Genotoxic Damage in Polychaetes: A Study of Species and Cell-Type Sensitivities.

Ceri Lewis * & Tamara Galloway

School of Biosciences, Hatherley Laboratories, University of Exeter, Prince of Wales Road, Exeter, UK, ES4 4PS.

Tel: +44 (0)1392 264672, fax 0(44) 1392 264672

Contact E-mail: c.n.lewis@exeter.ac.uk

Abstract

The marine environment is becoming increasingly contaminated by environmental pollutants with the potential to damage DNA, with marine sediments acting as a sink for many of these contaminants. Understanding genotoxic responses in sediment dwelling marine organisms, such as polychaetes, is therefore of increasing importance. This study is an exploration of species specific and cell-specific differences in cell sensitivities to DNA damaging agents in polychaete worms, aimed at increasing fundamental knowledge of their responses to genotoxic damage. The sensitivities of coelomocytes from three polychaetes species of high ecological relevance; the lugworm *Arenicola marina,* the harbour ragworm *Nereis diversicolor* and the king ragworm *Nereis virens* to genotoxic damage are compared, and differences in sensitivities of their different coelomic cell types determined using the comet assay. *Arenicola marina* was found to be the most sensitive to genotoxic damage from the direct acting mutagen methyl methanesulfonate (MMS), and showed dose dependent responses to MMS and the polycyclic aromatic hydrocarbon benzo(a)pyrene. Significant differences in sensitivity of the different types of

coelomocyte were also measured. Eleocytes were more sensitive to DNA damage than amoebocytes in both *Nereis virens* and *Nereis diversicolor*. *Arenicola marina* spermatozoa showed significant DNA damage following *in vitro* exposure to MMS, but were less sensitive to DNA damage than coelomocytes. This investigation has clearly demonstrated that different cell types within the same species and different species within the Polychaeta show significantly different responses to genotoxic insult. These findings are discussed in terms of the relationship between cell function and sensitivity and their implications for the use of polychaetes in environmental genotoxicity studies.

Keywords: DNA damage; coelomocytes; sperm; *Nereis virens*; *Nereis diversicolor*; *Arenicola marina*

1. Introduction

A significant proportion of the chemicals entering aquatic environments have the potential to induce DNA damage or interfere with the processes involved in cell division [1, 2]. These include, amongst others, persistent organics such as polycyclic aromatic hydrocarbons (PAHs) and metals, which can damage DNA either directly or indirectly via the production of free radicals or via metabolic activation. Whilst the body of evidence documenting these effects in vertebrates is high, there has been less attention given to the invertebrate species that occupy key ecological niches in marine habitats and for which genetic damage might have great potential for harm. The increasingly widespread aquatic distribution of chemicals with genotoxic potential has meant that the measurement of genotoxicity in the marine environment is fast becoming an area of great concern [3-7]. Sediments have long been recognised as a sink for organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), which by virtue of their hydrophobic nature can strongly adsorb onto sediments affecting the benthic community inhabiting them. Sediment dwelling macrofauna (infauna) are important vectors for the transfer of sediment-associated contaminants to higher trophic levels since they form the primary food source for many commercial fish and crustacean species. Understanding and monitoring the genotoxic impacts of pollutants in sediment dwelling organisms is therefore of great importance for both environment and human health.

Polychaete worms tend to form the dominant sediment dwelling fauna of most mud flats and estuaries, yet despite their obvious importance for environmental monitoring purposes, they have received surprising little attention with regards to their genotoxic responses to environmental pollutants. The main study is that of De

Boeck and Kirsch-Volders [8], who investigated the use of *Nereis virens* as a sentinel species for measuring genotoxic responses to PAH's. The authors used an intra-coelomic injection of benzo(a)pyrene (0.3-45 mg/ml), harvesting coelomocytes for assessment using the comet assay one hour after injection. Based on the negative results obtained using this protocol, De Boeck and Kirsch-Volders conclude that this species is tolerant to PAHs and therefore not suitable for environmental monitoring. Studies using alternative end points in other polychaete species, however, have suggested certain polychaetes are highly sensitive to genotoxic damage (e.g. *Platynereis dumerilii* [9,10]; *Pomatoceros lamarckii* [11]). Studies using the terrestrial earth worm *Lumbricus terrestris* and *Eisenia foetida* [12-15] also found increased comet tail lengths in response to PAH's, benzene and dioxins in the soil. A greater understanding of the genotoxic responses of marine polychaetes is required to aid our understanding of their survival in polluted environments and hence in environmental monitoring.

The *in vivo* comet assay in its alkaline form (pH > 13) is increasingly used in genotoxicity testing of substances such as industrial chemicals, biocides and pharmaceuticals [16]. Genotoxic studies using the comet assay are often based on an organisms' free cells, i.e. blood cells or sperm in humans and vertebrates or haemocytes or coelomocytes (invertebrate equivalents to blood cells) in invertebrate species, due to their ease of collection. Polychaetes are morphologically and physiologically an extremely diverse group of animals and their free cells exhibit a similar diversity which has confounded attempts to apply a simple classification system to describe the cell types of the group [17]. Certain types of coelomocytes are present throughout the group however, the most widely used terms to describe them are 'amoebocytes' and 'eleocytes'. *Nereis virens* and *N. diversicolor* both have

several types of amoebocytes, which have varying functions such as immunity and wound healing, as well as nutritive eleocytes that produce vitellogenin for developing oocytes and nucleotides for developing spermatids [17,18].

