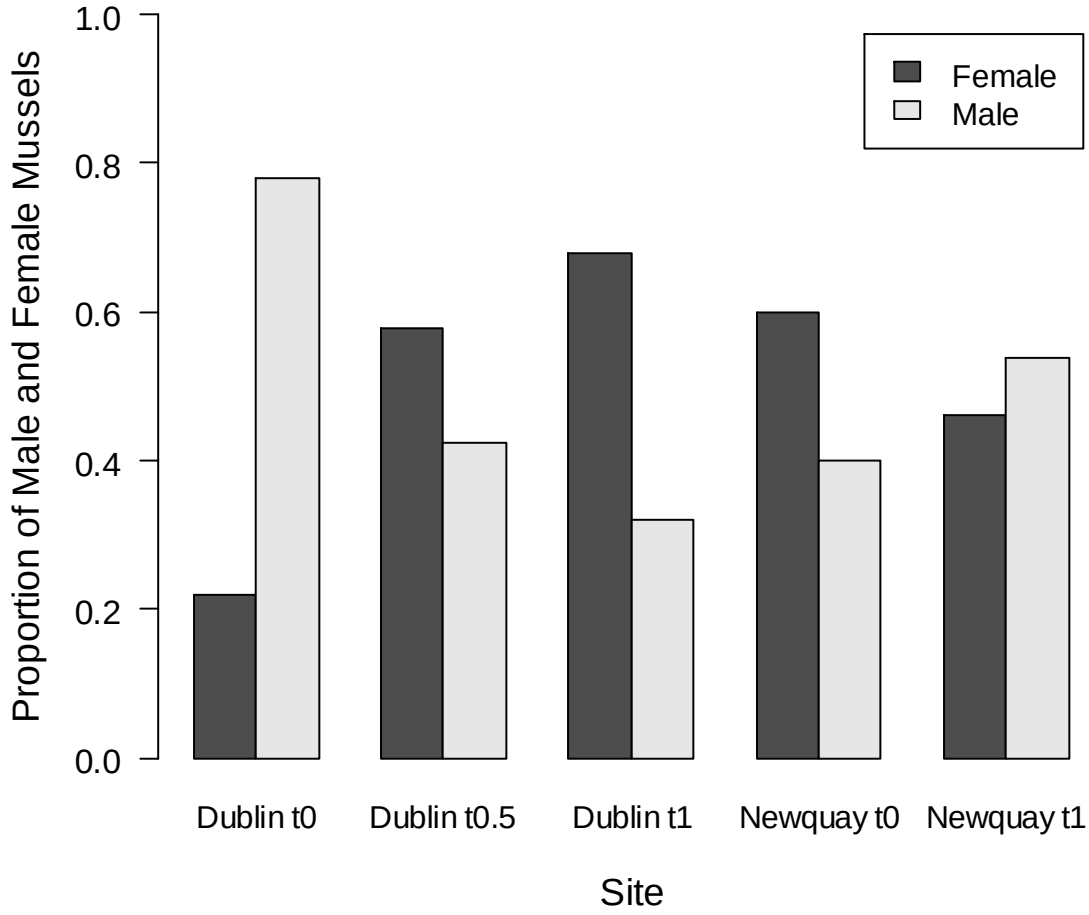

Figure 5: Paired bar chart showing the proportions of male and female mussels in the different sites. The dark bars show proportion of female mussels and the lighter bars show male mussels.

There was a significant difference between the groups with respect to the sex of the mussels (X-squared = 24.2151, df = 4, p-value = 7.232e-05). The reasons for this are unknown but could be as a result of a skewed sex ratio in the population of mussels from which the original samples came since the sex of the mussels could only be identified after dissection (Lenderink, 2010). The differences in proportions of male mussels between the initial samples (Dublin t0 - Newquay t0, difference = 0.38, critical range = 0.114) may also explain some of the differences between the sites with respect to other variables measured. A two-way ANOVA was used to test the effects of site and sex on the histopathological score of the mussels. While no interaction between site and sex was found (df = 4, Sum of Squares = 10.1, p = 0.36057), a significant effect of sex on histopathological score was found (df = 1, Sum of Squares = 16.4, p = 0.00833). As

can be seen in the paired bar chart below (Fig. 6) there is a significant difference in the mean histopathological score of male and female mussels from Dublin t1.

