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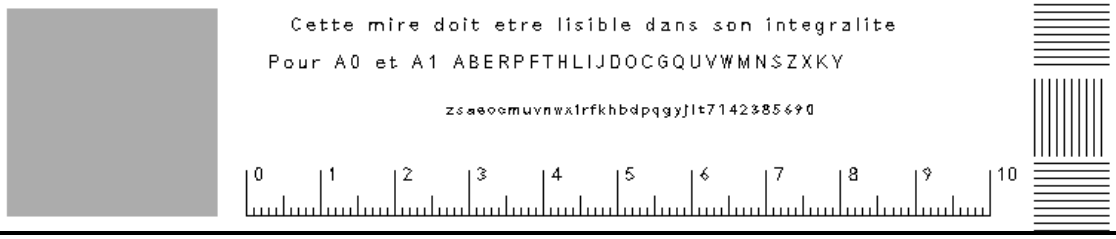

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Investigation of hydrazine toxicity to marine species

Investigation of hydrazine toxicity to marine species

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Executive summary

Hydrazine is a reducing agent which is used in very low concentrations to prevent corrosion in the boiler water of Nuclear Power Plants (NPP) and consequently a liquid effluent containing residual hydrazine may be released periodically to the environment through the cooling water system.

The fate of hydrazine in the aquatic environment is dependent on dilution/dispersion, chemical and biological degradation as well as processes such as volatilisation and sedimentation with hydrazine ultimately degrading to form nitrogen.

There is evidence that hydrazine is harmful to aquatic organisms at low concentrations with the lowest reported acute six day EC_{50} of 0.4 µg l^{-1} for growth inhibition of a marine alga, *Dunaliella tertiolecta* (Dixon *et al*., 1979). Some initial hydrazine toxicity tests were conducted under the BEEMS programme on a range of marine species including selected marine algae (BEEMS Technical Report TR175). However, some species showed toxicological effects at hydrazine concentrations below the detection limit of the then available analytical method of 10µg l^{-1} e.g. the microagal species *Isochrysis galbana* showed sensitivity to hydrazine with a 72-hours EC_{50} <10µg l^{-1}.

To supplement hydrazine toxicity data and to determine whether a wider range of species are as sensitive to hydrazine as indicated by the study on *D. tertiolecta* conducted by Dixon et al., 1979, toxicity tests were conducted on a range of plant and animal species of relevance to either or both Sizewell and Hinkley Point. The analysis technique used for these tests had a limit of quantitation for hydrazine of <1.5 ng l^{-1}.

Marine algal species

Four species of microalgae belonging to different groups were chosen for testing:

- *Thalassiosira weissflogii* and *Skeletonema* sp. representative of diatoms. Both are important components of the phytoplankton at Sizewell (BEEMS Technical Report TR326). Benthic diatoms are a group that is dominant in the microphytobenthos (MPB) on the mudflats at Hinkley Point. (MPB is responsible for most primary production at Hinkley Point, BEEMS Technical Report TR368). *Skeletonema* sp. are also standard regulatory toxicity test species.

- *D. tertiolecta,* a single celled marine green flagellate that has previously been shown to be particularly sensitive to hydrazine. Several attempts to culture and test this species were unsuccessful.

- *Micromonas pusilla,* a single celled marine micro flagellate green algae which is in the same phylum (*Chlorophyta*) as *D. tertiolecta* and is a major component of the pico-phytoplankton

The three species tested showed a marked range in their sensitivity to hydrazine. Two different endpoints were considered for all the tests; these were area under the growth curve (AUGC), indicative of biomass and growth rates compared to the control groups. The most sensitive was *M. pusilla* with a 96 hours EC_{50} of 1.27 µg l^{-1} hydrazine for inhibition calculated with AUGC and 1.80 µg l^{-1} hydrazine when calculated with growth rate. This species is in the same phylum as *D. tertiolecta* and also with thin walled or naked cells which may make it more susceptible to the effects of hydrazine than for example diatoms that have silica in their cell walls and which are one or two orders of magnitude less sensitive e.g. *Sketetonema* sp (72 hour EC_{50} 37.61 µg l^{-1} hydrazine AUGC), and *T. weissflogii* (72 hour EC_{50} of 140.5 µg l^{-1} hydrazine AUGC)

For *Ceramium tenuicorne* a filamentous red macro algal species previous studies had shown that in a 7 day test its growth was reduced to 20% of the control at a nominal hydrazine concentration of 10 µg l^{-1}. However, in this report *C.tenuicorne* exposed to hydrazine up to a measured concentration of 0.7 µg l^{-1} had no detectable effect upon growth. These data suggest that effect concentrations for this species are between

these values and therefore *C.tenuicorne* is of intermediate sensitivity compared to the microalagal species tested.

Marine animal species

Three species of marine animals belonging to different groups were chosen for testing:

- *Lanice conchilega* larvae, a tube dwelling marine polychaete worm, common in European waters.
- *Acartia tonsa adults. Acartia* sp. are marine copepods that are an important component of the zooplankton community at Hinkley Point and Sizewell. Copepods are the principal prey species for many commercially important planktivorous fish.
- *Crassostrea gigas* embryos. The pacific oyster was chosen to be representative of other bivalve species that are present at Hinkley Point and Sizewell. It is also widely used as a standard toxicity test species.

For the animal species tested late stage larvae of the polychaete worm *Lanice conchilega* were the most tolerant to hydrazine exposure with no significant mortality relative to the control group up to an exposure concentration of 1307 µg l^{-1} hydrazine (overall survival was ≥60% in control and treatment groups). Oyster larvae were also relatively resistant to hydrazine toxicity with a LOEC of 10 µg l^{-1}. The copepod *Acartia tonsa* had a 48 hour NOEC for hydrazine of 50 µg l^{-1} based on survival. An attempt to consider the effect of hydrazine on feeding rate in *Acartia* led to some significant changes in feeding rate but further data is required to fully evaluate the validity of these data.

Conclusions

Evaluation of the toxicity of hydrazine based on available literature data has previously calculated an acute Predicted No Effect Concentration of 4 ng l^{-1} hydrazine, based on the hydrazine toxicity of the most sensitive species, *D. tertiolecta* with a six day EC_{50} of 0.4 µg l^{-1} for growth inhibition (ELIER0600773). The most sensitive marine microalgae tested for this report was *M. pusilla* which produced a similar 96 hour EC_{50} of 1.27 µg l^{-1} for inhibition calculated with AUGC. Based on this value a derived acute PNEC value would be 12.7 ng l^{-1} if the same expansion factor of 100 is applied as previously reported (ELIER0600773).

The data in this study confirm that microalgae are the most sensitive group to hydrazine exposure but also that there is a considerable range in sensitivity amongst different species. With the tested microalgae, the most sensitive was *M. pusilla*. This species is in the same phylum as *D. tertiolecta* and also with thin walled or naked cells which may make it more susceptible to the effects of hydrazine than for example diatoms that have silica in their cell walls and which are one or two orders of magnitude less sensitive e.g. *Sketetonema* sp (72 hour EC_{50} 37.61 µg l^{-1} hydrazine AUGC), and *T. weissflogii* (72 hour EC_{50} of 140.5 µg l^{-1} hydrazine AUGC). Other plant and animal species tested were similarly less sensitive.

1 Background

Hydrazine (N_2H_4) is highly reactive, weak base and reducing agent which to prevent corrosion is used in very low concentrations in the boiler water of Nuclear Power Plants (NPP), most commonly in the form of Hydrazine hydrate ($N_2H_4.H_2O$). Cooling water circuits of boilers of nuclear reactor facilities generate a liquid effluent containing residual hydrazine, which may be released periodically to the environment through the cooling water system.

The fate of hydrazine in the aquatic environment is dependent on dilution/dispersion and chemical and biological degradation as well as processes such as volatilisation and sedimentation (Kuch, 1996).

Studies of the degradation rate of hydrazine in sea water collected from Hinkley point indicate a half-life value around 8.5 hours for an initial concentration of 50 µg l^{-1} with an increased degradation rate at lower concentrations (BEEMS Technical Report TR146). Other laboratory based decay studies using natural seawater but collected at Sizewell showed that hydrazine concentrations of ~30 ng l^{-1} were reduced by around 75% after 1 hour (BEEMS Technical Reports TR352 and TR390).

The chronic (daily) source term is currently being investigated but a worst case daily discharge during operation has been calculated to result in a dose in the Cooling water system of 69 ng l^{-1} for 2.2 hrs each day (BEEMS Technical Report TR353).

Where a substance present in the discharge has the potential to harm marine habitats a Predicted No Effect Concentration (PNEC) is usually derived so that this may be compared to the Predicted Exposure Concentration (PEC) with the resulting PEC/PNEC comparison indicating the potential areas over which effects may occur. Derivation of substance PNECs is based on available toxicity data for relevant species and the application of safety factors that are consistent with the nature and quality of the available data and the guidance given (i.e. section 3.3.1 of Part II of 'Technical guidance document in support of Commission Directive 93/67/EEC (TGD, 2003) on risk assessment for new notified substances and Commission Regulation (EC) No 1488/94 on risk assessment for existing substances'). The PNEC values derived from acute short term test data are applied as 95 percentiles and chronic PNECs based on longer term chronic toxicity test data are expressed as an annual average value providing protection against long-term exposure.

There is evidence that hydrazine is harmful to aquatic organisms at low concentrations and the lowest acute six day EC_{50} of 0.4 µg l^{-1} for growth inhibition of a marine alga, *Dunaliella tertiolecta* (Dixon et al, 1979) was used to derive a PNEC for hydrazine (ELIER0600773). The acute and chronic PNEC values estimated were, respectively, 0.004 µg l^{-1} and 0.0004 µg l^{-1}. Although PNEC values provide a reference point for assessing the likelihood that a chemical may impact or not the aquatic environment other factors such as persistence and potential for bioaccumulation as well as the sensitivity range of relevant species will influence the likely extent of any effects.

This report assesses the sensitivity of a range of species to hydrazine that are ecologically important and relatively representative of those present at Sizewell and Hinkley Point:

Toxicity studies were performed on *M. pusilla*, *I. galbaba, Skeletonema* sp, *T. weissflogii*, *C. tenuicorne*, *L. conchilega, A. tonsa and C. gigas*.

- Marine microalgae

Marine microalgae are primary producers and at the bottom of the food chain therefore impacts upon this group of organisms could, have significant effects on higher trophic levels and to the wider ecosystem. Toxicity data for hydrazine on marine microalgae are scarce and evaluations of toxicity of hydrazine and

derivation of PNEC have been based on data obtained for *D. tertiolecta* which is the species which showed highest sensitivity.

This study evaluated the toxicity of hydrazine on different species of marine microalgae representing different taxa and with different ecological relevance (*M. pusilla*, *I. galbana, Skeletonema* sp, *T. weissflogii*, *C. tenuicorne*,).

- Lanice conchilega

Lanice conchilega, commonly known as the Sand Mason Worm is a tube dwelling marine polychaete worm. It lives in the benthos and is common in waters all around the United Kingdom and Europe. They produce planktonic larvae, with spawning taking place from April to October (Kuhl, 1972). They spend one to two months in the plankton prior to settlement, this facilitates larval dispersal.

- Acartia tonsa

A. tonsa (Copepoda) are widely distributed in estuarine and marine environments. Adult *A. tonsa* feed on phytoplankton and zooplankton assemblages. They are an important component of the food web and are the principal prey species for many commercially important planktivorous fish.

A. tonsa are commonly recorded during BEEMS plankton surveys at Sizewell. They are the most abundant species of copepod recorded at Sizewell, peaking in abundance during the summer months (May – September) with estimates of up to 1272 individuals per m^3 (BEEMS Technical Report TR326).

- Crassostrea gigas

The Pacific Oyster, *Crassostrea gigas* is native to the inshore and estuarine waters of the far east, due to a combination of a rapid growth rate and its ability to grow in a range of different environmental conditions, it has become one of the most common species of oyster in global aquaculture. In the UK it was deliberately introduced into the River Blackwater in Essex and is now farmed commercially at several other locations.

Surveys of the benthos around Sizewell have not recorded any adult *C. gigas*, however 13 different species of bivalves have been recorded, of which *Nucula nucleus* and *Nucula nitidosa* occur in notably high abundance (BEEMS Technical Report TR338). Pacific oyster is chosen as a species of interest in this report because of the difficulty in collecting and culturing locally abundant species. *C. gigas* is often used in marine ecotoxicology as an indicator species to determine any potential biological effects of a wide range of water samples, such as from anthropogenic discharges. The OSPAR Joint Assessment Monitoring Plan (JAMP) uses a *C. gigas* bioassay in this manner to measure the proportion of normal vs abnormal development in early stage larvae. As such there is a large volume of data on the effects of water quality and chemicals on the development of *C. gigas* larvae. As a result, it has been selected as a good surrogate indicator species that can be used to infer any potential impacts of hydrazine on larval bivalves.

1.1 Aims and Scope

As hydrazine has been shown to be particularly toxic to algae, this report evaluates the toxicity of hydrazine to several species of marine algae. Different species of microalgae belonging to Prasinophyceae (*Micromonas pusilla*), Bacillariophyceae (*Thalassiosira weissflogii* and *Skeletonema* sp.) and Prymnesiophyceae (*Isochrysis galbana*) were used to investigate the toxicity of hydrazine over a period of 72-96 hours. *T. weissflogii* and *Skeletonema* sp. representative of diatoms, a group that is dominant on the mudflats at Hinkley Point were selected for testing. *Skeletonema* sp. also represent standard regulatory toxicity test species. Several attempts to culture and test *D. tertiolecta,* the species shown to be particularly sensitive to hydrazine, were unsuccessful so a species of micro flagellate green algae *Micromonas pusilla* (which is in the same phylum as *D. tertiolecta* and also has naked cells) was also tested.

The main aim of the tests was to establish the sensitivity of a range of algal species to hydrazine exposure, along with several other taxonomic groups of zooplankton, including the subphylum crustacea and phyla mollusca and annelida.

2 Methods

2.1 Test organisms and culture conditions

2.1.1 Marine algae

Marine microalgae of three different taxa were used as test organisms. The marine diatoms, *Thalassiosira weissflogii* (CCAP 1085/1) and *Skeletonema* sp. (NIVA BAC-1), and the Prasinophyceae *Micromonas pusilla* (CCAP 1965/4) and the prymnesiophycea *Isochrysis galbana* (CCAP 92711) were chosen for the experiments. *Dunaliella tertiolecta* (CCAP 19/6B) was cultured but not used in the definitive tests because it did not show sufficient growth for the toxicity tests (Appendix A.2).

