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Effects of algal toxicity on the clearance rate of the blue mussel (*Mytilus edulis*)

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Abstract

The purpose of this study was to investigate the effect of toxic strains of *Karlodinium veneficum* on the clearance rate of *Mytilus edulis*. Suspensions of the toxic strain and the non-toxic strain of *K. veneficum*, mixed with an alternative non-toxic species, *Prorocentrum micans*, were made and performed as parallel experiments in order to compare their effects on clearance rate. Clearance rate was measured by taking samples from suspensions every five minutes up to 30 minutes, and counting the algal cells found within each sample using a Sedgewick rafter slide. No significant difference was found between the two experiments ($F(9.322, 79.237) = 0.743$, $P>0.05$). Further tests were performed in order to test the effects of varying concentrations of toxic *K. veneficum* on the clearance rate of *M. edulis*. Analysis of these experiments also disproved the hypothesis, indicating an insignificant difference between experiments ($F(2.962, 23.696) = 1.265$, $p>0.05$). Due to wide ranges of standard error within collected data, a control experiment was performed using no mussels in order to show that changes in algal concentration were in fact caused by mussel filtration and not sampling error. The results of this experiment were significantly different from those involving mussels ($F(3.870, 61.926) = 0.636$, $P>0.05$), proving that results were valid. As a result of analysis, the hypothesis of this study cannot be accepted, and further research is considered to be necessary.

Key words

Filtration - toxic algal blooms - bivalve - algal cultures - algal cell density

Introduction

The blue mussel, *Mytilus edulis*, has long been used as a biological indicator, with its popularity stemming from the Mussel Watch Program (Goldberg, 1975). This and other *Mytilus* spp. have since been used to indicate and monitor anthropogenic pollution events such as oil (Alyakrinskaya, 1966), detergents and emulsifiers (Foret-Montardo, 1970; Swedmark et al, 1971), heavy metals (Manga & Hughes, 1981; Zatta et al, 1992), radioactive waste (Nagaya & Folsom, 1964; Young & Folsom, 1967) and halogenated hydrocarbons (Koeham & van Genderen, 1972; Roberts, 1973). Natural environmental changes, for example in temperature (Pörtner, 2001) and salinity, have also been assessed using *Mytilus* spp. *M. edulis* is considered to be such an advantageous biological indicator as it is found to be highly tolerant to environmental changes (O'Sullivan, 1971). Being an accumulation bioindicator also allows for the species to readily reflect these changes detected in the surrounding seawater (Lee et al, 1972; Fossato & Siviero, 1974). Bivalves such as *Mytilus* spp. are able to concentrate certain compounds to levels which may be 1000 to 10 000 times that of surrounding seawater concentrations (Serafim, 2008; Swackhamer et al, 2009). These contaminants are also not at all or only minimally altered after uptake, allowing for simple analysis (Gilek, 1996).

Mussels are known to access toxins which may be accumulated through filter feeding. Some species of algae, which mussels commonly feed on, are known to produce harmful toxins. *M. edulis* is an extremely tolerant organism and is often thought to cope with such algal toxins, as the species shows no apparent harm (Alyakrinskaya, 1966). Focus is more often placed on the effects of such toxins on consumers of the mussels. However, studies have investigated potential threats and significant effects of toxic algal species on *M. edulis*. Effects of exposure to toxic algae include restriction of shell growth (Nielsen & Strømgren, 1991) and inhibition of gene expression and cell functionality (Buratti et al, 2011). Within these studies, the factors examined are considered to be indicators of the health of mussels.

