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Spatial and temporal variation of three biomarkers in *Mytilus edulis*

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Abstract :

Environmental conditions and xenobiotic exposure can be sources of stress to living organisms. Biological markers are measurable indicator of changes which may happen at any biological level and which can be considered an early warning signal of some biological or environmental state or condition. A structured field study was undertaken to investigate the relationship between three biomarker assays and the spatial and temporal variation of each biomarker in samples of *Mytilus edulis*. The three biomarkers were the neutral red retention assay, micronucleus assay and comet assay, which indicate damage at different cellular/molecular levels. Three sites in Poole Harbour, an area on the South coast of the UK were sampled on six separate occasions at least three weeks apart. The results for the comet assay showed a significant difference between sites and between sampling dates whereas the results for the other two assays did not show a significant difference for either factor. There was no significant correlation between the results of any pair of the three biomarkers. The results of the micronucleus assay showed a significant correlation with water temperature. This temperature effect, as well as induced repair, may contribute to explain the lack of a strict correspondence between pollution gradients and biomarkers responses.

Highlights

► The CA results, but not those of NRR or MN, showed a significant variation with respect to sampling site and date. ► No significant correlation between NRR or CA and any of the measured environmental variables (temperature, salinity, pH). ► The MN Index showed a significant correlation with water temperature but no significant correlation with salinity or pH. ► There was no correlation reported between the results of the three biomarkers (NRR, MN and CA).

Keywords : *Mytilus edulis*, Biomarkers, Stress, Neutral red retention, Comet assay, Micronucleus assay, Poole Harbour

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1. Introduction

Poole Harbour, located in the South of England, is a large natural harbour which contains numerous active commercial bivalve harvesting areas (Food Standards Agency, 2017). The northern and western region of the harbour contains industrial and urban centres and more

sources of anthropogenic pollution compared to the southern sections. Discharges of industrial waste have resulted in high concentrations of metals including cadmium (Cd), mercury (Hg), copper (Cu), silver (Ag), nickel (Ni) and zinc (Zn) in the northern part of the harbour (Bryan & Langstone, 1992; Turner A. 2000; Poole Harbour Steering group, 2011). Additional contamination arises from leisure marine activities and the commercial port operation, again concentrated on the northern side of the harbour. In the past, these activities have contributed to the historic contamination of the harbour by tributyltin (TBT) (Langston et al., 2015). The presence of many of these compounds in Poole Harbour has been described by Cefas in the SEA8 final report (2007), which focused on the assessment of major contamination inputs and on their resulting contamination of the environment. The south side of the harbour is potentially impacted by two oil production facilities: one located on land and the other on an island within the harbour. However, PAH and metal values in sediments from Poole harbour, while exceeding certain sediment quality guidelines, are relatively low when compared with other heavily industrialised ports and harbours around the coast of England and Wales (Cefas, 2007; Nicolaus 2015). The western side of the harbour is mainly impacted by agricultural activity resulting in elevated concentrations of inorganic nitrogen levels, which are about six times higher in this section of the harbour (Environment Agency, 2013).
Biological effect markers are represented by any physiological and structural alteration that may occur at any level of the organism as response to environmental changes, both natural and anthropogenic. Biomarkers are defined as quantitative measures of any biochemical, physiological or behavioural changes occurring in an organism in response to external factors that can be a source of stress as xenobiotic exposure. The changes may be measured in cells, body fluids, tissues or organs and are typically intended to provide an early warning signal of biological effect due the fact that the sub-cellular or biochemical responses measured tend to precede those that occur at an organism or population level (Lam & Gray, 2003; Martin-Diaz et al., 2004; Schettino et al., 2012). Due to the dynamic equilibrium of the marine environment, the use of a battery of biomarkers allows an integrated assessment of effects at chemical, cellular, sub-cellular, tissue and whole organism levels. Bivalve mussels have a long history of use to indicate the extent of environmental contamination by a wide range of chemical compounds (International Mussel Watch Committee; Intergovernmental Oceanographic Commission, 1992). More recently they have also been used in the field to determine the effects of such contamination using biomarkers (Brooks et al., 2009; Shaw, *et al*., 2011). A combination of three different biomarkers in *Mytilus edulis* (common or blue mussels) was selected for the present study, neutral red retention (NNR) assay provides an indicator of general cellular stress, while the comet assay and micronucleus assay are both markers of genotoxicity and represent a more specific indicator of DNA damage. The NRR assay had been developed to determine the lysosomal membrane stability of mussel haemocytes (Zhao et al., 2011). It is basically a stress assay, which consist in verifying the stability of the lysosomal membrane by putting it in contact with the neutral red dye. When the lysosomal membrane, or possibly the H+ ion pump, is destabilized, neutral red leaks into the cytosol of the cell much more quickly than in an unstressed cell (Zhao et al., 2011). The damage detected by this assay may be due to several factors such as exposure to heavy metals (Cu, Cd, Hg) or organic pollutants as well as natural factors such as extremes variations of temperature or salinity (Dailianis et al., 2003; Loayza-Muro & Elias-Letts, 2007; Aguirre-