Species were cultivated in F2 medium as described by Guillard & Ryther (1962), in liquid medium. Natural sand filtered seawater is pumped from coastal waters adjacent to the laboratory through the Cefas seawater circulation system. The seawater was then filtered through a 0.2 µm filter and autoclaved for 20 minutes at 121°C before use for the cultures. Cultures were maintained at 15°C under constant light (35 µmol s^{-1} m^{-2}).

Cultures of the filamentous red microalgae *Ceramium tenuicorne* were held in sterile seawater with added nutrients according to methods described by Ekelund, 2005 and ISO 10710:2010, at 122 ± 2°C, salinity of 20 ppt and a light:dark cycle of 16:8 hours at a light intensity of 35 µmol m^{-2} s^{-1}. Salinity was increased by 3 ppt every other day to reach a salinity of 30 ± 1 for test conditions. Cultures were acclimatised for at least 1 week before testing in natural seawater.

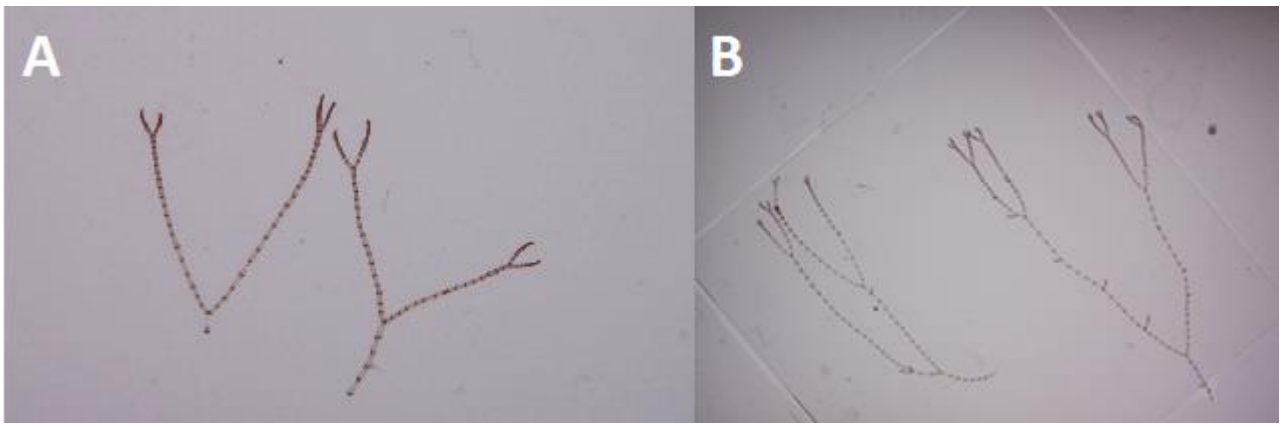

Figure 1 Tips of *Ceramium tenuicorne* at the beginning of a test (left) and after seven days' growth (right).

2.1.2 *Lanice conchilega*

Plankton samples were collected after sunrise from the fishing vessel Our Josie Grace, on the 15/06/2016. Sampling was conducted from inshore waters in the Dunwich / Sizewell area, using a 1 m diameter ringnet with a 270 µm diameter cod end mesh containing a built in solid panel. This was towed vertically from the bottom to the surface, see Appendix A.2 Figure 16 for sampling locations.

Sampling took place over several hours in order to accumulate a sufficient number of organisms. Organisms were held in glass one pound jars with seawater until sampling was completed. Upon arrival back at Cefas Lowestoft laboratory they were transferred into flow-through tanks, where non-target and dead organisms were removed. The polychaete worms were identified as late stage *Lanice conchilega* larvae. They were held for four days prior to testing to allow for any mortality as a result of capture to become apparent before testing. During this time, they were fed a mixed algal culture once daily.

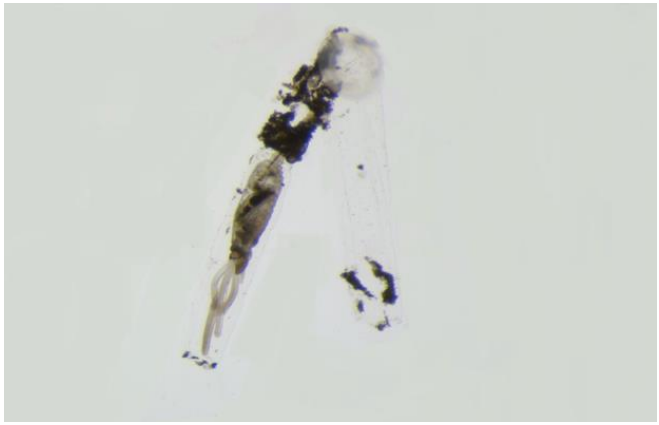

Figure 2 An individual *Lanice conchilega* larvae

2.1.3 Acartia tonsa

Adult *Acartia tonsa*, approximately 13 days old were obtained from Reefshotz (Reefshotz, 14 Nant Y Mynydd coed Y cwm, CF37 3JH, Pontypridd, UK) and were acclimated to test temperature (20°C) before use. They were sieved to 180 µm before use and were transferred between culture and test vessels using glass pipettes.

2.1.4 Crassostrea gigas

The test organisms were embryos produced from mixing the sperm and eggs of conditioned adult males and females. The batch of *C. gigas* used in the test was obtained from Guernsey Sea Farms (Parc Lane, Vale Guernsey GY3 5EQ) and acclimated in the laboratory to test conditions before use.

2.2 Toxicity tests design

2.2.1 Marine algae

Toxicity tests were performed according to the guideline ISO 10253 (2006). Briefly, pre-cultures were started 3 to 5 days prior the test in order to have cells in exponential growth. These pre-cultures were kept at the same conditions as those used in the test (20 ± 2°C, 60-100 µmol s^{-1} m^{-2} and 100 RPM shaking). Tests were performed using 250 ml conical flasks containing 100 ml test solution. Six replicates were used for the control and three replicates for each test concentration. Test solutions were prepared immediately before the beginning of the test, an algal inoculum was added to these and then the test solutions were divided into the test vessels. The experiment started once the inoculum was added to the test vessels and continued for 72 hours. Growth, measured as changes in chlorophyll fluorescence, was checked at 0, 24, 48 and 72 hrs (± 4hrs) using a fluorescence plate reader (BMG Omega Fluostar).

Modifications to the method were necessary for all species except *Skeletonema* sp. All the species except *Skeletonema* sp. were tested in F2 media. Also, the initial inoculum was increased to 1 x 10^5 cells ml^{-1} for *M. pusilla* and to 5 x 10^4 cells ml^{-1} for *I. galbana*. *M. pusilla* and *I. galbana* were tested for 96 hours in order to achieve sufficient growth in the control to enable good discrimination between the treatments.

All *Ceramium* toxicity tests were carried out following the ISO 10710:2010 guideline with modifications. Growth was used as the measure of chemical effect. Briefly, growing tips of length between 0.6 and 1.2 mm were cut from plants the day before the start of the test. Two tips were placed into 6 well polystyrene culture

plates with 5 ml of solution (with the addition of nutrients). Tests were run with a 14:10 light:dark cycle and light intensity of 70 ± 7 µmol m^{-2} s^{-1}. Hydrazine solutions were renewed every 48 hours. Water qualities were measured for fresh solutions and aged solutions each time these were renewed.

At the end of the test *Ceramium* tips were preserved in 0.4% buffered formalin and stored at 5 ± 3°C until the measurement of the samples. Tips were measured using images captured with a stereo-microscope connected to a camera while they were within the test well plates, in solution. These were then analysed using *Leica Application Suite Version 4.7.0 (Leica microsystems, UK Limited).* A reference study was also conducted in parallel with the definitive study using zinc sulphate to check the sensitivity of the test organisms. The reference test LC_{50} should be between 0.01 to 0.08 mg l^{-1}.

Growth rates of microalgae were calculated according to ISO 10253 (2006) following the formula:

$$\mu = \frac{lnN_L - lnN_0}{t_L - t_0}$$

Where

t_0 is the time of test start

t_L is the time of test termination

N_0 is the initial fluorescence

N_L is the fluorescence at test termination

Biomass was also calculated using the Area Under the Growth Curve (AUGC) because this is considered a more sensitive endpoint (Environmental Agency, 2009) using the following formula:

$$\text{AUGC} = \frac{N_1 - N_0}{2} t_1 + \frac{N_1 + N_2 - 2N_0}{2}(t_2 - t_1) + \frac{N_2 + N_3 - 2N_0}{2}(t_3 - t_2)$$

t_1 is the time (for example 24 hours) of the first cell density (or surrogate) estimate after the beginning of the test;
t_2 is the time (for example 48 hours) of the second cell density (or surrogate) estimate after the beginning of the test;
t_3 is the time (for example 72 hours) of the third cell density (or surrogate) estimate after the beginning of the test;
N_o is the initial cell density (or surrogate) estimate;
N_1 is the cell density (or surrogate) estimate at time t1;
N_2 is the cell density (or surrogate) estimate at time t2;
N_3 is the cell density (or surrogate) estimate at time t3.

For C. tenuicorne, growth rates were calculated according to ISO 10710:2010 as follows:

$$\mu = \frac{l_7 - l_0}{t_7 - t_0}$$

Where

l_7 is the length in millimeters at day 7

l_0 is the length in millimeters at day 0

t is time in days

2.2.2 Lanice conchilega

The test vessels used were 25 ml glass beakers, containing 15 ml of the test solution. The organisms were gently removed from their tubes and transferred to the test vessels using a Pasteur pipette. Five animals were added in each test vessel. The tests were conducted in a controlled temperature room at 15 ± 2°C with a light:dark cycle of 16:8 hours at ambient light. The experiment started once the test organisms were added to the test vessels and continued for 48 hours. Test solutions were renewed after 24 hours, the lowest and highest concentrations were sampled for chemical analysis at 0 and 24 hours. Observations of mortality were made at 24 and 48 hours (± 4 hours). The test organisms were added and observed under a dissection microscope with dark field illumination. Six replicates were used for the control and three replicates for each test concentration.

2.2.3 Acartia tonsa

Toxicity tests were performed according to the guideline ISO 14669 (1999). The test solutions were prepared in 1 l glass volumetric flasks. The test was performed using 25 ml beakers containing 25 ml test solution. The tests were conducted in a controlled temperature room at 20 ± 2°C with a light:dark cycle of 16:8 hours. Five animals were added in each test vessel using a glass Pasteur pipette under a stereomicroscope with dark field illumination. Observations of mortality were made at 24 and 48 hours (± 4 hours). The test was terminated after 48 hours at which point the water quality was tested and recorded. Eight replicates were used for the control, six replicates for the lowest three concentrations and four replicates for the highest three concentrations. Additional replicates of the lowest concentrations were included to allow sufficient numbers of test organisms for post exposure assessment.

A reference study was also conducted in parallel with the definitive study using 3,5-dichlorophenol to check the sensitivity of the test organisms.

After termination of the test, organisms from the control and two hydrazine exposure treatments were used in a post exposure feeding study. *Thalassiosira weissflogii* was cultured two weeks prior the test in order to have cells in lag phase and not dividing exponentially. This culture was kept at 15°C (40 µmol m^{-2} s^{-1} F2 media). The *T. weissflogii* culture was diluted with fresh 0.45 µm filtered seawater to a cell density of 5000 cells ml^{-1} in a 1 l volumetric flask. 50 ml was dispensed into each test vessel using a measuring cylinder. Five live organisms were transferred from the 25 ml mortality test beakers using glass pipettes into 50 ml filtered seawater. Eight replicates were used for the control, six replicates for the lowest concentration and three replicates for the highest concentration. The tests were conducted in a controlled temperature room at 20 ± 2°C in complete darkness. The experiment started once the test organisms were added to the test vessels and continued for 24 hours. The test was terminated after 24 hours (± 4 hours) at which point any mortality was recorded. Cell density was calculated after termination, using a flow cytometer. Blank samples containing algae, but no organisms, were analysed at time 0 and after 24 hours., The feeding assessment data was based on analysis of these blank samples.

2.2.4 Crassostrea gigas

Toxicity tests were performed based on the guideline: ICES Techniques in Marine Environmental Science No. 54 Oyster embryo-larval bioassay. Upon delivery to the laboratory, the adult oysters were acclimated individually overnight in 1 L glass beakers, with natural seawater at 30 ± 5 ppt salinity and aeration at 15°C. The following day the water was replaced with seawater at 23°C and the oysters were observed over a further period of 1-2 h to allow natural spawning to occur. To encourage natural spawning, the seawater temperature was raised and lowered between 20 and 24°C and the water volume was varied. The eggs and sperm were checked for viability and the three most viable batches were then selected. Fertilisation was carried out by diluting the egg suspension to a density of 6000 eggs ml^{-1} (±500) adding sperm at 10 ml l^{-1}. The fertilised eggs were kept at 24°C, mixed every 15 minutes, and observed every hour until they reached the 16-32 cell development stage. The batch which reached 16-32 cell stage and appeared to be most viable was then used for the test.

The study was conducted in 12 well test plates. Hydrazine test solutions (4.5 ml) were added to each replicate well. Control groups were set up in the same way, but contained only 4.5 ml seawater with no hydrazine. Eight replicates were used for the control and four replicates for each test concentration.

A concentration of approximately 50 oyster embryos ml^{-1} of embryo solution (at the 16-32 cell stage of development) was added to each well and the plates were incubated for 24 hours at 24 ± 2°C. Embryos were preserved after 24 ± 2 hours in 0.4% buffered formalin. The numbers of normal and abnormal embryos were then assessed under a light microscope using a Sedgewick Rafter counting cell. At the beginning and end of the test, physico-chemical measurements (dissolved oxygen, pH, salinity and temperature) were taken from water quality beakers that were kept under the same environmental conditions as the test plates.

A reference test using zinc sulphate was conducted in parallel. The EC_{50} for the zinc test should be between 0.03 – 0.44 mg l^{-1}.

From the raw counts of oyster embryo numbers, the 'mean control Percent Normal Development (PND)' and the 'mean control Percent Abnormal Development (PAD)' results were calculated as shown below:

PND = 100 x (number of normal larvae/mean number of control embryos counted*)

**Unfertilised eggs are not included*

PAD = 100 - PND

If the mean control PND is less than 60%, then the test is regarded as invalid. The Percent Normal Rate (PNR) was then determined using the ICES guideline equation below:

$$\textbf{PNR} = [(PAD_{m(TC)} - PAD_{m(C)})/(100 - PAD_{m(C)})] \times 100$$

Where $PAD_{m(TC)}$ is the mean PAD value for the test concentration and $PAD_{m(C)}$ is the mean PAD value for the control. This shows the rate of oyster development minus the background level of abnormality in the controls. The data were transformed to give a 'real PNR' by subtracting each PNR from 100.