Clearance rate, which refers to "the volume of water cleared from suspended particles per unit of time" (Riisgård, 2001), is a well studied indicator of the health of *M. edulis* (Duchemin et al, 2008; Scarlett et al, 2011; Brooks et al, 2009). It is therefore possible that exposure to toxic algae may have a detrimental effect on clearance rate, as on other studied indicators of mussel health. This study therefore aims to research this potential relationship by exposing *M. edulis* to toxic and non-toxic strains of an algal culture. The effects of this exposure on clearance rates of the mussels will then be measured and compared. It is hypothesised that there will be a significant difference between clearance rates for non-toxic and toxic algae. Clearance rate is suspected to be substantially reduced when mussels are exposed to toxic algae. Extending from this, *Mytilus* has been known to show a limit to its tolerance to some contaminants (Widdows et al, 1982). It therefore may be possible for *M. edulis* to present a concentration threshold at which the species cannot tolerate uptake of toxic algal cells.

Materials and Methods

Study organisms and conditions

Mussels identified as *M. edulis* were collected from a rocky region of Whitsand Bay beach, south east Cornwall, UK, during low tide. Mussels were selected by size, measuring 3 cm in length (maximum anterior-posterior measurement). They were placed in a large plastic bucket and covered with brown seaweed (Laminaria) to prevent drying out during transport to the laboratory. The organisms were placed in a holding tank filled with seawater and provided with a pump which continuously flushed fresh seawater into the system. As the water used contained natural levels of phytoplankton, no supplementary food was given. The tank was emptied of water and pseudofaeces, cleaned and refilled every few days. The mussels were in the tank for several weeks before experimentation commenced. During experiments, selected mussels were transported in a bucket with a small amount of seawater, to glass beakers within a controlled temperature room set at 12 ºC.

The study took place at the Marine Biological Association in Plymouth, Devon. This location houses the Plymouth Culture Collection which supports the growth and maintenance of algal cultures. *Karlodinium veneficum* was selected for this study as it is a natural food source for *M. edulis*, and was also available in two strains; toxic and non-toxic. However, these two strains are indistinguishable from each other when viewing with a microscope, so another species, *Prorocentrum micans*, which is non-toxic and morphologically different, was also selected. This species was to act as an alternative, allowing for parallel experiments comparing effects of the two algal treatments.

Experimental design

This study aimed to test the hypothesis that there is a difference between the clearance rates of toxic and non-toxic strains of the alga, *K. veneficum* in the mussel, *M. edulis*. In order to test this hypothesis, two main experiments were performed; the toxic experiment and the non-toxic experiment. To ensure that any differences found were due to cell clearance by the mussel and no other factor, a further experiment was completed, repeating the toxic experiment conditions, but with the mussel removed. It was expected that cell density would remain the same throughout this experiment. Finally, another repeat of the toxic experiment was performed using a higher density of toxic algal cells. This was to determine whether *M. edulis* had a threshold up to which it would feed upon toxic algae.

Several factors were controlled during the experimental process including temperature, mussel size, volume of liquid and length of experiment. Five replicates were achieved for each experiment.

Data collection procedures

All experiments took place in the controlled temperature room and involved five mussels individually placed within five 500 ml glass beakers. Glass pipettes attached to oxygen lines were placed in each beaker, creating a circulation within the solution. All solutions had varying amounts of algal culture which were diluted with seawater to create equal concentrations within each experiment. All solutions were made up to equal volumes of 200 ml in measuring cylinders. Experiment time began as soon as these solutions were added to the beakers containing mussels. The replicates were staggered at one minute intervals in order to allow for all replicates to be tested at once. A 1 ml sample was taken from a similar location in each solution using a

pipette at five minute intervals for 30 minutes. This sample was released into a labelled eppendorf tube and fixed using Lugol's iodine solution. These samples were each later discharged onto a Sedgewick Rafter slide, and the cells counted using a light microscope. Mussels used in all experiments had their flesh removed and placed into individual labelled foil cups. The flesh was placed into an oven at 60 ºC overnight, and then, in order to obtain each mussel's dry weight, was transported using a 'dessicator' containing silica gel crystals, to a Sartorius analytical balance set to three decimal places.

Toxic experiment
Each of the five replicate suspensions contained 73 ml toxic *K. veneficum* culture, 54 ml *P. micans*, and 73 ml seawater. This created a toxic and alternative algal concentration of 20 000 and 5 000cells/ml respectively.