Martìnez, 2013) and the final stage of gametogenesis and spawning, which is a natural stressful process.

The micronucleus assay (MN) detects fragments of chromosomes that result from anomalies during the cell division phase (Barsiene et al., 2006). Their presence is an indicator of genotoxic effect and may indicate the presence of organic and inorganic substances such as polycyclic aromatic hydrocarbons, heavy metals and organochlorinated compounds (Brunetti et al., 1992; Magni et al., 2006; Dailianis et al., 2003; Bolognesi & Fenech, 2012). The frequency of MN formation is considered to be a suitable index of a time-integrated response to cumulative stress following exposure to environmental contaminants.

Similar to MN assay, the comet assay (CA) is a measurement of genotoxicity that may result from exposure to numerous chemical contaminants (Frenzilli et al., 2009). The assay is rapid, simple and produces a linear dose response to exposure e.g. ultraviolet (UV)-induced pyrimidine dimers, oxidized bases, and alkylation damage (Collins et all. 1996; Azqueta et al., 2011), or to a fuel oil spill (Lewis et al., 2010). The direct measurement of DNA damage is due to the evaluation of tails formed during the electrophoretic migration of the nuclear material. The degree of damage can be evaluated as either the tail moment (a function of tail length and fraction of DNA in tail) or the percentage of DNA in tail (also known as tail intensity) (Kwok et al., 2013; Mamaca et al., 2005; Lyons et al., 2012; Rank et al., 2005).

The NRR assay therefore gives an indication of general cellular stress and it has been used widely as a biomarker of the impact of chemical contaminants or other adverse environmental conditions (Dondero et al., 2006; Shaw et al., 2011). The MN and CA are both markers of genotoxicity and therefore give a more specific indicator of DNA damage, if compared to NRR. Increases in the frequency of micronuclei and/or increased tail length in the CA are expected in response to specific chemicals with genotoxic properties. Although the biomarkers chosen for the present study have been previously demonstrated in laboratory studies to respond in a concentration-dependent manner to several compounds (Galloway et al., 2002), they are not specific for the effects of one single pollutant. Moreover, they reflect the impact of a suite of environmentally relevant contaminants, able to affect the exposed animals (Lewis et al., 2010).

The purpose of the present work was to compare the biological effect on the shellfish at site assessed as being exposed to differing levels of sewage and chemical pollutants under a range of environmental conditions. NRR, MN assay and CA were used to evaluate the health condition of the mussels and the occurrence of a stress syndrome. It was planned to locate mussels in bags at three different locations within one area with sampling undertaken approximately every 15 days over a four-month period. After each sampling occasion mussels were replaced with other bagged depurated mussels.