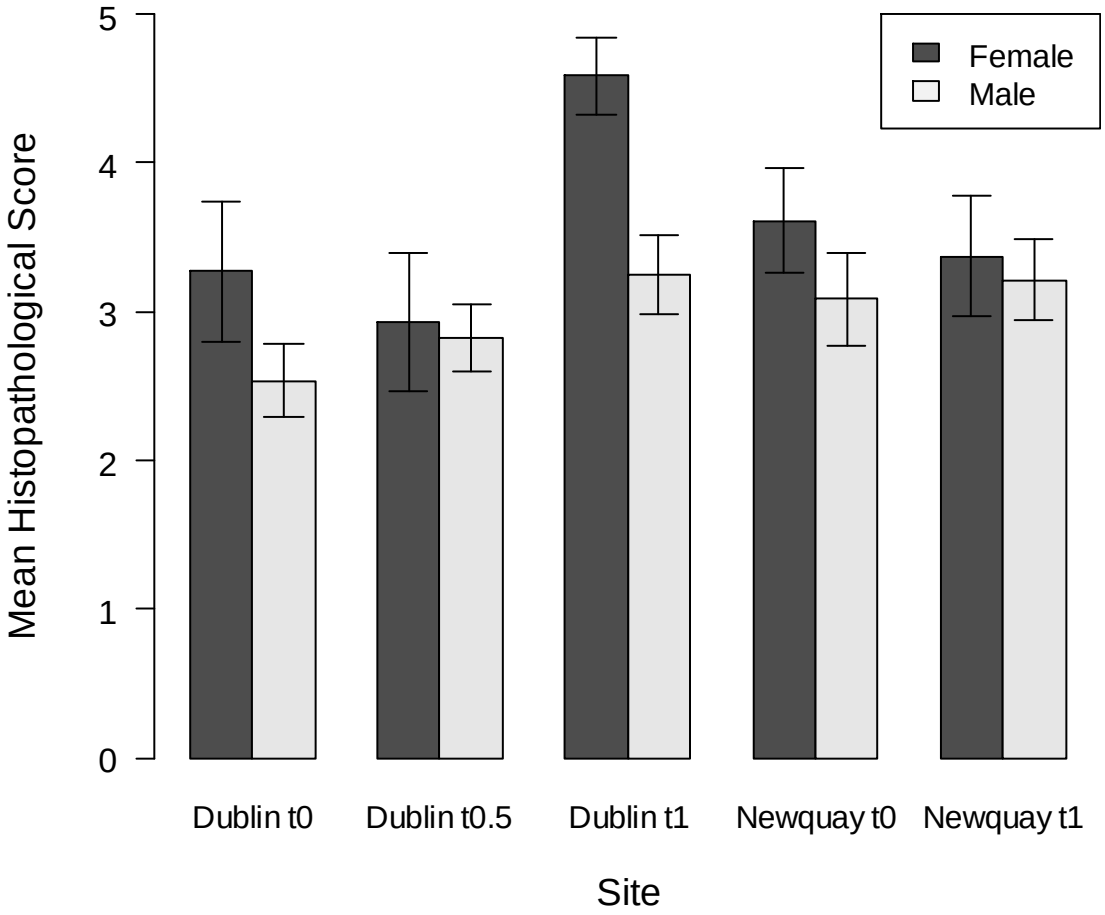

Figure 6: Paired bar chart showing the mean histopathological score for male and female mussels from the different sites. The dark bars show the mean score of female mussels and the lighter bars show the mean score of male mussels. The error bars show standard error.