Non-toxic experiment
Five replicate suspensions contained 121 ml non-toxic *K. veneficum* culture, 54 ml *P. micans*, and 25 ml seawater. This created a non-toxic and alternative algal concentration of 20 000 and 5 000cells/ml respectively.

Control experiment
The exact same suspensions as in the toxic experiment were repeated, but no mussels were used.

Threshold experiment
Solutions contained 200 ml toxic *K. veneficum*. This created a toxic algal concentration of 80 000cells/ml.

Data analysis
Statistical analysis for collected data was performed in SPSS. The mean cell density of all replicates at each time of sample was used in analysis. An ANOVA was used to test the equality of these means, and since samples were taken over time, a repeated measure ANOVA was required.

The significance level α=0.05 was used to analyse data.

Results
The results of Mauchly's Test of Sphericity for the preliminary analysis allow sphericity of the data to be assumed (X^2_{14} =14.176, p>0.05). The results of the repeated measures ANOVA indicate that time does not have a statistically significant relationship with trial (F (5, 35) =0.829, P>0.05). All outcomes may therefore be compared, irrespective of trial effect.

The results of Mauchly's Test of Sphericity in the first analysis indicate that sphericity may not be assumed (X^2_{14}= 24.568, p<0.05). The highest value of Epsilon must therefore be considered, which in this case is Huynh-Feldt (P=1.000). The corresponding Huynh-Feldt results for the repeated measures ANOVA must then be used (Table 1).

The results of the repeated measures ANOVA for the first analysis (Table 1) (Fig.1) show that time has a statistically significant effect on mussel clearance rate (F (4.661, 79.237) =4.249, P<0.005). However, the relationship between time and treatment is not statistically significant (F (9.322, 79.237) =0.743, P>0.05). This result indicates that the difference between clearance rates of toxic and non-toxic

strains of algae over time is not significantly different. However, tests of between-subjects effects show that independently, treatment does have a significant effect on clearance rate (F (2, 17) = 8.773, P<0.005). Post Hoc tests expand on this, indicating that treatment 2 (non-toxic *K. veneficum*) has a significantly different effect on clearance rate than treatments 1 (toxic *K. veneficum*) and 3 (*P. micans*).

Table 1: Results of Repeated Measures ANOVA for the first analysis

Source		df	Mean Square	F	Sig.
TIME	Huynh-Feldt	4.661	0.229	4.249	0.002
TIME * TRTMT	Huynh-Feldt	9.322	0.040	0.743	0.673
Error(TIME)	Huynh-Feldt	79.237	0.054		