2. Materials and methods

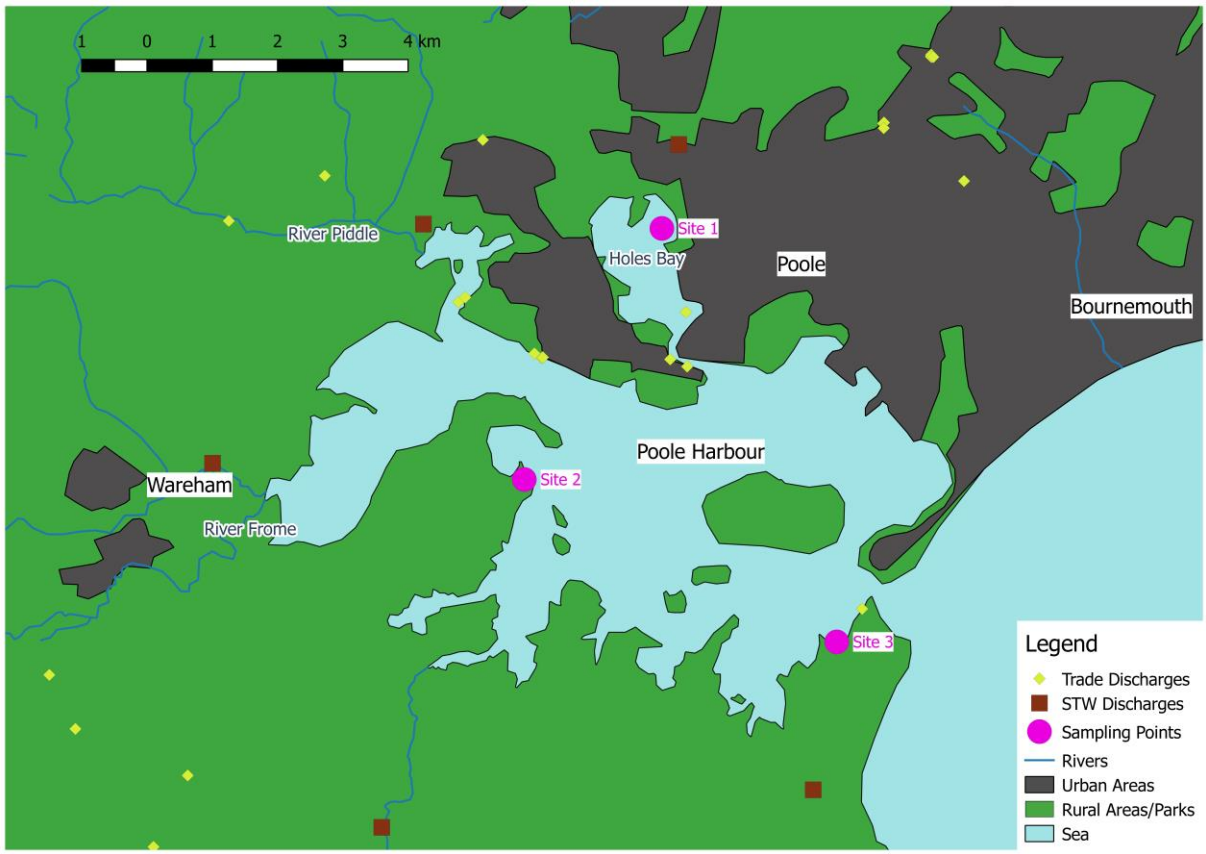
2.1 Sampling sites

Three sampling sites were selected within the harbour intended to represent different contamination sources and environmental influences (see Figure 1):
Site 1: it was located within a bay in the northern part of the harbour. This bay is subject to the greatest amount of historical and current contamination from historical and current industrial, maritime and sewage sources.

Site 2: it was located on the eastern side of Arne Peninsula which contains a nature reserve with an associated farm. This was expected to be subject to moderate amounts of anthropogenic contamination arising from the northern side of the harbour as well as some sewage and agricultural contamination arising from the western side of the harbour.

Site 3: it was located near to the entrance of the harbour and was selected as likely to be the least contaminated site. The only identified local sources were a small sewage discharge located approximately 2 km to the south-west and the Furzy Island oil operation located approximately 2 km to the north-west. The large tidal flows through the nearby mouth of the harbour were considered likely to reduce the effect of any impacts from those sources.

All sites were therefore potentially subject to impact to different extents from known sources of contamination and there was the possibility of other unknown sources. Therefore, it was considered that it was not appropriate to consider any of the sites as a control for comparative purposes.



STW = Sewage Treatment Works

Figure 1. Study area showing sampling sites and potential sources of contamination

2.2 Sampling and on-site measurements

As previously described (Strubbia et al., 2016), bagged depurated Poole Harbour mussels (approximately 1.5 kg) obtained from a local depuration center, were placed intertidally at each site 15 days prior to each planned sampling occasion. Immediately after sampling, any mud or other sediment adhering to the shells was removed by scrubbing/rinsing with seawater from the immediate vicinity of the sampling site while avoiding re-immersion. Sampling of mussels, together with collection of physicochemical measurements, was undertaken on six occasions between November 2013 and January 2014, generally around low water springs in order to facilitate access to the intertidal sampling locations. The mussels in each bag were split into three equal quantities and placed into appropriately labelled plastic bags. Transport of samples was undertaken in insulated boxes with cool packs in order to maintain a temperature ranging between 1°C and 8°C. The duration from the time of sampling to the arrival at the laboratory was always less than 6 hours and analyses started within 24 hours of sampling. Between arrival at the laboratory and the start of analysis, all samples were stored at 4°C.Seawater salinity was measured at the time of sampling using either a YSI Professional Plus meter with conductivity and temperature probe or a YSI castaway Conductivity/Temperature/Depth (CTD) recorder calibrated using a 35 practical salinity unit standard.