In an effort to correct for this effect the calculations for the histopathological score were run again, this time omitting the data collected for atresia since it is only found in female mussels. Similar results to the original histopathological score were found, as can be seen below (Fig. 7).

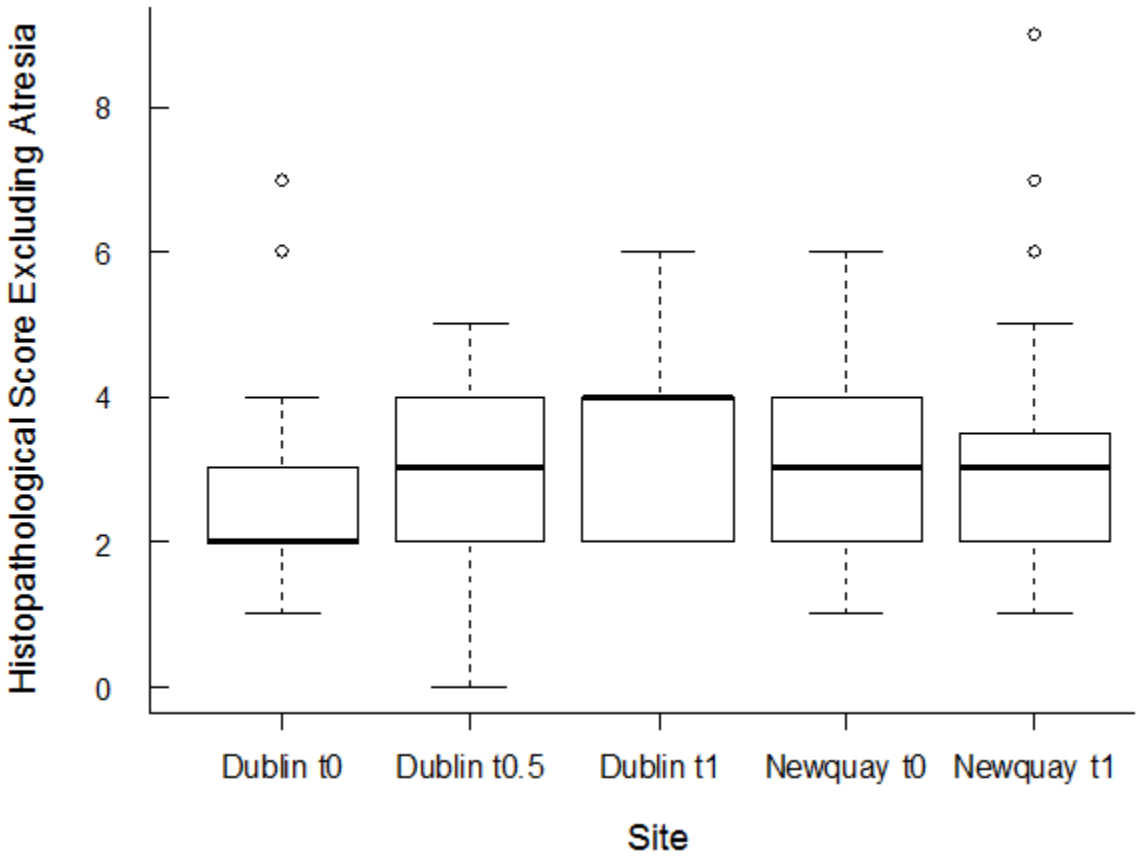

Figure 7: Boxplots showing the histopathological scores calculated for mussels from the different sites, excluding the data collected for atresia. A lower score indicates healthier mussels; the maximum score for any mussel is 18.

An ANOVA found a significant difference between the sites ($p = 0.00998$, $df = 4$, sum of squares = 28.0). However Tukey's HSD post-hoc test found that there was only a significant difference between Dublin t0 and Dublin t1 (difference = 0.98, $p = 0.0067297$). When tested with a two-way ANOVA no significant effect of sex on histopathological score was found ($df = 1$, sum of squares = 0.0, $p = 0.8781$).