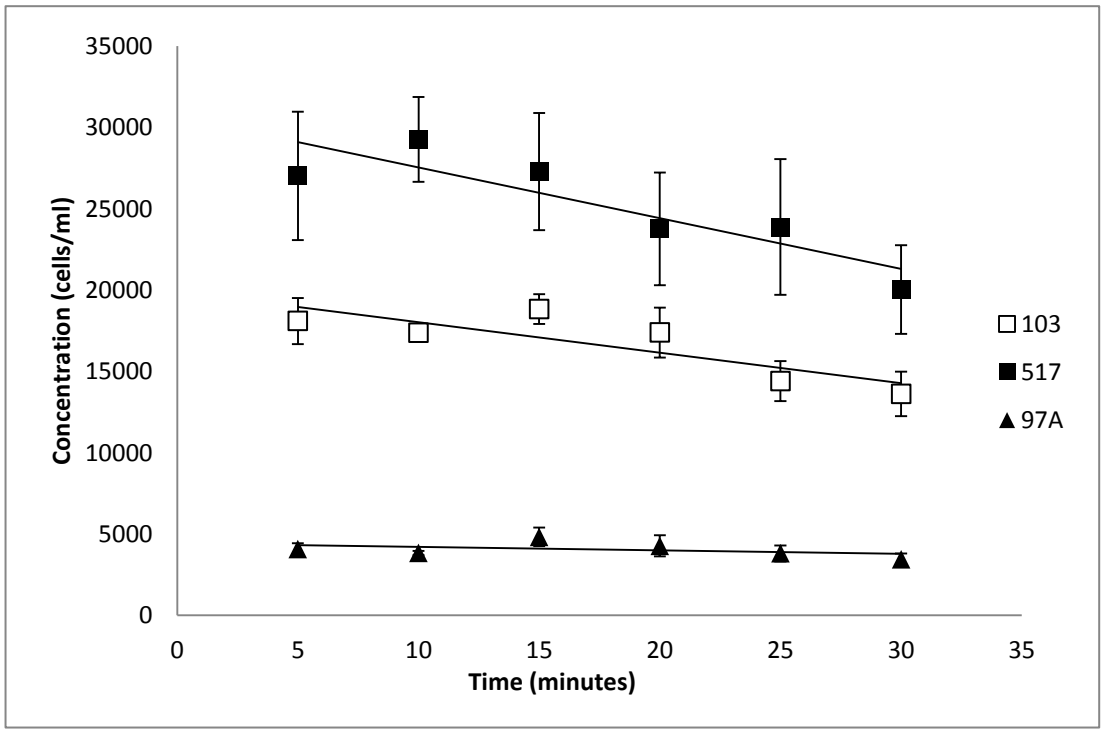

Figure 1: Change in clearance rate over 30 min of *M. edulis* exposed to a) 20 000cells/ml algal culture 103 (toxic *K. veneficum*), b) 20 000cells/ml algal culture 517 (non-toxic *K. veneficum*), c) 5 000cells/ml algal culture 97A (*P. micans*). Error bars show ±SEM.

Mauchly's Test of Sphericity in the second analysis indicates that sphericity cannot be assumed (X^2_{14}= 35.080, p<0.005). The highest value of Epsilon is then to be used, which is Huynh-Feldt (p=0.592). The corresponding Huynh-Feldt results for the repeated measures ANOVA are to then be referred to (Table 2).

The results of the repeated measures ANOVA for the second analysis (Table 2) (Fig 2) show that time does not have a statistically significant effect on mussel clearance rate (F (2.962, 23.696) = 0.996, p>0.05). Time also does not have a statistically

significant relationship with trial (F (2.962, 23.696) = 1.265, p>0.05). This result shows that the clearance rates for the two trials presenting different concentrations of algal cells are not significantly different.

Table 2: Results of Repeated Measures ANOVA for the second analysis

Source		df	Mean Square	F	Sig.
TIME	Huynh-Feldt	2.962	1.545	.996	.411
TIME * TRIAL	Huynh-Feldt	2.962	1.962	1.265	.309
Error(TIME)	Huynh-Feldt	23.696	1.551		