2.3 Haemolymph extraction for biomarker assays

Haemolymph was extracted from a total of 12 mussels for each sample following the method reported by Moore et al. (Moore, et al., 2004). Briefly, the mussel valves were prised apart along the ventral surface, using a solid scalpel and 0.1 ml of haemolymph from the posterior adductor muscle was extracted into a hypodermic syringe containing 0.1 ml of physiological saline, withdraw. The contents were transferred to a 1.5–2.0 ml siliconized microcentrifuge tube and stored in a refrigerator for up to 20 minutes prior to use. The haemolymph from each sample of mussels was stored in four separate vials, each containing the haemolymph from three different animals. The use of pooled samples of haemolymph allowed the examination of a large number of animals while keeping the number of individual biological marker test replicates to a practical level. A sample size of at least 10 mussels per site has been determined as being sufficient for the assessment of the environmental genotoxicity levels and evaluation of the existence of genetic risk zones (Baršienė, et al., 2012).

2,4 NRR assay

The method used in the present work was previously described by Moore et al. (ICES 2004). Fifty microlitres of the collected haemolymph were placed on a microscope slide and placed in a dark room for 15 min to allow adherence to occur. Fifty microliters of a 200 µM neutral red solution were then added and incubated for 50 minutes, again in the dark. Those cells showing release of dye into the cytosol were deemed to be showing lysosome membrane damage. Fifty cells were counted per slide and results were expressed as the number of

damaged cells out of fifty. A proportion of 50% or more of the cells exhibiting lysosomal leakage is considered to show evidence of a stress condition (Ringwood et *al*, 2005).

2.5 MN assay

The MN assay was undertaken by the method of Bolognesi and Fenech (2012). One hundred microliters of pooled haemolymph were placed onto a microscope slide and immediately placed in a humid chamber for 15 min; the microscope slides were left to dry on the bench for at least 5–10 min. The cells were fixed with 100% methanol for 10 min followed by further air-drying for 10 min at room temperature. Dried slides were stained with 3% (vol/vol) Giemsa solution for 5 min at room temperature, then rinsed in washing solution, prepared by adding 3 ml of Sorensen buffer to 196 ml of distilled or deionized water, and air-dried. Coverslips were applied and slides were examined using transmitted light microscopy at ×1,000 magnification.

The ICES Cooperative Research Report (Baršienė, 2012) diagnostic criteria for micronucleus identification were used. These are:

- the micronucleus is less than 1/3 of the size of the main nucleus.
- micronuclei are round- or ovoid-shaped, non-refractive chromatin bodies located in the cytoplasm of the cell and can, therefore, be distinguished from artefacts
- micronuclei are not connected to the main nuclei, and the micronuclear boundary should be distinguishable from the nuclear boundary.

One thousand cells were examined per slide, with four replicate slides per sample, each representing a separate haemolymph pool. The MN index for the sample was determined as the mean number of micronuclei per thousand cells over the four slides.

2.6 Comet Assay

The method used in the present work was performed according to guidelines published by ICES (Lyons, et al., 2012). Samples were cryopreserved using the method described by Kwok et al. (2013) in order that the assay could be undertaken when convenient. Prior to the assay, samples were left defrost at ambient temperature. Ten microlitres of haemolymph were placed in 160µl of low melting point (LMP) agarose (0,7% w/v in Phosphate-buffered saline (PBS) (Ca^{++}, Mg^{++} free): Dulbecco's PBS pH 7.4) previously heated and kept warm in the incubator at 37°C; 50µl were placed onto a separate circle on a Trevigen Comet Slide®. During the placement of the agarose the slide was kept in the dark and on ice, and then left in fridge for 10 minutes to set. A lysis working solution was prepared thirty minutes prior to use adding 1% Triton X-100 and 10% DMSO to the stock solution (2.5M NaCl, 100nM EDTA, 10nM Trizma base in distilled water).Slides with the embedded cells were immersed in the lysis working solution for 30 minutes in the fridge in order to ensure the digestion of cellular and nuclear constituents. Slides were then placed in an electrophoresis tank with Alkaline Electrophoresis Solution pH>13 (300 mM NaOH, 1mM EDTA) and incubate for 30 minutes. Electrophoresis was performed at 0.7 volts per cm and 300mA for 30 minutes. At the end of the run slides were washed 3 times in ultra-pure water and twice with 100µl of neutralisation