4. Discussion

4.1 Individual Variables

Of the individual variables with significant results, statistically significant differences were found between the mussels from t0 in Newquay and t0 in Dublin Bay for 6. Since these initial mussel samples were taken from the same population which had been exposed to the same conditions of pollution in Newquay before the study began any differences between the groups with regard to these variables cannot be due to the pollution the mussels were exposed to and therefore calls into question their use as biomarkers of pollution. One of these variables was the sex of the mussels. A skewed sex ratio in the initial sample population may explain some of the differences between the groups of mussels in Newquay and Dublin Bay at t0 regarding the other individual variables since male and female mussels can concentrate pollutants differently under the same conditions (LaTouche & Mix, 1982). The only variable that had a statistically significant difference between the sites but no statistically significant difference between Newquay t0 and Dublin Bay t0 was the length of the mussels. It was found that the mussels at t0.5 in Dublin Bay were slightly larger than those at t0 in Newquay. The reasons for this are unknown since an effort was made to collect mussels of similar size (Lenderink, 2010), as specified in the OSPAR guidelines (Davies & Vethaak, 2012). However it is still important to note since the size of a mussel can affect its ability to concentrate pollutants and therefore act as a bioindicator (Mubiana *et al.*, 2006).

4.2 Histopathological Score

In contrast, when the histopathological score was calculated for each mussel by summing all the categorical variables, including those that were statistically insignificant, the mean score for each group followed the pattern predicted by the hypothesis. As predicted, the mean score of mussels from Dublin Bay at t1 was significantly higher than the mean score of mussels from Dublin at t0. This indicates less healthy mussels, which is logical since mussels collected from Dublin Bay at t1 had been exposed to higher pollution levels. However due to the differences in the initial sample groups regarding the individual variables the accuracy of the histopathological score as a biomarker is questionable, even when the effects of sex on the histopathological score are taken into account.

4.3 Potential of Mussel Histopathology

Mussel histopathology is recommended by OSPAR as part of their integrated approach to marine monitoring (Davies & Vethaak, 2012). Recently, it has been found that mussel histopathology is realistic, sensitive and robust in Irish waters. However it is not specific, takes time, requires skill and has a high cost in relation to the benefits. Despite the problems associated with mussel histopathology, it still scored 4 out of 5 for its use as a biomarker (Giltrap *et al.,* 2014). If the use of mussel histopathology as a biomarker in Irish waters was perfected it could increase the number of ways mussels, and their tissue, could be used as bioindicators of water pollution. This would allow for greater confidence in the results of such monitoring programmes since there would be more evidence provided. It could also increase the accuracy of 'mussel watch' programmes since chemical tests of the tissue of the mussels cannot identify the presence of every possible pollutant. By examining the mussels for the combined effects of all the pollutants and contaminants in an area an overall picture of the water quality and purity can be formed as opposed to the identification of the levels of a small variety of individual pollutants, as in the original Mussel Watch analysis (Farrington *et al.,* 1983).

4.4 Further Research Recommendations

If the project was to be repeated a greater effort to choose mussels of a similar size would be recommended. A balanced ratio of male to female mussels in all of the sample groups is also strongly recommended but, since the sex can only be determined after dissection, an increased sample size should be used to allow for the selection of a balanced number of male and female mussels. For unknown reasons there were different numbers of mussels in each sample group collected by the original experimenter (Lenderink, 2010), even though this was taken into account by the use of means and proportions in the data analysis this should be rectified in further research for ease of analysis. Further recommended research would also include a number of different sample sites around the coast of Ireland in order to avoid the problems associated with pseudoreplication. In addition, the mussels should be left at the sites for a longer exposure period. It has been recommended in the past that mussels be left in an area for at least 90 days or 3 months in order to obtain an accurate picture of the major pollutants present (Salazar & Salazar, 1995). This is confirmed by the results

from the histopathological score which only showed a significant difference at the end of the study, after 4 months, while the mussels from the middle sample after 2 months showed no significant difference.