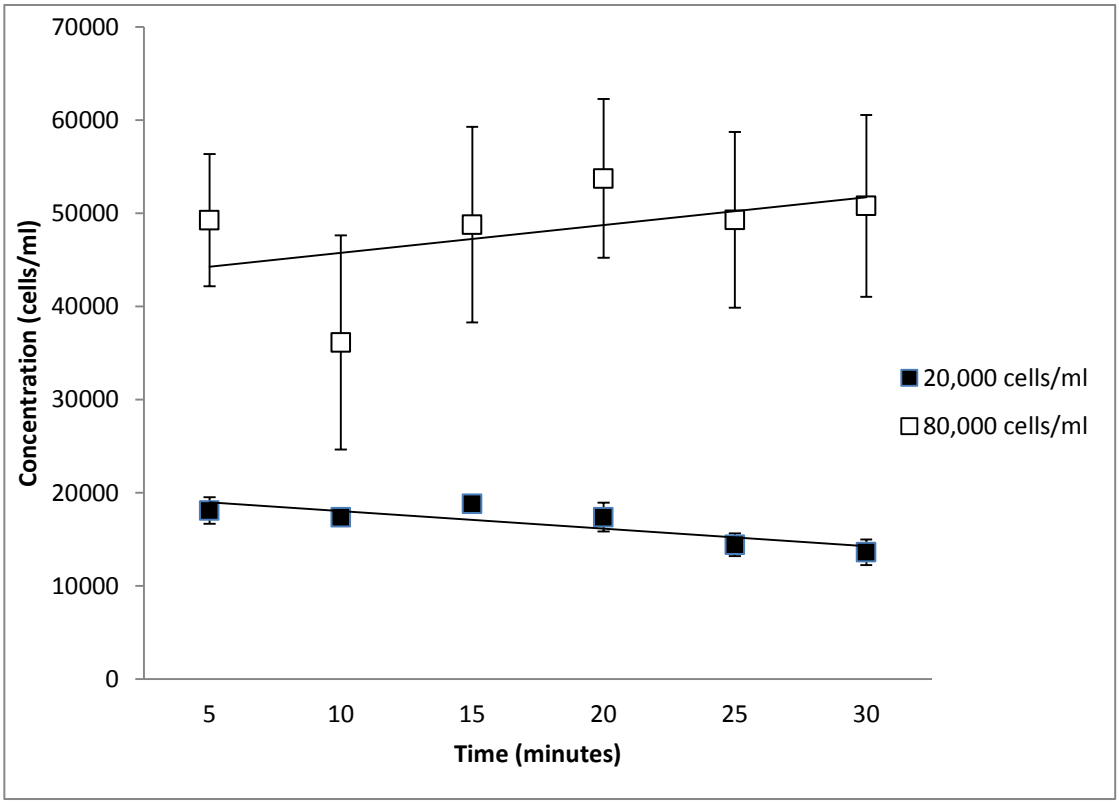

Figure 2: Change in clearance rate over 30 min of *M. edulis* exposed to a) 20 000cells/ml algal culture 103 (toxic *K. veneficum*), b) 80 000cells/ml algal culture 103. Error bars show ±SEM.

Mauchly's Test of Sphericity in the third analysis denotes that sphericity of the data cannot be assumed (X^2_{14}= 35.519, p<0.005). The highest value of Epsilon will therefore be used, which is Huynh-Feldt (P=0.774). The corresponding Huynh-Feldt data for the repeated measures ANOVA must be referenced (Table 3).

The results of the repeated measures ANOVA for the third analysis (Table 3) showed that time is a significant factor within the experiment ($F(3.870, 61.926) = 2.982$, $p<0.05$). Time was also shown to have a statistically significant relationship with trial ($F(3.870, 61.926) = 4.624$, $P<0.005$). This indicates a significant difference in clearance rate between trials where no mussel is present and trials where a mussel is present (Fig 3. & Fig 4.). However, time does not have a statistically significant relationship with treatment ($F(3.870, 61.926) = 0.636$, $P>0.05$).This shows that results are not significantly different between differing strains of algae over time.

Table 3: Results of Repeated Measures ANOVA for the third analysis

Source		df	Mean Square	F	Sig.
TIME	Huynh-Feldt	3.870	.084	2.982	.027
TIME * TRIAL	Huynh-Feldt	3.870	.131	4.624	.003
TIME * TRTMT	Huynh-Feldt	3.870	.018	.636	.634
TIME * TRIAL * TRTMT	Huynh-Feldt	3.870	.016	.553	.692
Error(TIME)	Huynh-Feldt	61.926	.028		