2.3 Test chemical

A hydrazine dihydrochloride stock solution in reverse osmosis water was prepared from hydrazine dihydrochloride (> 99.99%) (Sigma Aldrich, Poole, Dorset). The main stock was prepared immediately before the beginning of the test and used for the preparation of the relevant test solutions.

2.4 Analysis of hydrazine concentrations

Due to the rapid degradation of hydrazine in the aqueous environment, test solutions were collected and derivatised into a stable azine using the following procedure. A 250 ml sample was poured into a 500 ml amber glass Duran bottle with a PTFE lined cap which contained 0.25 ± 0.03 mg of anhydrous sodium sulphite (Fisher Scientific, Loughborough, Leicestershire) and 2.5 ± 0.025 g of potassium hydrogen phosphate (Fisher Scientific, Loughborough, Leicestershire). The 250 ml sample was immediately spiked with 25 µl of 0.5 mg l^{-1} isotopically labelled ($^{15}N_2$) hydrazine surrogate solution and 2.5 ml of acetone (HPLC grade, > 99.9%) (Sigma Aldrich, Poole, Dorset). This procedure was performed for all test solutions at 0 hours (at the beginning of each test) and after 24 hours (*M. pusilla*, *I. galbaba, L. conchilega, A. tonsa and C.gigas*) and 48 hours (*Skeletonema* sp, *T. weissflogii and C. tenuicorne*). The samples were stored under conditions ensuring stability if analysis could not commence immediately (room temperature for up to 3 weeks). Samples were extracted following Cefas Standard Operating Procedure (SOP 2153 Rv2). Briefly,

hydrazine was determined in sea water by GC/MS Triple Quadrupole. Each batch of samples analysed included a procedural blank and a quality control (QC). Five calibration solutions were chosen according to the concentrations of the test solutions. These were used for hydrazine quantification and were also derivatised with each batch of samples. The limit of quantitation for hydrazine was <1.5 ng l^{-1}.

2.5 Quality assurance

All the tests were carried out in controlled temperature rooms or incubators. Water qualities (pH, salinity, dissolved oxygen and temperature) were checked at the beginning and at the end of each test or at any point in which solutions were renewed. In this case water qualities were recorded for both, old and new solutions. Full water quality records are reported in Appendix A.1.

2.6 Statistical analysis

All statistical analyses for effect concentrations were performed in CETIS v1.8.0 (Tidepool Scientific, USA). EC_{10}s and EC_{50}s were calculated using the linear interpolation method. NOEC and LOEC values were calculated using the Wilcoxon/Bonferroni Adj test.

Data obtained with the *A. tonsa* feeding test were analysed using GraphPad Prism 6 Version 6.04 (GraphPad Software, Inc.). Normality was tested using Kolmogorov-Smirnov test and homogeneity of variance with Barlett's test. Analysis of Variance (ANOVA) was performed to detect differences between groups followed by Tukey's multiple comparisons test.

3 Results

3.1 Physical readings of samples

3.1.1 Marine algae

Temperature, pH and salinities were measured in all of the treatments at the beginning and at the end of the test. pH was always 8.0 ± 0.2 in the control treatments as recommended in the relevant guidelines. Temperature was monitored in the incubator throughout the test and always fell within acceptable limits (20 ± 2°C). Also, light levels were measured and were within acceptable limits (60-120 µmol s^{-1} m^{-2}). Growth of *T. weissflogii* was tested before the experiment. This species showed a better growth at higher light intensities and for this reason the test was run at a light intensity of 150-200 µmol s^{-1} m^{-2}. Full records of test conditions are provided in Appendix A.1.

Hydrazine concentrations were measured in test solutions at time 0 and 24 or 48 hours. Results are shown in Table 1 and 2. Hydrazine was detected in the control solutions but a separate re run of control samples confirmed that the controls were below the method detection limit. The initial detection of residual hydrazine in the controls may therefore have been due to some carryover between samples during analysis. Some values in the *M. pusilla* tests (Table 2) were not available. In these cases, the hydrazine peak area was compared to the method blank resulting in a much smaller peak. The internal standard was also lower than expected resulting in an underestimate of actual concentrations. For this reason, these results were not calculated using the internal standard and nominal concentrations were used as the effective concentrations in these cases.

Hydrazine results for *I. galbana* were not available due to the very low recovery in the internal standard which was more than 250 times lower than what was found in natural seawater. This could have been due to an interference of the algal cells or the test medium with the standard. Nominal concentrations were therefore used for the calculation of effective concentrations in this latter case.

Table 1 Nominal and verified hydrazine concentrations (µg l^{-1}) in the test solutions used in the toxicity tests performed with *Skeletonema* sp and *T. weissflogii*.

Nominal (µg l^{-1})	Verified 0 hour (µg l^{-1})	Verified 48 hours (µg l^{-1})
0	<0.0015	<0.0015
21.3	22.42	5.14
47.7	44.29	10.22
100.6	77.58	46.44
213.4	204.14	38.74
457.3	439.19	76.80

Table 2 Nominal and verified hydrazine concentrations (µg l^{-1}) in the test solutions used in the toxicity test performed with *M. pusilla*.

Nominal (µg l^{-1})	Verified 0 hour (µg l^{-1})	Verified 24 hours (µg l^{-1})
0	<0.0015	-
0.3	0.65	-
1	1.27	0.19
3	3.38	0.46
10	12.09	0.88
30	35.43	8.42
100	-	42.02

Table 3 Nominal and verified hydrazine concentrations (µg l^{-1}) in the test solutions for *C. tenuicorne*.

Nominal (µg l^{-1})	Verified 0 hour (µg l^{-1})	Verified 24 hours (µg l^{-1})
0	<0.0015	-
0.001	0.00058	0.00027
0.003	-	-
0.01	-	-
0.1	0.0589	0.0039
0.3	-	-
1	0.7335	0.1307

3.1.2 Lanice conchilega

Table 4 Nominal and verified hydrazine concentrations (µg l^{-1}) in the test solutions used in the toxicity test performed with *L. conchilega*.

Nominal (µg l^{-1})	Verified 0 hour (µg l^{-1})	Verified 24 hours (µg l^{-1})
0	-	-
15	13.56	11.51
50	-	-
150	-	-

1500	1309	1306

3.1.3 Acartia tonsa

Table 5 Nominal and verified hydrazine concentrations (µg l^{-1}) in the test solutions used in the toxicity test performed with *A. tonsa*.

Nominal (µg l^{-1})	Verified 0 hour (µg l^{-1})	Verified 24 hours (µg l^{-1})
0	<0.0015	-
0.07	0.13	0.03
5	-	-
15	-	-
50	-	-
150	***0.56****	***0.34****
500	529.21	2.27

**problem with detection of hydrazine in the solutions. These have been excluded from the calculations of the EC_{50}s*

3.1.4 Crassostrea gigas

Table 6 Nominal and verified hydrazine concentrations (µg l^{-1}) in the test solutions used in the toxicity test performed with *C. gigas*.

Nominal (µg l^{-1})	Verified 0 hour (µg l^{-1})	Verified 24 hours (µg l^{-1})
0	<0.0015	-
10	-	-
100	89.94	0.00
1000	869.47	5.74

3.2 Toxicity tests results

3.2.1 Marine algae

Growth rates and AUGC relative to control groups for *Skeletonema* sp. exposed to different concentrations of hydrazine for 72 hours are shown in Figure 3. AUGC wasa more sensitive endpoint compared to the growth rate. In fact, results for the lowest concentrations tested (nominal values 21.3, 47.7 and 100.6 µg l^{-1} hydrazine) where always lower for the AUGC. For example, in the 21.7 µg l^{-1} hydrazine treatment group a small enhancement of growth compared to the control was observed for growth rate calculations but not for the AUGC (respectivelly, 1.02 ± 0.11 for growth and 0.77 ± 0.02 for AUGC). Differences in the endpoints are also found in the effective concentrations of hydrazine (Table 7). EC_{10} was 10 times lower for AUGC compared to growth. These were respectively, 3.31 (1.99 - 4.99) µg l^{-1} and 33.73 (n/a -53.99) µg l^{-1} hydrazine for AUGC and growth. The difference between the two endpoints was smaller for EC_{50}s which were only 2 times lower values for the AUGC compared to growth (89.75 and 37.61 µg l^{-1} hydrazine).

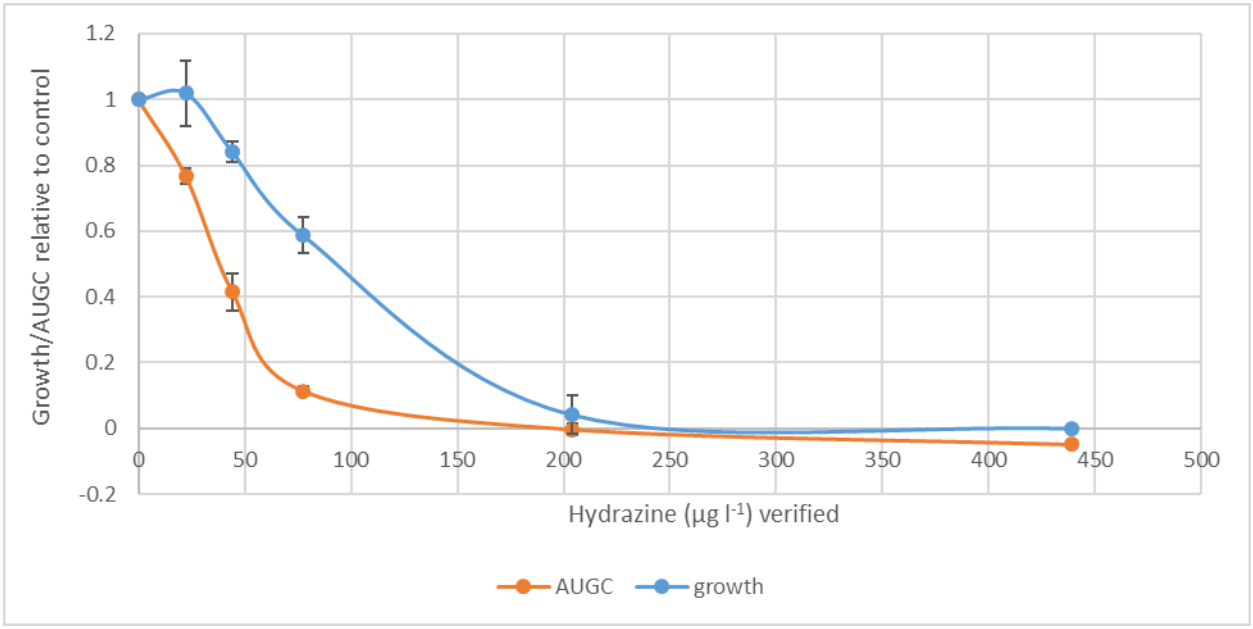

Figure 3 Growth rate (day^{-1}) and Area Under the Growth Curve (AUGC) relative to control groups for *Skeletonema* sp. Exposed to different concentrations of hydrazine for 72 hours. Error bars indicate standard deviation (n=6 control group and n=3 treatment groups).

Table 7 No effect concentrations (NOEC), Low effect concentrations (LOEC), 10 and 50% Effective concentrations of hydrazine (EC_{10} and EC_{50}) calculated for *Skeletonema* sp. Verified concentrations in µg l^{-1} were used for the calculations based on changes in chlorophyll fluorescence over 72 hours. LCL and UCL indicates lower and upper confidence limits.

Hydrazine (µg l^{-1})	Growth	AUGC
NOEC	44.3	<22.4
LOEC	77.6	22.4
EC_{10} (95% LCL-UCL)	33.73 (n/a-53.99)	3.31 (1.99-4.99)
EC_{50} (95% LCL-UCL)	89.75 (63.87-108.20)	37.61 (32.49-45.41)

Hydrazine toxicity to *T. weissflogii* was lower than what was observed for *Skeletonema* sp. AUGC was a more sensitive endpoint also in this case (Figure 4). Contrary to what was observed for *Skeletonema* sp. the biggest difference between the two endpoints was found at the highest concentrations tested (100.6, 213.4 and 457.3 µg l^{-1} hydrazine). The AUGC relative to the control group was, in these cases, 0.71 ± 0.04, 0.36 ± 0.02 and 0.08 ± 0.06 while the growth relative to the control group was 1.03 ± 0.12, 0.64 ± 0.09 and 0.27 0.17. The calculated effect concentration endpoints also showed the same findings with EC_{10} and EC_{50} almost 2 times lower for the AUGC endpoint compared to those calculated with growth rates (Table 8).

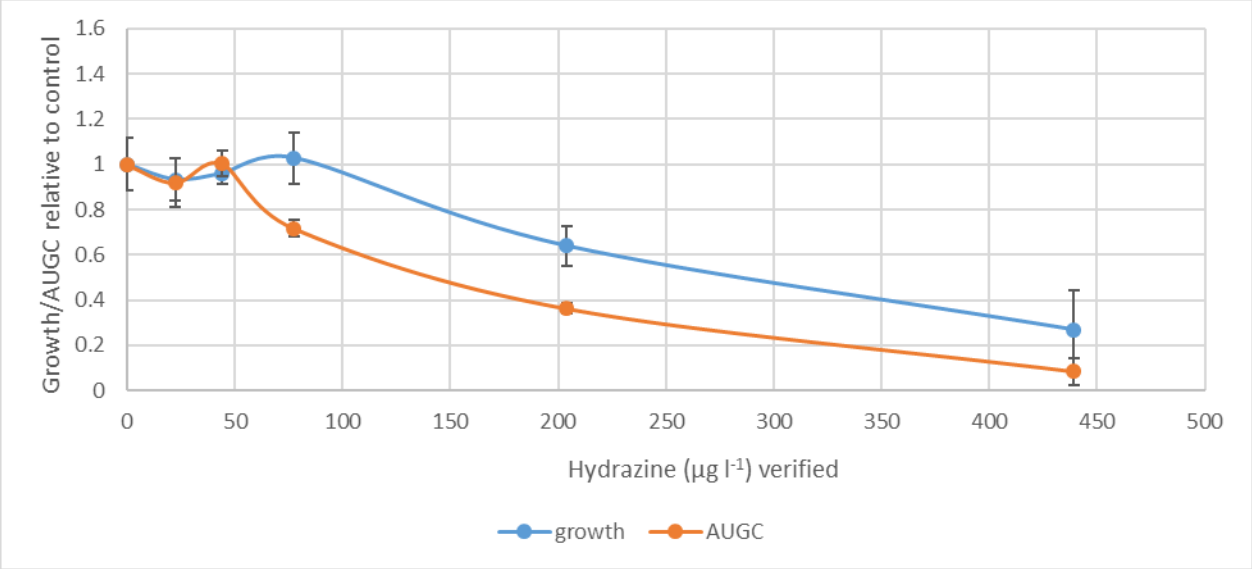

Figure 4 Growth rate (day^{-1}) and Area Under the Growth Curve (AUGC) relative to control groups for *T. weissflogii*. Exposed to different concentrations of hydrazine for 72 hours. Error bars indicate standard deviation (n=6 control group and n=3 treatment groups).