buffer (0.4M Tris base; prepared with 48.44g of Tris made up to 1000 mL with distilled water and adjusted to pH 7.5 using concentrated hydrochloric acid). They were then fixed with 100% methanol for 5 minutes. Immediately before reading the slides were rehydrated and stained with the DNA specific fluorescent dye, SYBR Gold, and read using 40x magnifications. 50 cells per slide were counted. Four slides per experimental point were scored at each sampling occasion. The tails were evaluated through Comet Assay IV analysis software (Perceptive Instruments Ltd) and Camera AVT smart view for, Fire Package version 1.6.2) to measure the total image fluorescence intensity, head intensity, tail intensity, head length and tail length. The results were recorded as the % tail intensity over 50 cells.

2.7 Data analysis

Graphs were prepared in Microsoft Excel 2016 (Microsoft Corporation) and statistical analyses were undertaken using Minitab v16 (Minitab Ltd). Probability plots were prepared for the data for each of the biomarkers and a fit to a normal distribution assessed using the Anderson-Darling test. The graphs (not shown) for all three biomarkers showed a reasonable fit to a normal distribution and the probability values for the Anderson-Darling test markedly exceeded 0.05 in each case. Therefore, non-transformed biomarker data were subsequently used for statistical analyses and graphical display. Analysis of the biomarker data by site and sample collection date was undertaken using General Linear Modelling. No assumptions were made about the distribution of the environmental variables and therefore Spearman's Rank Correlation was used to investigate any association between the results of the biomarker assays and the environmental variables.

2.8 Mapping

The map shown in Figure 1 was prepared using QGIS v2.18.13 (https://www.qgis.org/). A database of consented discharges to controlled waters was downloaded from the UK government data repository (https://data.gov.uk/dataset/consented-discharges-to-controlled-waters-with-conditions; downloaded 19/10/2017; metadata given on source page). Data tables were exported to Microsoft Excel 2016, duplicate entries removed and separate tables for discharges relating to sewage treatment works (STWs; which may contain allowed discharges of industrial effluents) and trade discharges (discharges from commercial premises, including those containing industrial effluents) were exported as comma separated variable (.csv) files to allow import into QGIS).

3. Results and discussion

3.1 Association between biomarkers

Statistical analyses showed the lack of a significant correlation ($p \geq 0.05$) between the three biomarkers. Other authors already reported similar results showing that living organisms may have different behaviour after exposition to environmental pollutants (de Lafontaine et al.

2000 ; Forbes et al. 2009). These observations support the importance to select a battery of biomarkers in order to detect pollution effects on the environment since different stressors may induce a different effect on the studied organisms. Sometimes the use of biological markers for environmental monitoring may be controversial. Buchelli and Fent (2009) showed correlation between the induction of cytochrome P4501A and various contaminant (e.g. PAHs, coplanar PCBs, polychlorinated dibenzofurans, and dibenzodioxins). But they also highlighted that multiple factors can inhibit, or modify, biomarker responses, such as species, age and reproductive stage of the living organism, or physical environmental parameters as temperature or the presence of possible inhibitors. Furthermore, the lack of any significant correlation between the results of any two of the three biomarkers complicates the assessment of the state of contamination of the different sites within Poole Harbour. As well as the effect of temperature on MN Index, considered above, another aspect that may have affected the results is that DNA repair mechanisms are often triggered as compensatory response to damage. Several authors, as Nicholson & Lam (2005) and Siu et al. (2004), discussed the presence of a threshold-dependent DNA repair system. Siu et al. studied the possible application of CA and MN assays to the detection of benzo[a]pyrene (B[a]P) in mussels. The authors suggested that a DNA repair system may be activated after the mussel tissue has accumulated sufficient toxicant above a threshold level, as previously observed also by other authors as Ching et al. (2001), Nicholson (2001) and Siu et al. (2003).