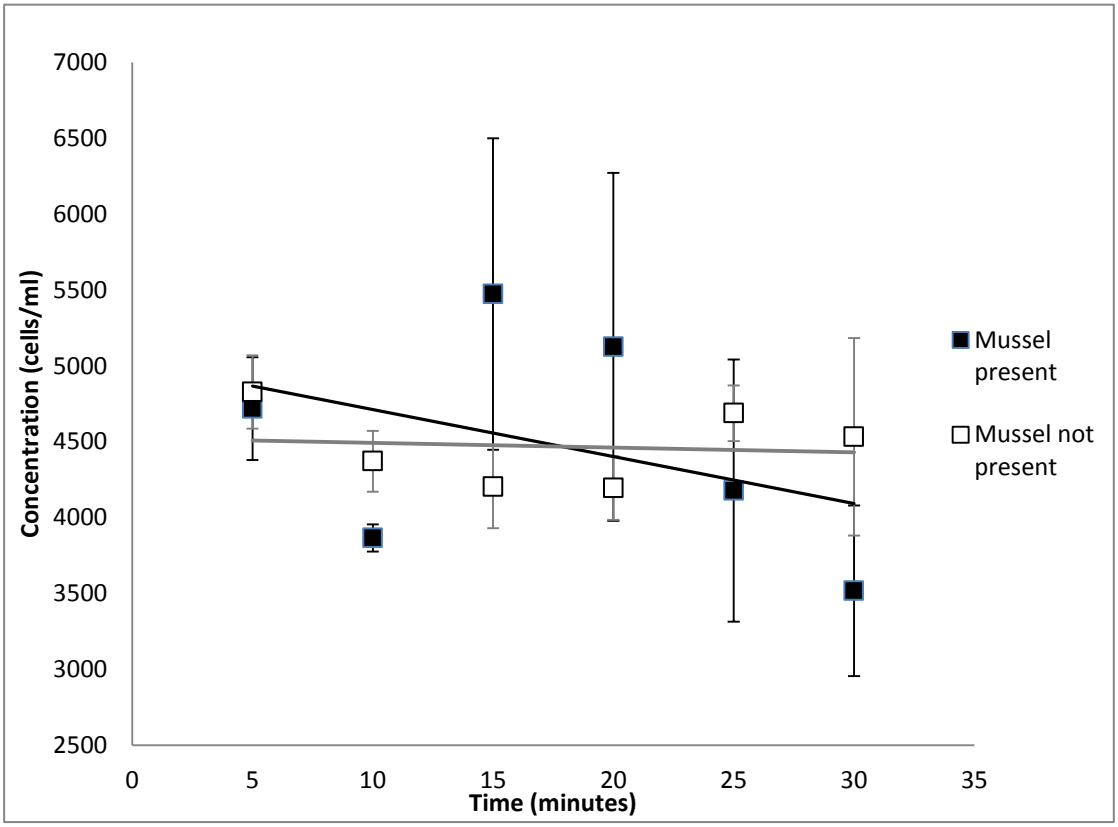

Figure 3: Change in clearance rate over 30 min of *M. edulis* exposed to 5 000cells/ml algal culture 97A (*P. micans*). a) Mussel present, b) Mussel not present. Error bars show ±SEM.