Table 8 No effect concentrations (NOEC), Low effect concentrations (LOEC), 10 and 50% Effective concentrations of hydrazine (EC_{10} and EC_{50}) calculated for *T. weissflogii*. Verified concentrations in µg l^{-1} were used for the calculations based on changes in chlorophyll fluorescence over 72 hours. LCL and UCL indicate lower and upper confidence limits.

Hydrazine (µg l^{-1})	Growth	AUGC
NOEC	77.6	44.3
LOEC	204.1	77.6
EC_{10} (95% LCL-UCL)	96 (n/a-128.6)	51.09 (n/a-61.48)
EC_{50} (95% LCL-UCL)	272.1 (186.8-399.6)	140.5 (113.2-166.7)

M. pusilla was the most sensitive of the species tested. Figure 5 shows a comparison of dose response curves based on growth rates and AUGC. These were equivalent only at the lowest concentration tested (0.3

µg l^{-1} hydrazine) where no effect was detected. The two highest concentrations tested (30 and 100 µg l^{-1} hydrazine) were excluded from the graph because they showed the same effect (total inhibition of growth) to the 10 µg l^{-1} treatment group. Lower values were found for the AUGC for all the other treatment groups except the highest treatments. In these cases, both the endpoints showed negative values due to lower fluorescence found at 96 hours compared to what was found at the beginning of the test (30 and 300 µg l^{-1} not shown in Figure 3).

Contrary to what was found for the other species tested, effect concentrations were similar for *M. pusilla* (Table 9). In fact, EC_{10}s were 0.71 (0.002-0.95) µg l^{-1} hydrazine for growth and 0.70 (n/a-0.79) µg l^{-1} hydrazine for AUGC. Slightly lower values were found for EC_{50}s which were 1.80 (1.42-2.19) µg l^{-1} hydrazine for growth and 1.27 (0.96-1.24) for AUGC. NOEC and LOEC values were the same for both the end points (respectively, 0.65 and 1.27 µg l^{-1} hydrazine)

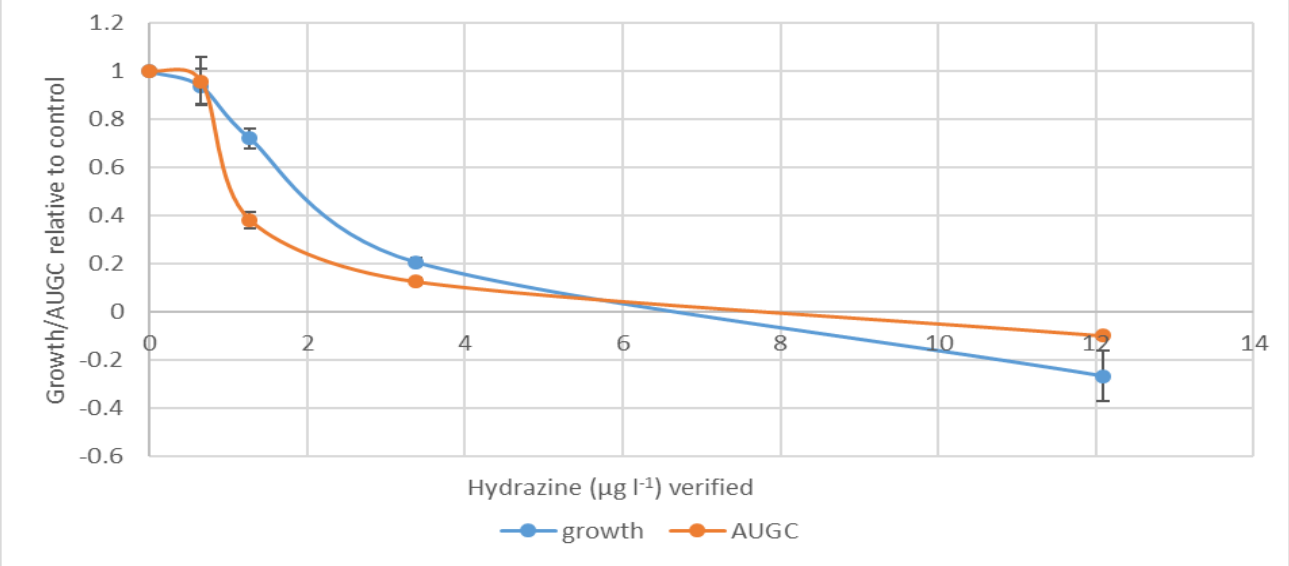

Figure 5 Growth rate (day^{-1}) and Area Under the Growth Curve (AUGC) relative to control groups for *M. pusilla*. Exposed to different concentrations of hydrazine for 96 hours. Error bars indicate standard deviation (n=6 control group and n=3 treatment groups).

Table 9 No effect concentrations (NOEC), Lowest observed effect concentrations (LOEC), 10 and 50% effect concentrations of hydrazine (EC_{10} and EC_{50}) calculated for *M. pusilla*. Verified concentrations in µg l^{-1} were used for the calculations based on changes in chlorophyll fluorescence over 96 hours. LCL and UCL indicate lower and upper confidence limits.

Hydrazine (µg l^{-1})	Growth	AUGC
NOEC	0.65	0.65
LOEC	1.27	1.27
EC_{10} (95% LCL-UCL)	0.71 (0.002-0.95)	0.70 (n/a-0.79)
EC_{50} (95% LCL-UCL)	1.80 (1.42-2.19)	1.27 (0.96-1.24)

I. galbana was tested at a range of hydrazine concentrations between 0.5 and 120 µg l^{-1}. A complete dose response was not observed during the 96 hour exposure. A slight enhancement of growth was observed in the 13 µg l^{-1} hydrazine treatment group. The maximum effect found was 45% inhibition compared to the control for AUGC (0.54 ± 0.22) at the highest concentration tested (120 µg l^{-1} hydrazine). For this reason, EC_{50}s could not be calculated from this dataset for this species and are >120 µg l^{-1} hydrazine. The top and bottom concentrations were analysed to confirm the concentrations of hydrazine at the beginning of the test, but an interference with the method prevented the confirmation of the concentrations. For this reason, the test was not repeated.

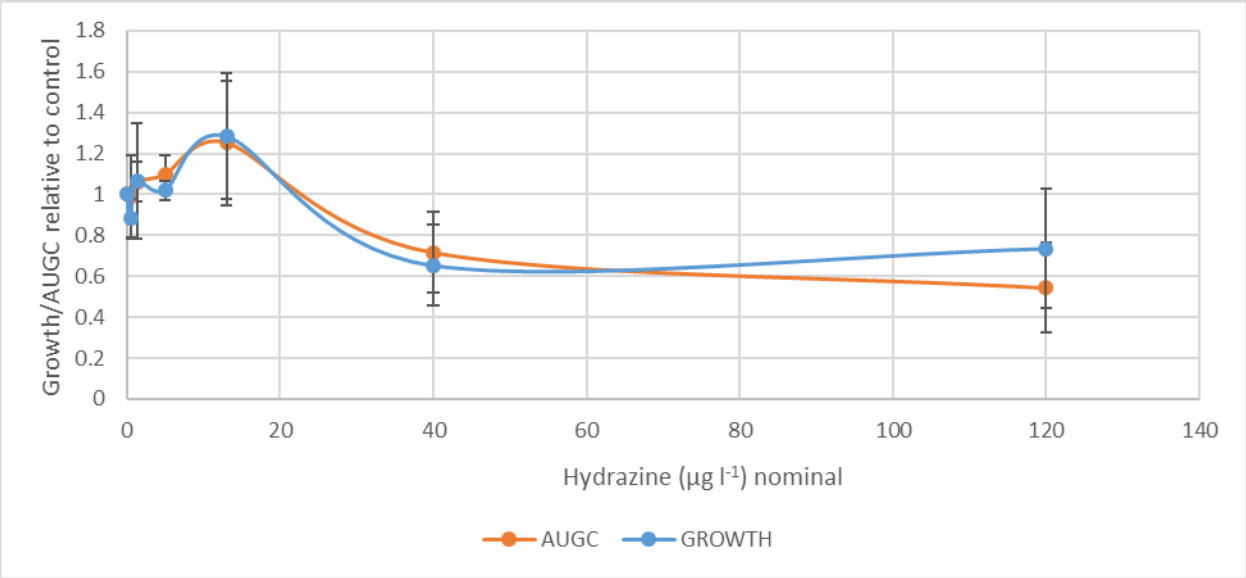

Figure 6 Growth rate (day^{-1}) and Area Under the Growth Curve (AUGC) relative to control groups for *I. galbana*. Exposed to different concentrations of hydrazine for 96 hours. Error bars indicate standard deviation (n=6 control group and n=3 treatment groups).

Table 10 No effect concentrations (NOEC), Low effect concentrations (LOEC), 10 and 50% Effect concentrations of hydrazine (EC_{10} and EC_{50}) calculated for *I. galbana*. Nominal concentrations in µg l^{-1} were used for the calculations based on changes in chlorophyll fluorescence over 96 hours. LCL and UCL indicate lower and upper confidence limits.

Hydrazine (µg l^{-1})	Growth	AUGC
NOEC	120	40
LOEC	>120	120
EC_{10} (95% LCL-UCL)	18.21 (13.58-34.18)	18.28 (14.43-60.10)
EC_{50} (95% LCL-UCL)	>120	>120

3.2.2 *Ceramium tenuicorne*

The EC_{50} from the zinc sulphate reference test was within acceptable limits (0.01 to 0.08 mg l^{-1}), therefore the test is regarded as valid i.e. the sensitivity of the culture of test species used in this study was comparable to that employed in previous studies. Average length in the control group after 7 days was 13.93 ± 2.10 mm. Increasing hydrazine concentration was not found to be correlated with changes in growth, compared to the control group (Figure 7). The highest level of growth as a percentage of the control in any treatment; 103.9%, was observed in the 0.01 µg l^{-1} group. The lowest level of growth as a percentage of the control; 81.9% was observed in the 0.001 µg l^{-1} group. The levels of growth were not sufficient to enable the calculation of an EC_{50} value.

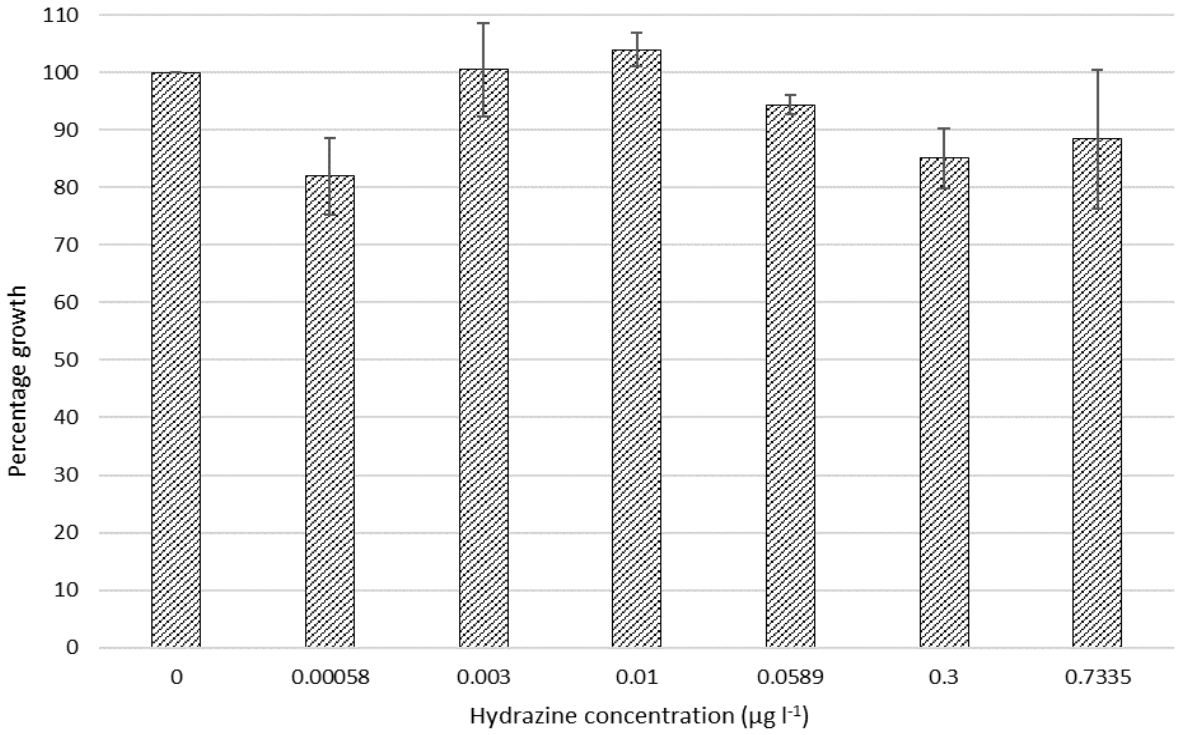

Figure 7 Bar graph showing the mean growth as a percentage of the control at different hydrazine concentrations for *C. tenuicorne*. Error bars indicate standard deviation (n=13 control group and n=8 treatment groups).

3.2.3 *Lanice conchilega*

Average mortality in the control groups was 10% after 24 hours, which is considered to be within acceptable limits for wild caught organisms. However, after 48 hours this had risen to a level of 33%. At both 24 and 48 hours increasing hydrazine concentration was not found to be positively correlated with increasing mortality (Figure 8). The highest total level of mortality in any treatment; 40%, was observed in the 15 µg l^{-1} group, after 48 hours. The mortality observed at 48 hours was not very different to that observed at 24 hours. The greatest percentage increase in mortality between 24 and 48 hours was 23.3%, observed in the control treatment.