3.2 Biomarker temporal and spatial variability

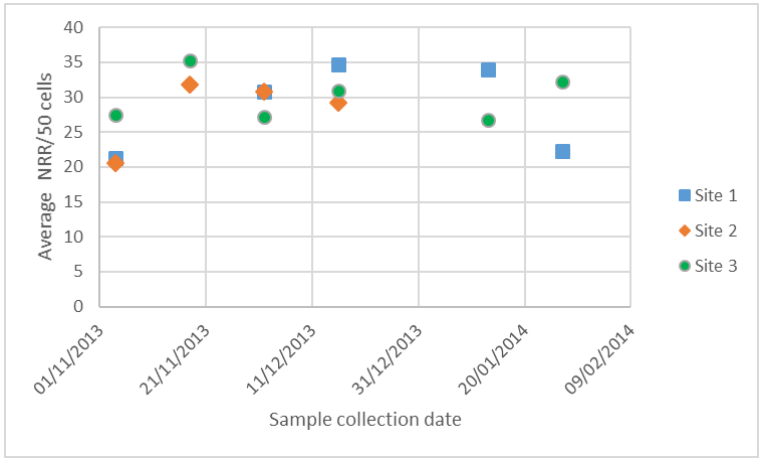
Scatterplots of the results of the three biomarker assays are shown in Figure 1 and a summary of the output of the General Linear Modelling is presented in Table 1. No significant difference was seen between either site or sampling date for either NRR or MN Index. On the contrary, with the comet assay, a statistically significant difference was seen between sites (0.031) and between sampling dates (<0.001). The highest mean value in the comet assay was seen at Site 3 (39.8 % tail intensity) although the maximum individual value was seen at Site 1 in November 2013 (53.8%). The lowest mean value was seen at Site 2 (38.5%). There was a tendency for the magnitude of the comet assay results at all sites to decline over the period of the study. Additional analysis using Tukey's method showed that, at the 95% confidence level, Site 1 had a significantly higher mean value than Site 2 and the two sampling occasions in November had significantly higher mean values than those in mid-December and January. On the three occasions that results were available for all three sites, site 2 yielded lower values than the other two sites (see Figure 2c).

The biomarker results for Site 1 were expected to be higher than the other two sites due to the concentration of historic and present-day sources of contamination in the vicinity. It has been assumed that site 3 would yield the lowest results with all assays due to its distance from most sources of contamination. However, it is possible that locations around the narrow harbour mouth are impacted from multiple sources within the harbour over the ebb tide, thus yielding the observed results with the comet assay.

The temporal pattern in the results of the CA did not result from the effects of any of the measured environmental variable (see below) and may reflect either other environmental

variables or changes in other stressors such as the concentration of contaminants in the water column. The latter could be due to variation in either the level of actual inputs or in the amount of sediment-associated contamination associated with resuspension events.

a.



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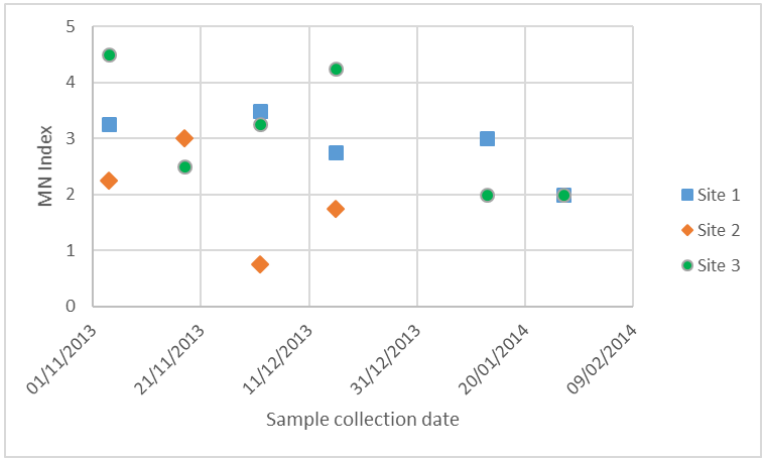