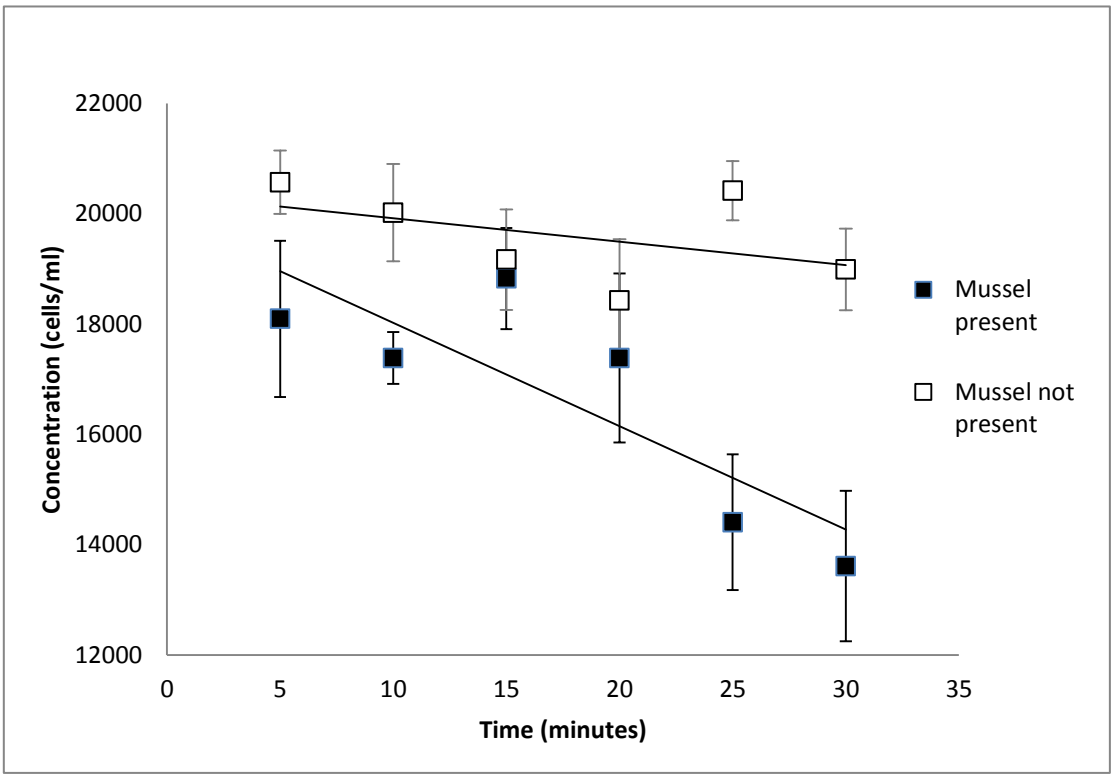

Figure 4: Change in clearance rate over 30 min of *M. edulis* exposed to 20 000cells/ml algal culture 103 (toxic *K. veneficum*). a) Mussel present, b) Mussel not present. Error bars show ±SEM.

No statistically significant relationship was found between clearance rate and dry weight of mussels across all treatments ($F(1, 20) = 0.101$, $p > 0.05$).

Discussion

Unfortunately it was not possible to distinguish between toxic and non-toxic algal cells of *K. veneficum* with a light microscope. In order to remedy this, parallel experiments were performed, mixing each strain of this algae with a non-toxic alternative; *P. micans*. This was a successful solution as statistical analyses were able to render the effects of using separate trials to be insignificant. In studies where similar identification problems have occurred, other methods such as fluorescent in situ hybridisation have been implemented (Hilton et al, 1989; Ernst, 1991; Jianjun et al, 2009). This involves binding specific probes to samples, and using a fluorescence microscope to measure emissions of cells at varying wavelengths. The process has been successfully used to differentiate between algal species and could have been considered for this study. New techniques and advancements of this approach are currently being investigated, and the future of this method is considered to be promising (Tsien et al, 2006; Borisy et al, 2011). If toxic and non-toxic *K.veneficum* cells could be distinguished from each other in this way, there would be no need for parallel run treatments. The response of *M.edulis* to a combination of both strains of algae could have been observed, which would more closely represent and investigate the conditions within the natural environment.

In analysis of the first experiment, time and clearance rate were found to have a statistically significant relationship. This was to be expected, as mussels would feed over the experimental time period. However, the relationship between time and treatment, which was not significant, suggests that *M. edulis* was feeding on the toxic and non-toxic algae in a similar way. This was not expected and does not support the original hypothesis that clearance rates between these algal strains would be significantly different. A possible explanation for this is that the mussels cannot detect the toxicity of algae at the densities used. Further analysis of this relationship suggests that there is some difference between clearance rates for different treatments after all. It is indicated that the non-toxic strain of *K. veneficum* has a significantly different effect on clearance rate than the toxic strain and the non-toxic alternative species, *P. micans*. This does provide support to what has been hypothesised, suggesting that *M. edulis* may in fact have a preference for feeding on non-toxic *K. veneficum* as opposed to toxic *K. veneficum* or non-toxic *P. micans*. However, examination of standard error of the mean for the data collected on each algal species (Fig.1) suggests that this preference is not certain. The data for toxic *K. veneficum* and non-toxic *P. micans* appear to have a high level of accuracy, whereas data for non-toxic *K. veneficum* suggests much larger potential for error. Further replicated of these experiments would need to be performed to investigate this relationship more closely. The size of algal cells is also an important consideration here, as *M. edulis* may have a preference. In using an alternative species such as *P. micans*, algal cell size could not be controlled for, and whilst the size of cells for toxic and non-toxic strains of *K. veneficum* was the same, the cells of *P. micans* were considerably larger. However, this should not have great detrimental effect on analysis as *P. micans* was not the focus algal species of the experiments and mussels within all experiments were exposed to the same cell density of this species.