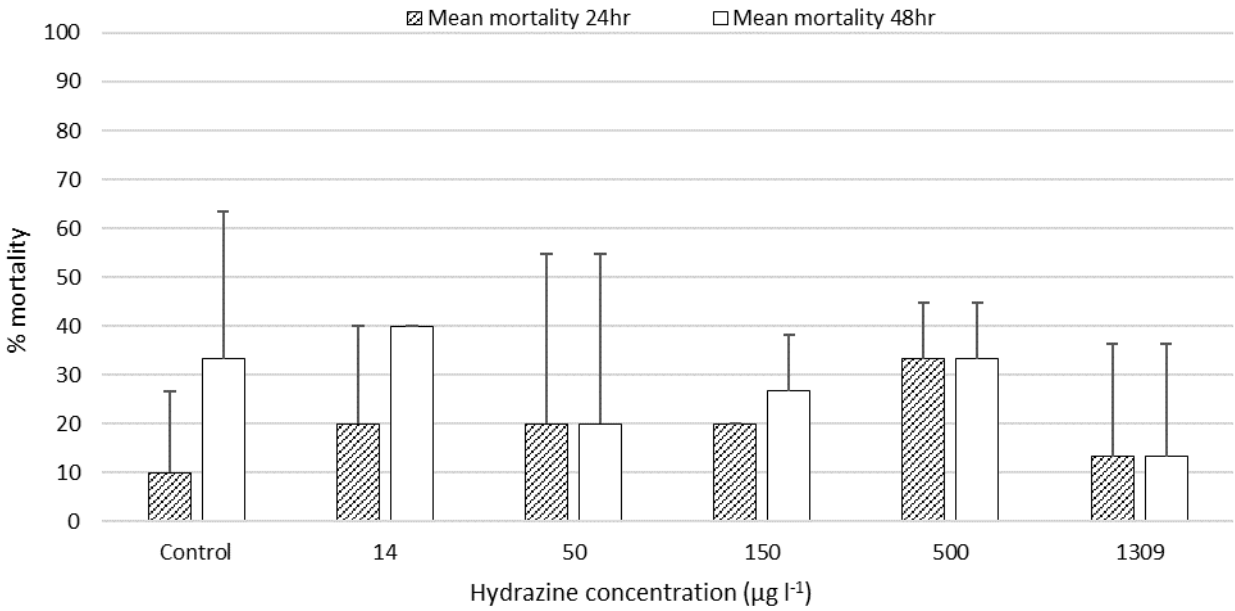

Figure 8 Bar graph showing the 24 and 48 hour mean mortality at different hydrazine concentrations for *L.conchilega*. Error bars indicate standard deviation (n=6 control group and n=3 treatment groups).

3.2.4 Acartia tonsa

The EC_{50} from the 3,5 dichlorophenol reference test was within acceptable limits (0.5 and 1.5 mg l^{-1}), therefore the test is regarded as valid. Significant mortality was observed in only one hydrazine treatment after the 48-hour exposure (Figure 9). Based on nominal concentrations the highest levels of mortality were found in the treatment group at the highest hydrazine concentration, 500 µg l^{-1} (529.21 µg l^{-1} verified). No mortality was detected in groups exposed to 150 µg l^{-1} hydrazine. No hydrazine was detected in samples belonging to this group so these were excluded from the calculations of the LC_{50}s (see Table 5). The levels of mortality were sufficient to enable the estimation of an LC_{50} value, 223 (186.5-231) µg l^{-1} hydrazine (Table 11).

Table 11 No effect concentrations (NOEC), Low effect concentrations (LOEC), 10 and 50% lethal concentrations of hydrazine (LC_{10} and LC_{50}) calculated for *A. tonsa* sp. Nominal concentrations in µg l^{-1} were used for the calculations based on mortality over 48 hours. LCL and UCL indicate lower and upper confidence limits.

Acartia tonsa Mortality results (48 hour)	Hydrazine (µg l^{-1})
NOEC	50
LOEC	500
LC_{10} (95% LCL-UCL)	67.57 (NA-68.05)
LC_{50} (95% LCL-UCL)	223 (186.5-231)

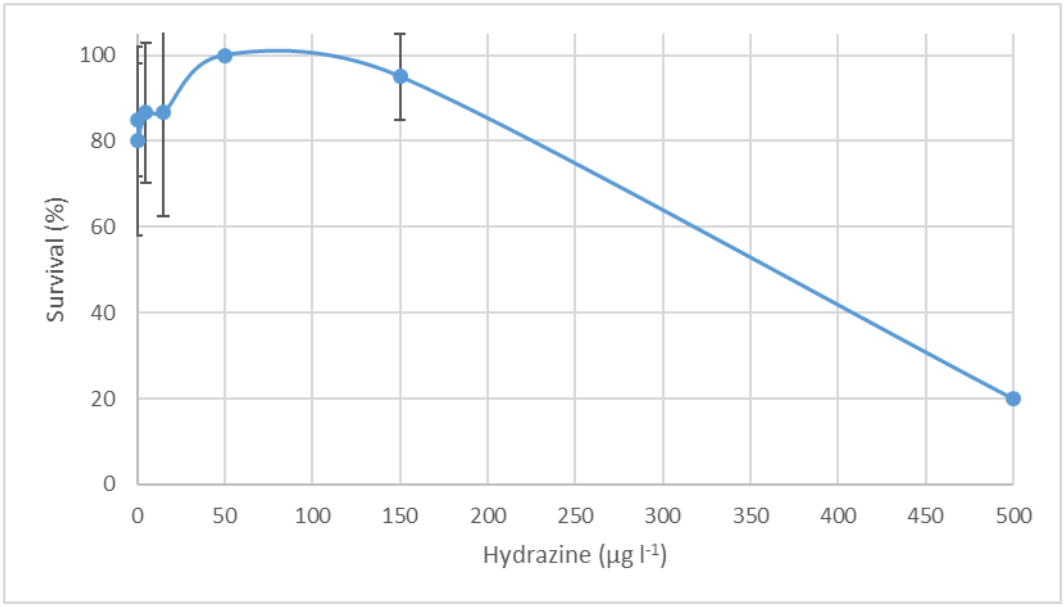

Figure 9 48 hours percentages of survival of *A. tonsa* exposed to different nominal concentrations of hydrazine. error bars indicate standard deviations (n=8 control and n=4 treatment groups).

The one-way ANOVA for the mean number of *T. weissflogii* cells consumed, per animal after 24 hours (Table 33) showed a significant difference between groups (P -value 0.0216) Therefore, a Dunnett's multiple comparisons test was used to compare each treatment to the control (Table34). The two exposure concentrations, 0.07 and 50 µg l^{-1} were significantly different to the control, with p-values ≤ 0.05. Whilst acute effects of hydrazine exposure upon *A. tonsa* were not found at these concentrations over 48 hours, feeding behaviour is significantly affected (Figure 10).

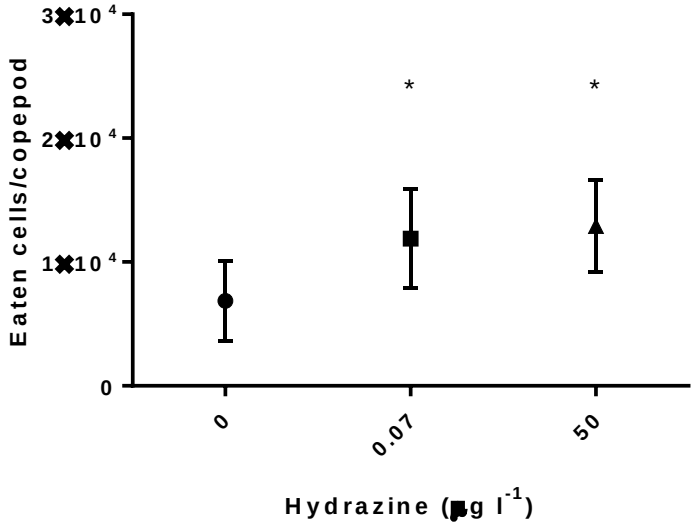

Figure 10 The mean percentage of *T. weissflogii* cells consumed, per animal after 24 hours at different hydrazine concentrations for *A. tonsa*. asterisks (*) indicate significant difference between nominal hydrazine concentrations and the control ($p<0.05$).

3.2.5 Crassostrea gigas

The EC_{50} from the Zinc reference test was within acceptable limits and the mean control PND was greater than 60%, therefore the test is regarded as valid.

Table 12 No effect concentrations (NOEC), Low effect concentrations (LOEC), 10 and 50% Effect concentrations of hydrazine (EC_{10} and EC_{50}) calculated for *C. gigas*. Verified concentrations in µg l^{-1} were used for the calculations based on embryo development results over 24 hours. LCL and UCL indicate lower and upper confidence limits.

C. gigas embryo development (24 hr)	Hydrazine (µg l^{-1})
NOEC	<10
LOEC	10
EC_{10} (95% LCL-UCL)	3.38 (0.64-146.1)
EC_{50} (95% LCL-UCL)	225 (156.7-266.8)

Significant abnormality was observed in only one hydrazine treatment after the 24-hour exposure (Figure 11). Based on measured concentrations the highest levels of abnormality were found in the treatment group at the highest hydrazine concentration, 869.47 µg l^{-1}. The levels of abnormality were sufficient to enable the calculation of an EC_{50} value, 225 µg l^{-1}.

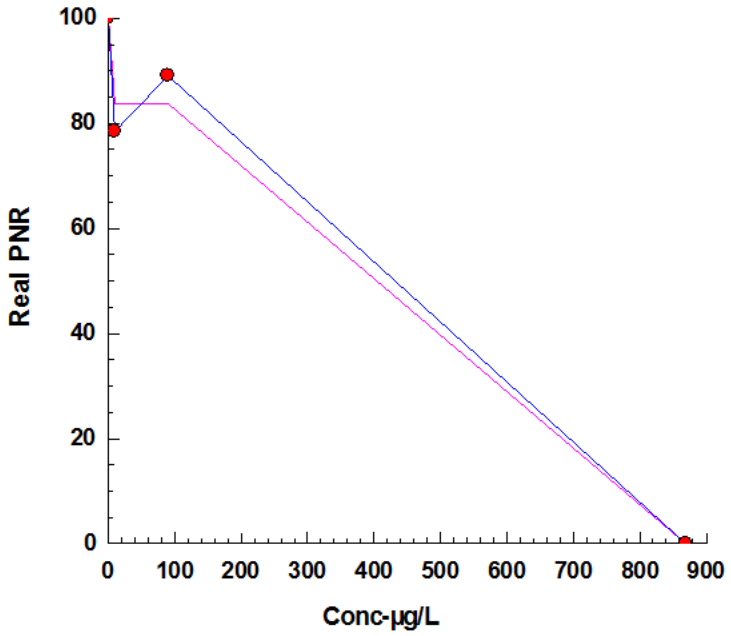

Figure 11 *C. gigas* Linear Interpolation based on hydrazine concentrations for present normal rate (real PNR) of oyster development, minus the background level of abnormality in the controls.

4 Discussion

Hydrazine is a reducing agent used in nuclear power plants to remove oxygen from the water and thus to minimise corrosion. Dependent upon the temperatures reached within the boiler system of the Nuclear power plant a significant percentage of the hydrazine used may breakdown to nitrogen, hydrogen and ammonia (Environment Canada, 2011 and references therein). Nevertheless, monitoring data show that some hydrazine is released from steam generator drain down and the concentrations vary according to the timing of sampling (BEEMS TR397). So hydrazine may be released into the marine environment periodically. The chronic (daily) source term is currently being investigated but a worst case daily discharge during operation has been calculated to result in a residual concentration in the Cooling water system of 69 ng l^{-1} for 2.2 hrs each day (BEEMS TR353). Hydrazine degradation rate in natural sea water is relatively rapid for concentrations up to 3000 ng l^{-1} e.g. half-life of approximately 38 minutes in sea water sampled from Sizewell (BEEMS TR352). Despite its natural degradation in seawater, all the toxicity tests with microalgae reported here used static renewal of the hydrazine concentration and so decay of hydrazine occurred over the test period and therefore test organisms received a diminishing exposure. Test solutions were renewed after 24 hours for tests conducted with the planktonic polychaete larvae *L. conchilega* and the copepod *A. tonsa*. For the red algae *C. tenuicorne* renewals were carried out every 48 hours. Algal cells and other planktonic organisms are likely to be exposed to hydrazine either as a result of primary entrainment in the power station cooling water system or through secondary entrainment in the discharge plume. Exposure under these circumstances is likely to be similar to that in a static test system (i.e. animals remain in a volume of water in which the hydrazine concentration decreases over time). Species associated with the benthos e.g. *C. tenuicorne* that are relatively fixed to the substrate are likely to receive a more variable exposure to any chemicals present in a discharge plume. In this latter case the exposure profile is likely to consist of a series of peaks and troughs in exposure concentration as the discharge plume mixes with new seawater and moves over the seabed at a given location.

A summary of the toxicity of hydrazine to all the plant species in this study is shown in Table 13

Table 13 10 and 50% Effective concentrations of hydrazine (EC_{10} and EC_{50}) calculated for *T. weissflogii*. Verified concentrations in µg l^{-1} were used for the calculations based on changes in chlorophyll fluorescence over 72 hours. LCL and UCL indicate lower and upper confidence limits.