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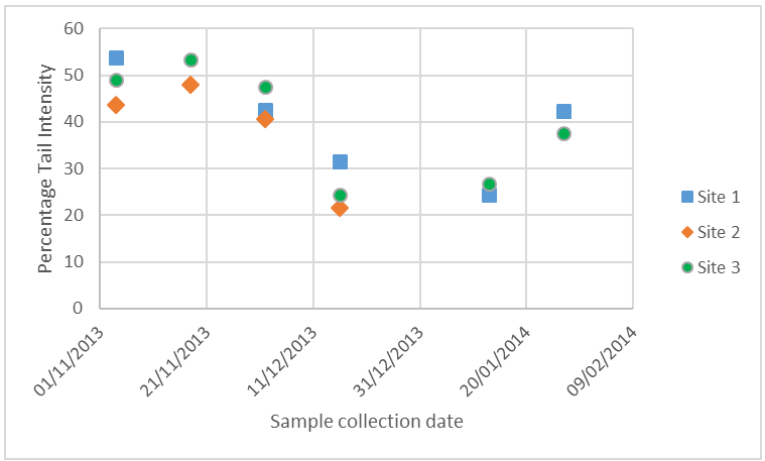

Figure 2. Results of biomarker assays by sampling site and date

a. NRR b. MN index c. CA

Table 1. Summary of GLM output from the analysis of biomarker results by site and date

Biomarker	Source of variation	F	P
NRR	Site	0.37	0.703
	Date	2.06	0.187
MN Index	Site	3.72	0.079
	Date	1.26	0.377
CA	Site	5.96	0.031
	Date	38.04	<0.001

3.2 Results of environmental variables

The mean, minimum and maximum values of temperature, salinity and pH obtained at the three sites are shown in Table 2.

Table 2. Mean, minimum and maximum temperature, salinity and pH values at the three sites

	Mean (minimum, maximum)		
Site	Temperature (°C)	Salinity (PSU)	pH
Site 1	10.2 (7.4, 11.9)	21.3 (14.6, 24.9)	7.4 (6.4, 8.5)
Site 2	8.9 (6.8, 10.3)	23.6 (18.5, 26.7)	7.7 (7.3, 8.5)
Site 3	9.1 (7.0, 11.3)	29.6 (24.0, 32.8)	8.0 (7.6, 8.7)

3.3 Relationship between environmental variables and biomarkers

No significant correlation was seen between the results of either NRR or CA and any of the environmental variables ($p \geq 0.05$). The MN Index showed a significant correlation with water temperature ($R_s=0.58$, $p=0.022$) but no significant correlation with salinity or pH.

The only significant association between the biomarker results and the three environmental variables that were measured was that between MN Index and temperature. The observed

correlation with water temperature is an effect previously reported by Bolognesi and Fenech (2012), who stated that temperature may be considered a confounding factor because it seems have a direct effect on the mitotic rate and consequently on the extent of MN expression. The temperature range recorded during the study period was relatively restricted (6.8 to 11.9) and the results do not preclude an effect of temperature on NRR or comet assay if the study was extended over a greater part of the year, and therefore a wider temperature range. The effect of temperature on MN Index in this study may have affected the ability to detect spatial differences with this biomarker and affected the relationship between it and the results of the comet assay: Siu et al (2004) had previously shown good correlation between MN Index and the CA results in haemolymph from *Perna viridis* exposed to benzo[a]pyrene under controlled laboratory conditions.

4. Conclusions

Holes Bay, an area subject to both historical and ongoing contamination, showed the highest mean CA level, which conformed to the expectation at the start of the work. However, the observation that the mean CA level at the site near the harbour entrance was not significantly lower than this was not expected: this could be due to unidentified local sources of one or more contaminants.

The lack of correlation between the results of the three biomarkers supports the importance of selecting a battery of biomarkers to detect the effects of pollutants in the environment. Multiple factors can inhibit, or modify, biomarker responses, such as species, age and reproductive stage of the living organism, or physicochemical parameters of the environment or the presence of possible inhibitors. Furthermore, the absence of correlation between sites may be due to levels of contamination too low to cause an effect in the assessed biological systems. Weak differences in concentrations between sites, may also explain the luck of significant effect over and above the underlying variability in the assay results.

HIGHLIGHTS

- The CA results, but not those of NRR or MN, showed a significant variation with respect to sampling site and date.
- No significant correlation was seen between the results of either NRR or CA and any of the three measured environmental variables (temperature, salinity, pH).
- The MN Index showed a significant correlation with water temperature but no significant correlation with salinity or pH
- There was no correlation between the results of the three biomarkers, neutral red retention (NRR), micronuclei index (MN) and comet assay (CA).

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