A possible explanation for the insignificant difference between *M. edulis* clearance rates of toxic and non-toxic *K. veneficum* is that the concentration of algal cells was not great enough for toxicity to be detected by the mussel. In order to investigate this, the second analysis tested *M. edulis* clearance rates of toxic *K. veneficum* at two cell densities; 20 000 cells/ml and 80 000 cells/ml. The results of this analysis show that the clearance rates of *M. edulis* feeding on these algal densities are not significantly different and therefore the original hypothesis is rejected. However the standard error of the mean for data in each trial may contradict this result (Fig.2). Standard error for toxic *K. veneficum* at 20 000 cells/ml is very small and so it is likely that the results of this trial are accurate. However, the standard error for toxic *K. veneficum* at 80 000 cells/ml is extremely large, allowing more great potential error in analysis. Further replicates of this experiment would need to be performed in order to better interpret this standard error. But a possible explanation for the wide degree of error is that clearance rate became more erratic and varied at the higher concentration of toxic algal cells. This would support the hypothesis that M. edulis clearance rate is significantly different between feeding on toxic and non-toxic *K. veneficum*. But would further suggest that there is a threshold of algal cell density at which this difference occurs.

As data was sometimes found to be highly varied and standard error of means was in some cases very large, a control experiment was performed, repeating the methods of the toxic experiment but without using *M. edulis*. Analysis of this experiment supported the accuracy of this study's findings, as the result of

experiments with and without mussels was significantly different. The results are particularly clear in Figures 3 and 4, where no significant decrease in concentration of algal cells was detected for experiments where no mussel was present. The standard error of the mean for data where no mussel was present was also relatively small, suggesting that these results are highly accurate.

No correlation was found between the dry weight of mussels and clearance rate across all treatments. However it is important to note that this analysis is unrepresentative and not overly significant given the small sample size. Experiments and analysis on individual mussels was overly time-consuming and due to time restrictions, sample size was not optimum to measure such an effect. Therefore, the differences in clearance rate for mussels of varying weight could not be accurately determined. However, many recent studies have found a notable relationship between mussel weight and clearance rate (Filgueira et al, 2008). If the sample size of this study was greatly increased, there would be potential for this also.

In addition to time being a great restriction of this study, the nature of the algal cultures also limited experimental scope. The cultures take a considerable amount of time to increase in cell density, and as they grow, they are gradually diluted in order to provide greater volumes of culture. These algae are also known to suffer from sudden decreases in cell density. This determined not only the densities that could be used within experiments, but also the number of experiments and replicates within them that could be performed.

Conclusion

The findings of this study are supportive of the original hypothesis to an extent, but further study would need to be performed in order to prove complete statistical significance. This study highlights the importance of accurately collecting data and performing an optimum number of replicates. In future studies, where time allows, the research presented here could be greatly extended. More replicates would be performed in every experiment allowing for improved accuracy of analysis. The second experiment testing the effects of differing algal concentrations on *M. edulis* clearance rate was particularly interesting and further experiments using a wider range of concentrations could be performed, investigating the potential for thresholds of tolerance of toxic algal species. Similar experiments could be performed for varying species of toxic algae, allowing for comparisons of potential for harm. The methodology of this study could also be improved by the use of modern research techniques such as fluorescent in situ hybridisation. This approach could allow for conditions of toxic algae which are found naturally to be tested. Extending from this, the reflection of the natural environment could promote accurate predictions of toxic algal bloom activity.

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