Species	Hydrazine (µg l^{-1})	Growth	AUGC
Skeletonema sp.	**EC_{10} (95% LCL-UCL)**	33.73 (n/a-53.99)	3.31 (1.99-4.99)
Skeletonema sp.	**EC_{50} (95% LCL-UCL)**	89.75 (63.87-108.20)	37.61 (32.49-45.41)
T. weissflogii	**EC_{10} (95% LCL-UCL)**	96 (n/a-128.6)	51.09 (n/a-61.48)
T. weisflogii	**EC_{50} (95% LCL-UCL)**	272.1 (186.8-399.6)	140.5 (113.2-166.7)
M. pusilla	**EC_{10} (95% LCL-UCL)**	0.71 (0.002-0.95)	0.70 (n/a-0.79)
M. pusilla	**EC_{50} (95% LCL-UCL)**	1.80 (1.42-2.19)	1.27 (0.96-1.24)
I.galbana	**EC_{10} (95% LCL-UCL)**	18.21 (13.58-34.18)	18.28 (14.43-60.10)
I.galbana	**EC_{50} (95% LCL-UCL)**	>120	>120
C. tenuicorne	**EC_{10} (95% LCL-UCL)**	>1.0	-

Table 14 10 and 50% Effective concentrations of hydrazine (EC_{10} and EC_{50}) calculated for three animal species. Verified concentrations in µg l^{-1} were used for the calculations

Species	Time	Hydrazine (µg l^{-1})	Survival	Other
Lanice conchilega	**48h**	**EC_{10} (95% LCL-UCL)**	>1307	-
Acartia tonsa	**48h**	**EC_{50} (95% LCL-UCL)**	223 (186.5-231)	-
Acartia tonsa	**48h**	**EC_{10} (95% LCL-UCL)**	67.57 (N/A-68.05)	-
Crassostrea gigas	**24h**	**EC_{50} (95% LCL-UCL)**	225 (156.7-226.8)	-
Crassostrea gigas	**24h**	**EC_{10} (95% LCL-UCL)**	3.379 (0.63-146)	-

In this study hydrazine toxicity was evaluated in four different species of microalgae representing different groups. *Skeletonema* sp. was chosen because it is one of the recommended marine microalgal species in the ISO guideline (ISO 10253, 2006) and as such one of the most common test species for regulatory toxicity testing. *T. weissoflogii* was selected as an example of a different taxonomic group, within the diatoms. *M. pusilla* was chosen because it is a naked photosynthetic flagellate, belonging to the picoplankton, commonly found in the English seas and with similar characteristics to *Dunaliella tertiolecta* (Cottrell & Suttle, 1991; Not et al., 2004). *D. tertiolecta* was one of the chosen species but could not be used as it did not grow enough in the control so as to allow reliable discrimination of any toxic effects if used in a full toxicity study. *I. galbana*, belongs to the group *Prymnesiophyceae* and was included as previous tests had shown it to be sensitive to hydrazine toxicity at concentrations <10 µg l^{-1} hydrazine (the detection limit of

the analytical method available at the time (BEEMS TR175). However, there was an incomplete dose response during the study with *I. galbana* and interference with the analytical method did not allow confirmation of the concentrations tested. For this reason, these results were not considered reliable.

Hydrazine concentrations in tests solutions were confirmed after 48 hours for toxicity studies using *Skeletonema* and *T. weissflogii* and after 24 hours for *M. pusilla*. All the test solutions showed a decrease of 70% or above over 24 hours for the lower concentration range used for *M. pusilla* (0.3-100 µg l^{-1} hydrazine nominal) and over 48 hours for the highest concentrations used for *T. weissflogii* and *Skeletonema* sp. (21.3-457.3 µg l^{-1} hydrazine nominal).

Data obtained for toxicity tests with microalgae can be analysed using different methods. These are based on the evaluation of growth inhibition induced by a chemical and the validity and significance of the different endpoints has been discussed in some detail in the scientific literature (i.e. Eberious *et al*., 2002). Different guidelines recommend different approaches for calculation of the test endpoint in toxicity tests with microalgae. All the tests with microalgae included in this report were carried out following the ISO 10253 (2006) guideline which recommends the calculation of growth rates. The same calculation is recommended in the OECD 201 guideline (2006). However, the Environment Agency method for direct toxicity assessment (2009) recommends the use of the Area Under the Growth Curve (AUGC) which is indicative of biomass and considered a more sensitive endpoint. For this reason, both the endpoints were calculated here. AUGC calculation takes in consideration all the time points between the beginning and the end of the test and was always shown to be the more sensitive endpoint. Among the chosen species, the most sensitive was *M. pusilla* with a 96 hours EC_{50} of 1.27 µg l^{-1} hydrazine for inhibition calculated with AUGC and 1.80 µg l^{-1} hydrazine when calculated with growth rate. This species also showed the lowest NOEC (0.65 µg l^{-1} hydrazine) and EC_{10} (0.70 µg l^{-1} hydrazine with growth rates). *Sketetonema* sp. showed less sensitivity to hydrazine but a wider difference in 72 hours-EC_{50}s was found when the two calculations were compared. These were 37.61 µg l^{-1} hydrazine for AUGC and 89.75 µg l^{-1} for growth rates. The least sensitive of the species tested was *T. weissflogii* which had a72 hour-EC_{50} of 140.5 µg l^{-1} hydrazine for AUGC and 272.1 µg l^{-1} hydrazine for growth rates.

The results here for different species of microalgae indicate that the lowest NOEC and EC_{50} for *M. pusilla* using a single addition of hydrazine were, 0.65 µg l^{-1} hydrazine and 1.27 µg l^{-1} hydrazine based on the calculation of AUGC (biomass). Based on this value a derived acute PNEC value would be 12.7 ng l^{-1} if an expansion factor of 100 is applied to this as was previously reported (ELIER0600773).

Ceramium tenuicorne is a filamentous red macro algal species that can grow up to 10 cm in length. It is widely distributed in temperate waters and is found in both brackish and marine waters (Ekelund, 2005). Whilst not identified in the Sizewell flora it has been chosen as it is also widely used as a standard toxicity test species. A previous study with *C. tenuicorne* reported growth inhibition in all measured concentrations, with only 20% of control growth at the lowest nominal concentration tested, 10 µg l^{-1} (BEEMS TR175). Results obtained in this report for nominal hydrazine concentrations up to 1 µg l^{-1} showed that growth was not significantly inhibited at any of the hydrazine concentrations tested. Therefore, an EC_{50} could not be calculated but the data here and in the previous report indicate that this lies between 1 and 10 µg l^{-1}. The measured concentrations of hydrazine in the test solutions were slightly lower than the nominal range and showed considerable decay over 24 hours. The concentrations tested cover a range that included concentrations representative of those expected in the vicinity of the cooling water discharge. Based upon these results there is a low likelihood that growth of *C. tenuicorne* will be significantly reduced at the predicted cooling water concentrations expected at Sizewell (0.069 µg l^{-1}) and in fact hydrazine concentrations are predicted to be >0.004 µg l^{-1} over no more than 1 Ha (BEEMS Technical Report TR303).

Lanice conchilega is a tube dwelling marine polychaete worm, common in European waters. They produce planktonic larvae, with spawning taking place from April to October (Kuhl 1972). Chemical analysis of the hydrazine solutions showed that the solutions were between 9.60 and 12.73% lower than the nominal range at 0 hours and surprisingly showed very little decay after 24 hours (15.12% in the 15 µg l^{-1} test solution and 0.23% in the 1500 µg l^{-1} solution). At both 24 and 48 hours increasing hydrazine concentration was not found to be positively correlated with increasing mortality. Survival was not significantly affected at any of the

hydrazine concentrations tested. Therefore, an EC_{50} could not be calculated. No standardised testing protocol exists for *L. conchilega*. Control mortality after 48 hours was more than 30%. Worms were removed from their tubes prior to testing and this may have been detrimental to survival, however no significant increase in mortality was observed compared to the control treatment, indicating low apparent sensitivity to hydrazine up to 1500 µg l^{-1}.

A. tonsa (Copepoda) is an ecologically important component of the marine food web and copepods are the principal prey species for many commercially important planktivorous fish. They are the most abundant species of copepod recorded at Sizewell, peaking in abundance during the summer months (May – September). A mortality of 80% was observed in the highest hydrazine treatment (500 µg l^{-1} nominal) after 48-hours exposure. No significant mortality was found in the lower treatment groups with the calculated 48 h-LC_{50} of 223 (186.5-231) µg l^{-1} and the NOEC 50 µg l^{-1} hydrazine, both endpoint results are significantly higher than the predicted discharge concentrations. Verified hydrazine concentrations were only available for the top concentration and this was within 10% of nominal (500 µg l^{-1} nominal, 529.21 µg l^{-1} verified). No hydrazine was detected in the 150 µg l^{-1} test solution and it is not clear why this was. However, confirmation was made that control samples were below levels of quantification and the lowest concentration tested was 0.13 µg l^{-1} and there is no reason to expect that other intermediate concentrations were not dosed. These data therefore suggest that the estimated 48 h EC_{50} of 223 (186.5-231) µg l^{-1} hydrazine for *A. tonsa* is reasonably representative and that as a consequence this species is unlikely to be significantly impacted at the concentrations predicted in the discharge and wider plume area at Sizewell (0.069 µg l^{-1}). The quantification of feeding rate as an ecologically relevant endpoint is important because it impacts upon other measures of fitness, such as growth, fecundity and survival at both the individual and population level (McWilliam and Baird 2002). This has been emphasised for primary producers, where sub-lethal levels of contaminants in the aquatic environment can facilitate eutrophication by reducing grazing rates of cladocerans (Jak *et al.,* 1996 and Taylor *et al.,* 1998). *A. tonsa* exposed to environmentally relevant concentrations of hydrazine (nominal 0.07 and 50 µg l^{-1}) perhaps surprisingly consumed significantly more cells per animal in a post exposure feeding assessment than control animals. It is not clear what may have contributed to this response and if it represents a real effect or is contributed to by the experimental design. Further evaluation of this endpoint is therefore necessary before firm conclusions can be made.

The Pacific Oyster, *Crassostrea gigas,* whilst not identified in Sizewell fauna has been chosen as representative of abundant species, such as *Nucula nucleus* and *Nucula nitidosa*. *C. gigas* is also widely used as a standard toxicity test species. Chemical analysis of the hydrazine solutions showed that the solutions were very close to nominal (between 10 and 13% difference) at 0 hours. After 24 hours, hydrazine concentrations were less than 1% or below detection. All embryos were abnormal in the highest hydrazine treatment only (nominal 1000 µg l^{-1}) after 24-hours exposure. At concentrations below 1000 and down to a nominal concentration of 10 µg l^{-1} there was 55 – 65% normal embryo development compared to 70% in the control. Linear interpolation of these data provided an estimate of EC_{50} of 225 µg l^{-1} and LOEC of 10 µg l^{-1}. However, the response of oyster embryos appears quite variable as a previous study on *C. gigas* exposure to hydrazine, reported 100% abnormality in most test concentrations with 61 - >98% abnormal development at the lowest nominal concentrations tested, 25 and 10 µg l^{-1} (BEEMS TR175). The present study suggests that whilst they are susceptible to hydrazine exposure, the threshold of effect is considerably above predicted concentrations in cooling water. It has also been previously reported that younger, early stage larvae generally exhibit greater sensitivity to toxicants than older larvae, at the settlement stage (Sheridan, 1981). Therefore, based upon the results reported here *C. gigas* is unlikely to be significantly impacted at the concentrations expected at Sizewell (0.069 µg l^{-1}). As a surrogate species, it was used in this report to infer potential for impacts on locally abundant species such as *N. nucleus* and *N. nitidos*. Whilst this report suggests that *C. gigas* are very tolerant of the predicted concentrations of hydrazine in cooling water, sensitivity to hydrazine exposure may vary between species in the same phyla. Therefore, an additional safety factor may be appropriate when extrapolating these data to other bivalve species.

All the tests have been conducted in either static or semi-static conditions. Hydrazine decayed at different rates in the different tests and this seems to be linked to test temperature. In fact, highest decay was found in tests with *C. gigas* for which the test temperature is 24°C while the lowest decay was observed for *L. conchilega* for which the test temperature was 15°C. Other water quality factors could have also influenced

the decay particularly where algal growth media are included but temperature (usually at much higher values) is known to affect decay rate in hydrazine.

Evaluation of the toxicity of hydrazine based on available literature data has previously calculated an acute Predicted No Effect Concentration of 4 ng l^{-1} hydrazine, based on the hydrazine toxicity of the most sensitive species, *D. tertiolecta* with a six day EC_{50} of 0.4 µg l^{-1} for growth inhibition (ELIER0600773). The most sensitive marine microalgae tested for this report was *M. pusilla* which produced a similar 96 hour EC_{50} of 1.27 µg l^{-1} for inhibition calculated with AUGC. Based on this value a derived acute PNEC value would be 12.7 ng l^{-1} if the same expansion factor of 100 is applied as previously reported (ELIER0600773).

The data in this study confirm that microalgae are the most sensitive group to hydrazine exposure but also that there is a considerable range in sensitivity amongst different species. With the tested microalgae, the most sensitive was *M. pusilla*. This species is in the same phylum as *D. tertiolecta* and also with thin walled or naked cells which may make it more susceptible to the effects of hydrazine than for example diatoms that have silica in their cell walls and which are one or two orders of magnitude less sensitive e.g. *Sketetonema* sp (72 hour EC_{50} 37.61 µg l^{-1} hydrazine AUGC), and *T. weissflogii* (72 hour EC_{50} of 140.5 µg l^{-1} hydrazine AUGC). Other plant and animal species tested were similarly less sensitive.

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A.1 Water Qualities and additional test conditions

A.1.1 Skeletonema sp.

Table 15 pH and salinity measured at the beginning of *Skeletonema* sp. test with hydrazine

Treatment-Hydrazine (µg l^{-1})	pH	Salinity (ppt)
0	8	30.5
21.3	7.9	30.6
45.7	8.1	30.5
100.6	8.1	30.6
213.4	8.1	31.1
457.3	8.1	31.3

Table 16 pH and salinities measured at the end of *Skeletonema* sp. test with hydrazine.

Treatment-Hydrazine (µg l^{-1})	pH	Salinity (ppt)
0	8.2	28.3
21.3	8.4	28.3
45.7	8.4	28.4
100.6	8.2	28.4
213.4	8.1	28.9
457.3	8.1	29.1

Table 17 Temperature and light intensities measured at the end of *Skeletonema* sp. test with hydrazine.

Set Temp °C	**20**		
Min Temp °C	20.3		
Max Temp °C	21.2		
Light (µmol s^{-1} m^{-2})			
Rear	103	104	96
Front	82	79	89

A.1.2 Thalassiosira weissflogii

Table 18 pH and salinities measured at the beginning of *T. weissflogii*. test with hydrazine.

Treatment-Hydrazine (µg l^{-1})	**pH**	**Salinity (ppt)**
0	8	30.5
21.3	7.9	30.6
45.7	8.1	30.5
100.6	8.1	30.6
213.4	8.1	31.1
457.3	8	31.3

Table 19 pH and salinities measured at the end of *T. weissflogii*. test with hydrazine.

Treatment-Hydrazine (µg l^{-1})	**pH**	**Salinity (ppt)**
0	8.2	28.3
21.3	8.4	28.3
45.7	8.4	28.4
100.6	8.2	28.4
213.4	8.1	28.9
457.3	8.1	29.1

Table 20 Temperature and light intensities measured at the end of *Skeletonema* sp. test with hydrazine.