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Appendix 1: Sample of Raw Data

Table showing sample of the raw data collected, showing one mussel from each site. The full data set is available from my supervisor, Professor Wilson, upon request.

Site	Dublin t0	Dublin t0.5	Dublin t1	Newquay t0	Newquay t1
Code on Slide	T0CS08-01	TICS09-01	CST2-01	IVT0-1	SwC-1
Length (mm)	15	15	10	13	11
Sex (female=0 male=1)	1	0	0	1	1
Gonadal Stage	D1	D2	S4	S4	S4
Normality of Gonadal Stage (expected=0, abnormal=1)	0	1	1	1	1
ADG Rate	3	3	3	3	3
Normality of ADG Rate (expected=0, abnormal=1)	0	0	0	0	0
Level of Digestive Epithelial Atrophy (0=<50% degenerated, 1=>50% degenerated)	0	0	1	0	0
Presence of *A. mytili* Parasites (absent=0, present=1)	0	1	0	1	0
Presence of Apoptosis (absent=0, present=1)	0	0	0	0	0
Presence of Atresia (absent=0, present=1)	0	1	1	0	0
Presence of Brown Cells in the Gill (absent=0, present=1)	0	0	1	0	1
Presence of Brown Cells in the Gonad (absent=0, present=1)	0	0	0	0	0
Presence of Brown Cells in the Vascular Connective Tissue or Digestive Diverticula (absent=0, present=1)	1	1	0	1	1
Presence of Granulocytomas (absent=0, present=1)	0	1	1	1	0
Presence of Gregarine Parasites (absent=0, present=1)	0	0	0	0	0
Presence of Intersex Characteristics (absent=0, present=1)	0	0	0	0	0
Presence of Inflammation (absent=0, present=1)	0	0	0	0	0
Presence of *Marteilia* sp. Parasites (absent=0, present=1)	0	0	0	0	0
Presence of Metacercarial Stages (absent=0, present=1)	0	0	1	0	0
Presence of *Mytilicola* sp. Parasites (absent=0, present=1)	0	1	1	0	0
Presence of Pearls (absent=0, present=1)	0	0	0	0	0
Presence of *Rickettsia*-Like Organisms or *Chlamydia*-Like Organisms in the Digestive Glands (absent=0, present=1)	0	0	0	0	0
Presence of *Rickettsia*-Like Organisms or *Chlamydia*-Like Organisms in the Gills (absent=0, present=1)	0	0	0	0	0
Histopathological Score (sum of categorical variables above)	1	6	7	4	3
Histopathological Score Excluding Atresia	1	5	6	4	3

Appendix 2: Proposal

Title: *Mytilus edulis* Histopathology as a Bioindicator of Pollution at North Bank Lighthouse, Dublin Bay and Newquay, Co. Clare

Section 1: Aim

The aim of my project is to carry out a correlative study to test the relationship between the histopathology of *Mytilus edulis* and the pollution level in the water at North Bank Lighthouse, Dublin Bay and Newquay, Co. Clare. It has been shown that mussels which have been exposed to higher levels of water pollution for a longer period of time will have the greatest amount of abnormal histopathology[1]. This means that mussels transplanted to the more polluted site at North Bank Lighthouse in Dublin Bay should show higher levels of abnormalities in their histopathology than those from the less polluted control site at Newquay, Co. Clare. The mussels collected from the North Bank site after 4 months should also have more abnormalities than those collected after 2 months. This can be seen in the graph below (Fig. 1) showing possible results for mean epithelial atrophy stage, one aspect of histopathology. The converse null hypothesis is that all the mussels will have similar histopathology regardless of the pollution level of the water they were exposed to.