Set Temp °C	**20**		
Min Temp °C	20.3		
Max Temp °C	21.2		
Light (µmol s^{-1} m^{-2})			
Rear	203	195	213
Front	132	175	159

A.1.3 Isochrysis galbana

Table 21 water qualities (temperature, pH, salinity and dissolved oxygen) measured at the beginning of *I. galbana* test with hydrazine

Hydrazine (µg l^{-1})	**Temp (°C)**	**pH**	**Salinity (ppt)**	**DO (%)**
0	20.8	7.7	28.8	96.7
0.5	21.2	7.9	28.3	99.6
1.3	21.2	7.9	28.3	100.3
5	21.2	7.9	28.3	100.7
13	21.2	7.9	28.3	100.8
40	21	7.9	28.3	100
120	21.1	7.9	28.3	100

Table 22 light intensity measured at the beginning of *I. galbana* test with hydrazine

Light intensity (µmol s^{-1} m^{-2})			
Rear	106	108	103
Front	99	105	101

A.1.4 Micromonas pusilla

Table 23 water qualities (temperature, pH, salinity and dissolved oxygen) measured at the beginning of *M. pusilla* test with hydrazine

Hydrazine (µg l^{-1})	pH	Salinity (ppt)	DO (%)	Temp (°C)
0	7.7	27.8	100.9	21.9
0.3	7.7	27.8	100.9	21.9
1	7.7	27.9	100.5	21.8
3	7.7	27.9	100.8	21.8
10	7.7	27.8	100.7	21.8
30	7.7	27.9	100.6	21.7
100	7.8	27.9	100.4	21.7

Table 24 water qualities (temperature, pH, salinity and dissolved oxygen) measured at the end of *M. pusilla* test with hydrazine

Hydrazine (µg l^{-1})	pH	Salinity (ppt)	DO (%)	Temp (°C)
0	8.4	28.1	99.8	20.2
0.3	8.4	28.3	99.8	20.4
1	8.3	28.3	99.8	20.5
3	8.2	28.2	99.8	20.4
10	8.2	28.1	99.9	20.8
30	8	28	100.6	21
100	8.1	28.1	100.4	21

Table 25 Light intensity measured at the beginning of *M. pusilla* test with hydrazine

Light intensity (µmol s^{-1} m^{-2})			
Rear	102	112	94
Front	73	74	70

A.1.5 Ceramium tenuicorne

Table 26 water qualities (temperature, pH, salinity and dissolved oxygen) measured during the *C. tenuicorne* hydrazine test (1 of 2).

Hydrazine (µg l^{-1})	Time from beginning of test (hours)	Solution age (hours)	Salinity (ppt)	pH	DO (%)	Temp (°C)
0	0.0	0	29.5	8.2	102.3	22.0
0.001	0.0	0	29.5	8.2	102.1	21.7
0.003	0.0	0	29.5	8.2	102.1	21.8
0.01	0.0	0	29.5	8.2	101.8	21.6
0.1	0.0	0	29.5	8.2	101.8	21.8
0.3	0.0	0	29.4	8.3	101.8	21.7
1	0.0	0	29.5	8.3	101.8	21.8
0	48.0	48	29.4	8.2	102.2	22.0
0.001	48.0	48	29.6	8.2	102.3	22.4
0.003	48.0	48	29.5	8.2	102.1	22.5
0.01	48.0	48	29.5	8.2	102.1	22.4
0.1	48.0	48	29.5	8.2	101.9	22.4
0.3	48.0	4	29.4	8.2	101.7	22.4
1	48.0	48	29.4	8.2	102.0	22.4
0	48.0	0	29.5	8.2	93.5	21.2
0.001	48.0	0	29.5	8.2	94.3	20.8
0.003	48.0	0	29.5	8.2	93.6	21.0
0.01	48.0	0	29.5	8.2	94.0	21.2
0.1	48.0	0	29.5	8.2	93.6	21.3
0.3	48.0	0	29.4	8.2	93.7	21.3
1	48.0	0	29.2	8.2	93.0	21.2
0	96.0	48	29.8	8.2	104.5	21.9
0.001	96.0	48	29.6	8.2	104.4	22.2
0.003	96.0	48	29.6	8.2	103.6	22.4
0.01	96.0	48	29.6	8.2	103.1	22.5
0.1	96.0	48	29.2	8.2	103.0	22.4
0.3	96.0	48	29.4	8.2	102.5	22.3
1	96.0	48	29.2	8.2	102.4	22.3
0	96.0	0	29.3	8.2	97.8	20.0
0.001	96.0	0	29.4	8.2	98.2	20.1
0.003	96.0	0	29.4	8.2	98.1	20.1
0.01	96.0	0	29.4	8.2	98.2	20.1
0.1	96.0	0	29.1	8.2	98.3	20.1
0.3	96.0	0	29.2	8.2	98.5	20.0
1	96.0	0	29.0	8.2	98.5	20.0

Table 27 water qualities (temperature, pH, salinity and dissolved oxygen) measured during the *C. tenuicorne* hydrazine test (2 of 2).

Hydrazine (µg l^{-1})	Time from beginning of test (hours)	Solution age (hours)	Salinity (ppt)	pH	DO (%)	Temp (°C)
0	144.0	48	29.4	8.1	100.8	21.7
0.001	144.0	48	29.5	8.2	101.8	21.7
0.003	144.0	48	29.4	8.2	100.8	21.8
0.01	144.0	48	29.4	8.2	99.9	21.8
0.1	144.0	48	29.4	8.2	99.8	21.8
0.3	144.0	48	29.3	8.2	100.2	21.6
1	144.0	48	29.1	8.2	99.8	21.6
0	144.0	0	29.5	8.1	101.0	21.4
0.001	144.0	0	29.4	8.1	101.0	20.7
0.003	144.0	0	29.4	8.2	100.5	20.9
0.01	144.0	0	29.4	8.2	99.9	20.9
0.1	144.0	0	29.2	8.2	99.8	20.9
0.3	144.0	0	29.3	8.2	99.7	20.9
1	144.0	0	29.2	8.2	99.9	20.9
0	168.0	24	28.2	8.1	102.6	22.0
0.001	168.0	24	28.2	8.1	101.5	22.3
0.003	168.0	24	28.2	8.1	101.1	22.4
0.01	168.0	24	28.3	8.2	100.5	22.3
0.1	168.0	24	28.0	8.1	100.0	22.2
0.3	168.0	24	28.2	8.2	100.0	22.1
1	168.0	24	28.0	8.2	100.2	22.1

Table 28 water qualities (temperature, pH, salinity and dissolved oxygen) measured during the *C. tenuicorne* zinc sulphate reference test.

Zinc sulphate (mg l^{-1})	Time from beginning of test (hours)	Solution age (hours)	Salinity (ppt)	pH	DO (%)	Temp (°C)
0.0033	0	0	29.5	8.2	101.9	22.1
0.01	0	0	29.5	8.2	101.8	22.1
0.033	0	0	29.4	8.2	101.7	22.1
0.1	0	0	29.5	8.2	101.7	22.1
0.33	0	0	29.4	8.2	101.6	22.1
0.0033	168	168	28.2	8.2	100.9	21.9
0.01	168	168	28.4	8.2	100.5	22
0.033	168	168	28.4	8.2	100.5	22.1

0.1	168	168	28.4	8.2	100.1	22.1
0.33	168	168	28.4	8.2	100	22.1

A.1.6 Lanice conchilega

Table 29 water qualities (temperature, pH, salinity and dissolved oxygen) measured during the *L. conchilega* hydrazine test.

Hydrazine (µg l^{-1})	Time from beginning of test (hours)	Solution age (hours)	Salinity (ppt)	DO (%)	pH	Temp (°C)
0	0	0	28.2	100.4	7.86	15.4
15	0	0	28.4	100.9	7.92	15
50	0	0	28.4	100.4	7.92	14.9
150	0	0	28.4	100.7	7.92	14.9
500	0	0	28.4	100.5	7.9	15
1500	0	0	28.4	100.7	7.83	15.1
0	24	24	28.5	102.1	7.92	14.9
15	24	24	28.5	102.1	7.97	14.8
50	24	24	28.5	102	7.97	14.7
150	24	24	28.5	102.3	7.96	14.7
500	24	24	28.4	102.1	7.95	14.7
1500	24	24	28.4	102.2	7.93	14.8
0	24	0	26.9	103.3	7.9	15.4
15	24	0	26.9	102	7.95	15.4
50	24	0	27.5	102.1	7.95	15.4
150	24	0	27.5	102.6	7.93	15.4
500	24	0	25.9	102.8	7.85	15.4
1500	24	0	28.5	103.4	7.94	15.2
0	48	24	28.4	102.7	7.92	15
15	48	24	28.2	103.2	8.09	15
50	48	24	28.6	103.5	8.12	14.9
150	48	24	28.5	103.4	8.12	14.9
500	48	24	28.6	102.7	8.12	14.7
1500	48	24	28.3	102.3	8.08	14.7

A.1.7 Acartia tonsa

Table 30 water qualities (temperature, pH, salinity and dissolved oxygen) measured during the *A. tonsa* hydrazine test.

Hydrazine (µg l^{-1})	Time from beginning of test (hours)	Solution age (hours)	Salinity (ppt)	DO (%)	pH	Temp (°C)
0	0	0	30.3	94.2	7.8	20.6
0.07	0	0	30.3	92.8	7.8	20.7
5	0	0	30.3	93.6	7.8	20.6
15	0	0	30.3	94.6	7.8	20.7
50	0	0	30.3	93.8	7.8	20.8
150	0	0	30.3	93.6	7.8	20.7
500	0	0	30.2	92.8	7.8	20.8
0	24	24	NA	NA	NA	NA
0.07	24	24	30.3	94.2	7.8	20.4
5	24	24	30.3	94.2	7.8	20.5
15	24	24	30.2	94.2	7.9	20.3
50	24	24	30.3	94.2	7.9	20.3
150	24	24	30.3	94.2	7.9	20.4
500	24	24	30.1	93.5	7.9	20.4
0	24	0	29.9	96.1	7.8	20.4
0.07	24	0	29.8	97.7	7.8	21.1
5	24	0	29.9	97.5	7.8	21.2
15	24	0	29.8	97.6	7.8	21.3
50	24	0	29.8	98	7.8	21.4
150	24	0	29.8	98.5	7.8	21.2
500	24	0	29.7	98	7.8	21.3
0	48	24	30.3	94.2	7.7	20.6
0.07	48	24	29.9	93.7	7.9	20.6
5	48	24	29.9	93.8	8	20.6
15	48	24	29.9	93.9	8.1	20.6
50	48	24	29.9	94	8	20.6
150	48	24	29.8	94	8	20.6
500	48	24	29.7	92.9	7.9	20.6

Table 31 water qualities (temperature, pH, salinity and dissolved oxygen) measured during the *A. tonsa* 3,5 dichlorophenol reference test.

3,5 dichlorophenol (mg l^{-1})	Time from beginning of test (hours)	Solution age (hours)	Salinity (ppt)	DO (%)	pH	Temp (°C)
1	0	0	30.3	96.1	7.8	21.3
0.22	0	0	30.3	95.9	7.8	21.4
0.46	0	0	30.3	96.3	7.8	21.5
1	0	0	30.3	96	7.9	21.5
2.2	0	0	30.3	96.4	7.8	21.5
4.6	0	0	30.3	96.1	7.9	21.4
1	48	48	30.3	93.1	8.1	20.3
0.22	48	48	30.3	93.5	8.1	20.6
0.46	48	48	30.3	93.7	8.1	20.6
1	48	48	30.3	93.9	8	20.6
2.2	48	48	30.3	93.8	7.9	20.6
4.6	48	48	30.3	94.6	8.1	20.6

A.1.8 Crassostrea gigas

Table 32 water qualities (temperature, pH, salinity and dissolved oxygen) measured during the *C. gigas* hydrazine test.

Hydrazine (µg l^{-1})	Time from beginning of test (hours)	Solution age (hours)	pH	Salinity (ppt)	DO (%)	Temp (°C)
0	0	0	7.8	28.7	104.3	21.4
0.01	0	0	7.8	28.5	103.7	21.8
0.1	0	0	7.81	28.5	103.2	22
0.3	0	0	7.82	28.5	103.1	22.1
1	0	0	7.81	28.5	103	22.2
3	0	0	7.82	28.5	102.8	22.1
10	0	0	7.83	28.5	102.9	22.2
0	24	24	8.1	29.1	105.2	23.1 – 24.2
0.01	24	24	8.1	29.4	105.4	23.1 – 24.2
0.1	24	24	8.1	29.4	104.7	23.1 – 24.2
0.3	24	24	8.1	29.1	105.1	23.1 – 24.2
1	24	24	8.1	29.4	105.1	23.1 – 24.2
3	24	24	8.1	29.8	105.4	23.1 – 24.2
10	24	24	8.1	29.9	105.6	23.1 – 24.2

A.2 Supplementary data

A.2.1 Culture monitoring data

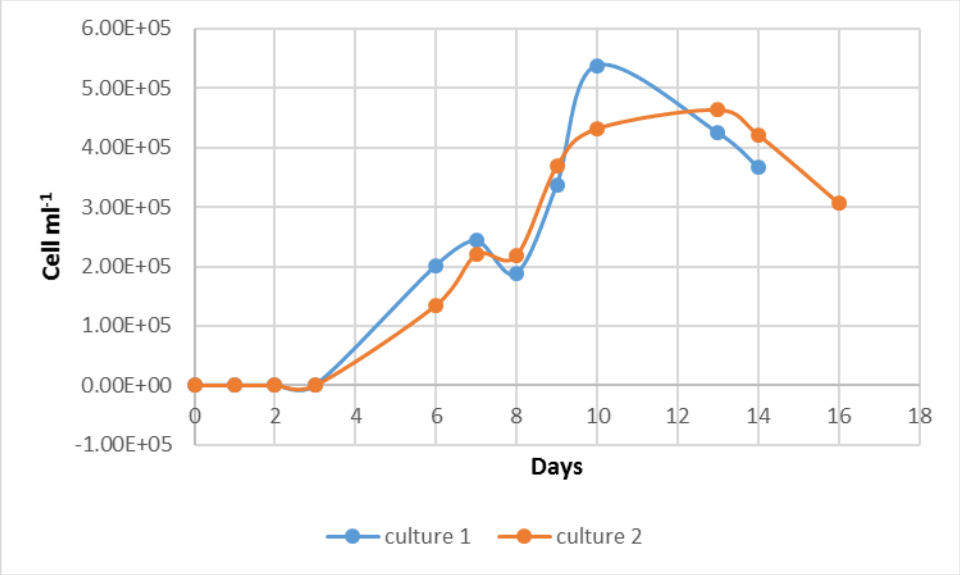

Figure 12 Example of growth in *D. tertiolecta* culture over a 16 days period. Cells were counted daily using a heamocytometer