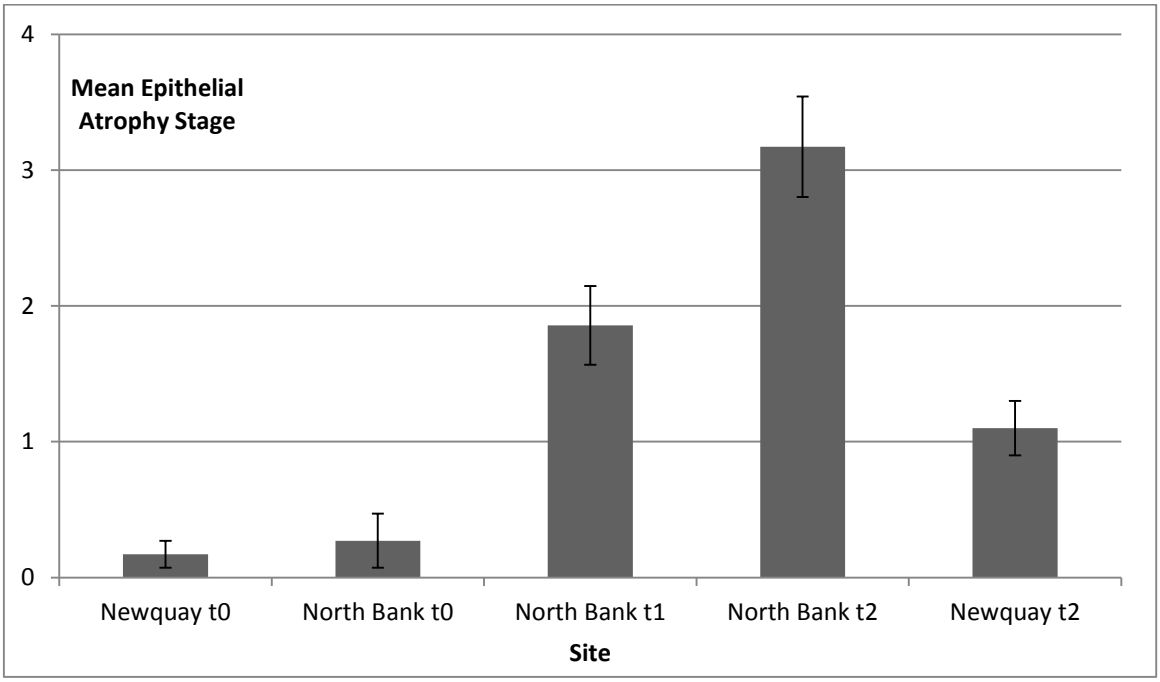

Figure 1: Bar chart showing possible results of mean epithelial atrophy stage for *Mytilus edulis* samples from sites with different water pollution levels.

Section 2: Background

Mytilus edulis, also known as the blue or common mussel, is an excellent bioindicator of water pollution. *M. edulis* and other *Mytilus* species have been used as indicators of various kinds of water pollution since the early 1970's[2]. In 1975 Edward D. Goldberg suggested a global 'Mussel Watch' where samples of mussels from sites all over the world would be analysed annually to track levels of water pollutants around the world[2]. The suggested study was carried out between 1977 and 1978 and a review of the results of the extensive analysis of pollutants and present in the samples was published in 1986[2]. In it a number of reasons why mussels are suitable as an indicator species are outlined. These reasons include the widespread geographic distribution of *M. edulis* which allows for easy comparisons between results from different areas. They are also sedentary organisms so give an accurate picture of the pollutant levels in a particular area. Since they are often found in large populations regular sampling is unlikely to have an adverse affect on their survival in an area. It is also easy to move samples from one area to another, as in caging studies, meaning that manipulative experiments are possible. Compared to other organisms bivalves are relatively pollution resistant, this means that samples can be obtained from areas that are too polluted for other bioindicator organisms to tolerate. They also concentrate the pollutants in the water by factors between 10^2 and 10^5. Since the pollutants are present in the water in such small amounts it is useful for the concentrations to be increased to a more easily detectable level. Bivalves also have relatively low levels of enzymatic activity that break down foreign chemicals when compared to other marine organisms including fish and crustaceans. As a result the biological half-lives of many pollutants in mussels can be several months[2]. As part of the 'Mussel Watch' programme *Mytilus edulis* and related species have been used as bioindicators of 4 major groups of pollutants. These include transuranic elements such as plutonium (^{239,240}Pu), halogenated hydrocarbons like polychlorinated biphenyl (PCB), petroleum hydrocarbons including benzopyrene and heavy metals like lead and cadmium[3]. The results from these studies were obtained through chemical analysis of the tissue of the mussels.

However this is not the only way they can be used as bioindicators. Since the exposure of *M. edulis* to pollutants and contaminants in the water can have a detrimental effect on the health of the mussel we can also examine the tissue of the mussels for biomarkers. Biomarkers are measured characteristics that can be used as indicators of the biological state of the organism of the organism and these effects can

be seen with a microscope. By examining the *Mytilus edulis* samples under a microscope we can observe the effect the pollutant has on the health of the mussel itself instead of testing for the presence of the pollutant using chemical tests. This is called histopathology and involves dissecting the mussels and examining cross sections of their tissue on slides. An example of this is examining the tissue for the presence of granulocytomas in the mussel[4]. Granulocytomas are a visible inflammatory response or a result of the presence of parasites formed by aggregations of granulocytes, a type of white blood cell. They can reach up to 1500µm in size and can be found as a single lesion or in groups scattered throughout the tissue of the organism. Granulocytomas can form as part of the inflammatory response of a mussel to pollution such as domestic and industrial waste in the water[4]. Another example of histopathology is the effect of pollutants on the digestive system[1]. Since the digestive system of the mussels comes into contact with contaminants from the external environment it is a good indication of the effect these contaminants can have on the mussel. When molluscs are exposed to pollutants there is visible thinning of the epithelial lining of the digestive system. This can be scored from 0, meaning normal epithelial thickness, to 4, meaning severe thinning and atrophy[1] (see Fig. 1 for possible results using this scale).

Section 3: Materials and Methods

The *Mytilus edulis* samples I will be examining were collected as part of a previous pilot study in 2009. As a result they have already been prepared and preserved in cross sections on slides in accordance with the method laid out in the International Council for the Exploration of the Seas Techniques in Marine Sciences *Mytilus edulis* histopathology manual[1]. This also means that there has already been data collected on the condition of the mussels which I can compare to my results[5]. Two sites with different levels of water pollution were chosen; the control or un-polluted site was in Newquay, Co. Clare and the more polluted site was at the North Bank Lighthouse in Dublin Bay[5]. The Dublin Bay site has been shown to have higher levels of pollution as a result of its location[5]. It is beside Dublin city and the associated suburbs which are home to more than 1 million people. Dublin Bay also contains Dublin Port which is the largest in the country, accounting for 2/3 of Irish port traffic. Dublin is also home to a large number of industries as well as the Ringsend Waste Water Treatment Works[5].

The mussels were placed in both of the sites as part of a caging study and left there for 4 months. This involved placing oyster bags containing mussels in the water at the chosen sites at a depth that will keep them submerged during low tides. The first samples of 30 Newquay mussels were collected at t0 in August when the mussels were initially placed in the sites. At t1 in October 30 mussels were removed from the North Bank site and 30 were collected at t2 in December from both the Newquay and North Bank sites.

For my study I will be examining the histopathology of the mussels by examining the cross sections on slides using a microscope. There are a number of different factors I could look at but the factors I plan to examine are presence and number of granulocytomas in the tissue, the level of digestive epithelial atrophy and the abundance and type of parasites present in the mussels. There are relevant scoring systems described for these factors in the ICES manual[1]. I will then use the computer programme R and GLMM to test for a statistically significant difference between the results from the different sites.

Section 4: References

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