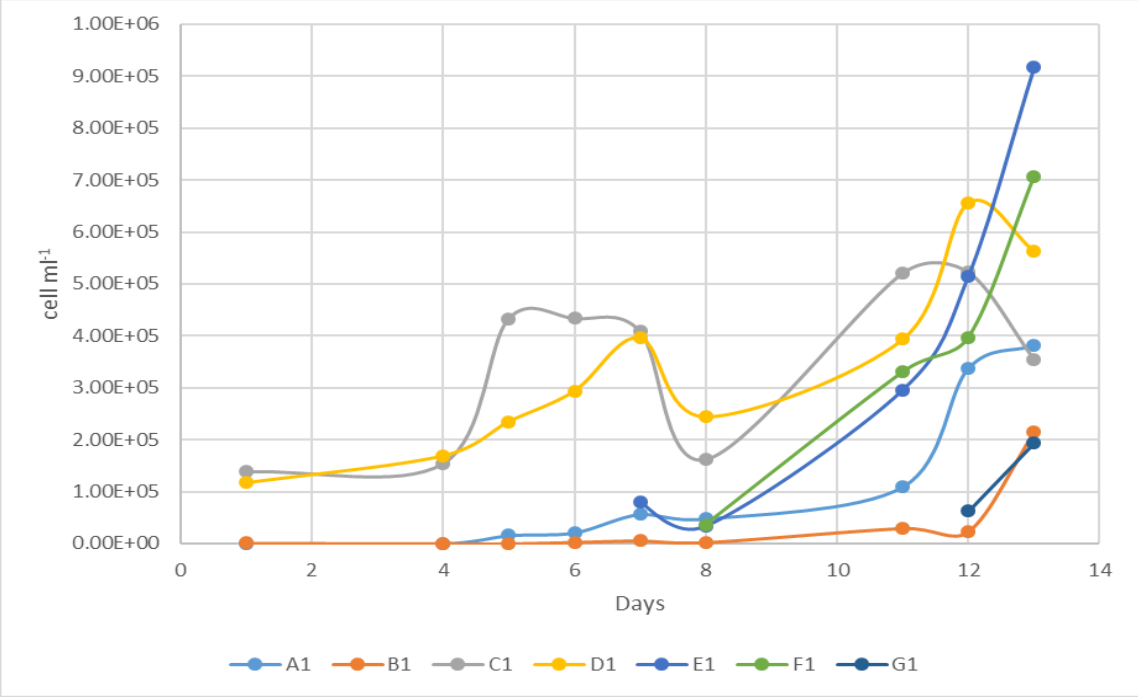

Figure 13 Example of growth in *D. tertiolecta* cultures over a 16 days period. Growth was measured using a plate reader and then fluorescence was converted in cell ml^{-1}. Cultures F1 and G1 were then used to monitor control growth over a 48 hours period. Despite these were in exponential growth they did not show any growth over this period and thus considered unsuitable for a test

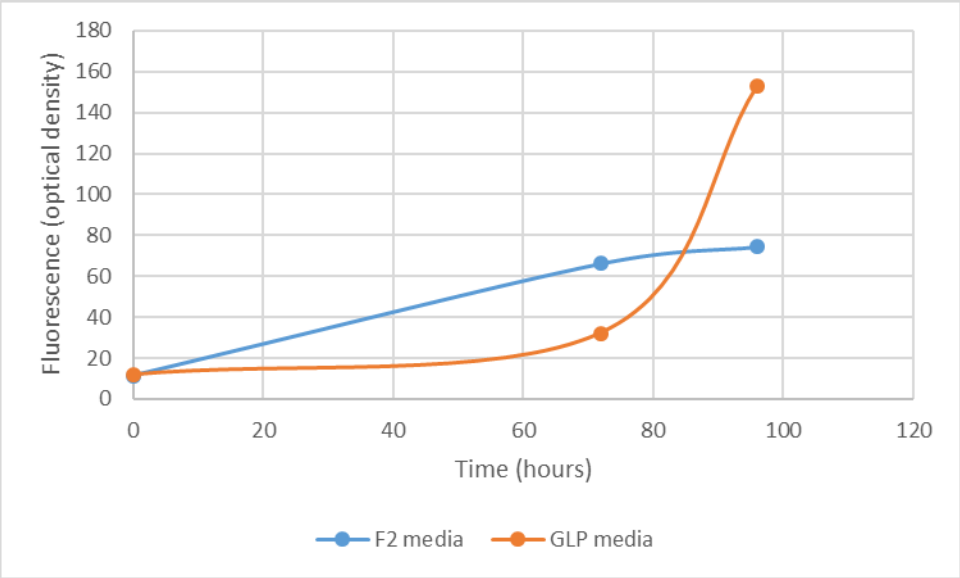

Figure 14 Example of growth in *I. galbana* controls with two different nutrient media.

Table 33 50% effective concentration (EC_{50}, mg l^{-1}) for *C. tenuicorne* exposed to zinc sulphate reference chemical test for 7 days.

Zinc sulphate Concentration (mg l^{-1})	95% confidence limits (mg l^{-1})	Statistical methods
0.061	0.046 - 0.082	Non-linear regression

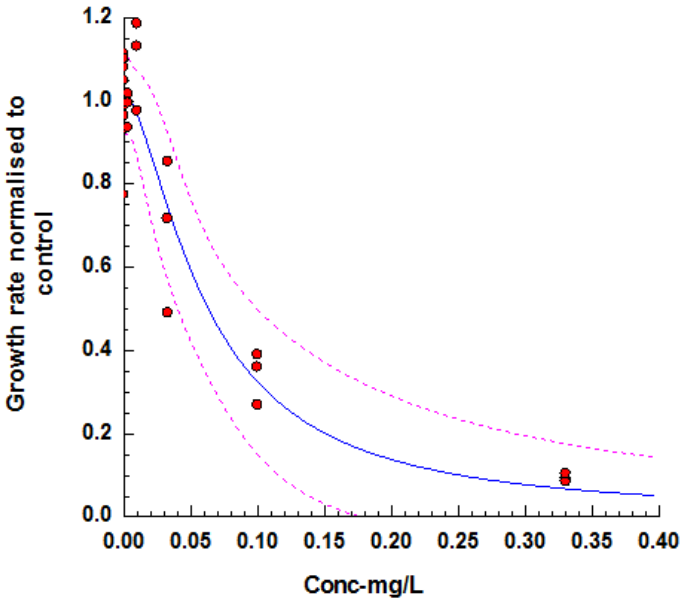

Figure 15 *C. tenuicorne* zinc sulphate reference test nonlinear regression, growth rate normalised to control.

4.1.1 *Lanice conchilega*

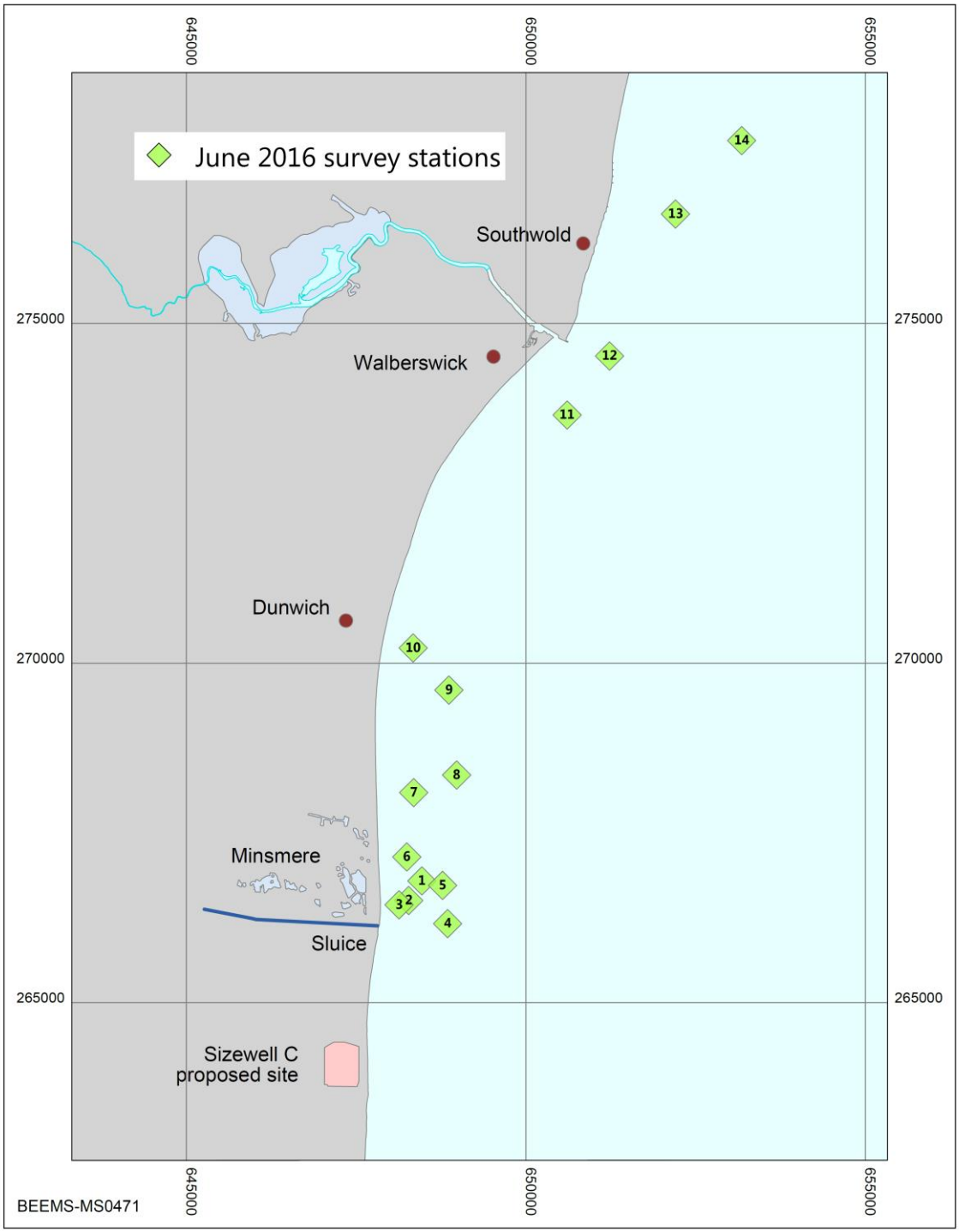

Figure 16 Survey sampling locations of *L. conchilega* aboard the fishing vessel Our Josie Grace, on the 15/06/2016.

4.1.2 Acartia tonsa

Table 34 50% lethal concentration (LC_{50}) results for *A. tonsa* exposed to 3,5 dichlorophenol reference chemical for 48 hours.

3,5 dichlorophenol LC_{50} (mg l^{-1})	95% confidence limits (mg l^{-1})	Statistical methods
0.53	0.3 – 0.78	Linear Interpolation

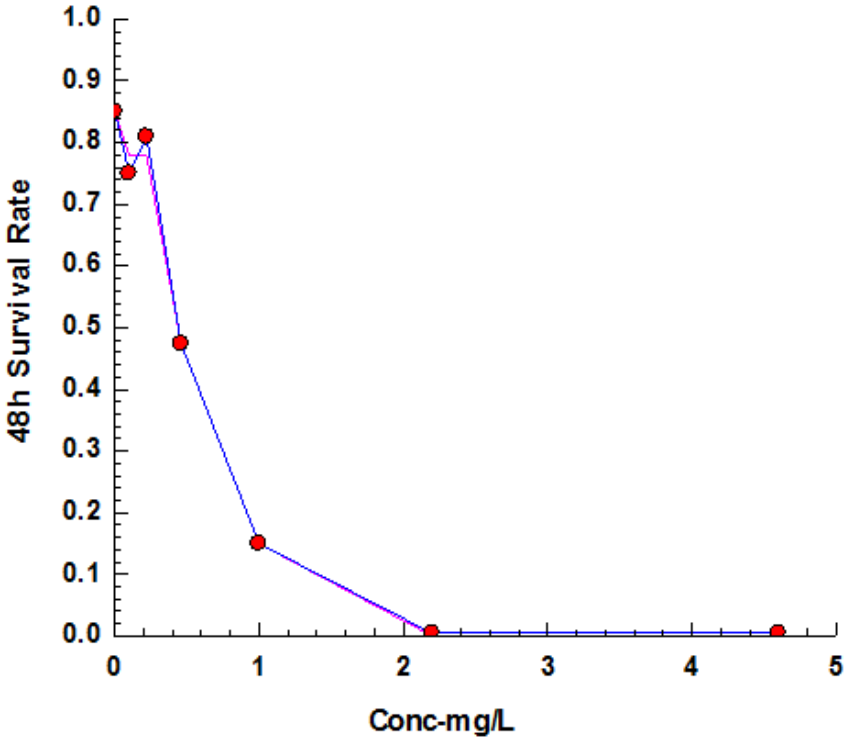

Figure 17 Linear interpolation showing the 48 hour mean survival at different 3,5 dichlorophenol concentrations for *A. tonsa*.

Table 35 One-way ANOVA for the mean number of *T. weissflogii* cells consumed per animal at different hydrazine concentrations for *A. tonsa*.

Feeding time	SS	DF	MS	F (dfn, dfd)	P value
24 hours	1.29E+08	2	64540000	F (2, 14) = 5.109	0.0216

Table 36 Dunnett's multiple comparisons test between different treatments and the control, for the mean number of *T.a weissflogii* cells consumed per animal after 24 hours for *A. tonsa*.

Comparison of control against hydrazine treatment	Mean Diff.	95% CI of diff.	Significant?	P value
0 vs. 0.07	-5024	-10003 to -45.28	Yes	≤ 0.05 (*)
0 vs. 50	-6032	-11380 to -684.3	Yes	≤ 0.05 (*)

4.1.3 Crassostrea gigas

Table 37 Results of zinc reference test (19th August 2016 – 20th August 2016)

Zinc sulphate Concentration (mg l^{-1})	**Average Real PNR (%)**
0 (control)	100
0.022	111
0.046	109
0.1	108
0.22	76
0.46	0
1.0	0

Table 38 50% effective concentration (EC50, mg l^{-1})) for C. gigas exposed to zinc sulphate reference chemical for 24 hours.

Zinc sulphate Concentration (mgl^{-1})	**95% confidence limits (mg l^{-1})**	**Statistical methods**
0.29	0.22 – 0.32	Linear Interpolation