The overall response of a cell to a genotoxic substance will result from a combination of exposure, uptake, metabolic activation, defence mechanisms and repair efficiency, and may differ significantly for different cell types. Any differences in the responses of these different cell types would lead to large individual variation in the genotoxicity data collected, making it more difficult to determine any significant effects caused by environmental exposure to a low genotoxic dose. The coelomic cavities of most polychaete species also contain developing gametes during some or most of the year, depending on species and location, which may also have differing responses from somatic cells, and therefore have the potential to interfere with investigations of genotoxicity if not taken into consideration when sampling cells from the coelomic cavity.

This study is an exploration of species specific and cell-specific differences in cellular sensitivity to DNA damaging agents in three polychaete species, aimed at improving the fundamental knowledge of genotoxic responses in polychaetes. Using three abundant polychaete species; the lugworm *Arenicola marina*, the king ragworm *Nereis virens* and the harbour ragworm *Nereis diversicolor*, the responses (i) between coelomic cell types (.i.e. amoebocytes, eleocytes and spermatozoa) and (ii) between species, to the direct acting genotoxins methyl methanesulfonate are compared to test the hypothesis that different cell types will exhibit different sensitivities to genotoxic damage. The time and dose responses of *A. marina* to methyl methanesulfonate (a direct acting mutagen) and the metabolically activated PAH benzo(a)pyrene are also

compared and a comparison of *Nereis virens* from different locations is made to address the hypothesis that this species have a high tolerance to DNA damage from PAH contaminated habitats.

2. Materials and Methods

2.1. Collection and maintenance of animals

Animals were chosen from sites considered to be relatively 'clean' and free of any significant contamination (Environment Agency 2007) and collected outside of their natural breeding season (except for the experiments using spermatozoa as described below). Adult *Arenicola marina* were collected from the beach at Mothercombe estuary, South Devon (50º18'41" N, 3º56'45" W), during September-December 2006. Individual animals were collected according to the methods of Lewis et al. [19] and returned to the laboratory were they were checked for maturity (gravid animals were not used except in the experiment using spermatozoa), then stored at 15ºC in individual containers in filtered (0.2µm) seawater (FSW). Animals were maintained in 10 litre glass aquarium tanks in well aerated FSW for two days post digging to allow their gut contents to be voided.

Adult *Nereis diversicolor* were dug from the muddy estuary at Exmouth, South Devon (50˚36'51" N, 3˚26'43"W) during September 2006. Animals were collected and returned to the laboratory, where they were maintained in aquaria in well aerated FSW at 15ºC until use. *Nereis virens* specimens were purchased from an aquaculture supplier (Seabait Ltd.), for the initial comet assay work, since local populations tend to occur in contaminated areas. For the population comparison

experiment, animals were also collected from the muddy shore at Torpoint, in the Tamar estuary, Cornwall (50˚22'14"N, 4˚11'44"W), and Poole harbour, Dorset (50˚42'14"N, 1˚58'43"W). Large immature adults were collected and returned to the laboratory, where they were maintained in aquaria in well aerated FSW at 15ºC until use. Torpoint and the lower regions of the Tamar estuary are well documented as being heavily contaminated with PAHs, metals and tributyltin (TBT) [20, 21], whilst Poole harbour appears to have much lower PAH levels [22].

2.2. Comet assay procedure

Coelomic fluid samples were collected using a 1ml syringe fitted with a 21g hypodermic needle (chilled prior to use), carefully inserted into the posterior region of the body avoiding the gut, and stored on ice until use. All samples were checked for cell viability prior to the comet assay procedure using Eosin Y staining. For each assay 50µL of coelomic fluid was used from each individual. Coelomic fluid was gently centrifuged at 78 x g (1000 rpm) for 4 minutes and the excess fluid removed. Cell concentrate was then gently mixed with 1% low melting point agarose (heated to 37°C) and dropped onto slides previously coated with 1% normal melting point agarose. The slides were protected with coverslips while they set for 10 minutes at 4°C, then the coverslips were carefully removed. The COMET assay was then performed according to the methods of Singh et al. [23] with modifications, using alkaline conditions at 5ºC. Briefly: 1hour lysis followed by 45 minutes denaturation in electrophoresis buffer (0.3M NaOH and 1mM EDTA, at pH 13) and then electrophoresis for 30 minutes at 25V and 300mA followed by neutralisation. Cells were stained with 20mgL^{-1} ethidium bromide and examined using a fluorescent microscope using a 420-490nm excitation filter and a 520nm emission filter. One hundred cells per preparation were quantified using Kinetic COMET Software.

2.3. Effects of using anticoagulant

Arenicola marina coelomocytes aggregate spontaneously to form large clumps as soon as they are removed from the body [17] and anticoagulants are often required for cellular based investigations. However, the use of anticoagulant in marine invertebrates has only previously been reported in Crustacea (where the pH of haemolymph is generally around 4.6,[24]) with no reports for polychaetes. Assay optimisation therefore included an investigation into the impact of anticoagulant on polychaete cells. Anticoagulant solution (0.45M sodium chloride; 0.1M glucose; 30mM sodium citrate; 10mM citric acid; 10mM EDTA) was made up according to Soderhall and Smith [24] at pH 4.6. Half of this solution was then adjusted to pH 7.3 (the pH of *Arenicola marina* coelomic fluid, [25]) via the addition of NaOH. To confirm the anticoagulant properties of these solutions, coelomic samples were collected from *Arenicola marina* using a 1ml syringe fitted with a 21g hypodermic needle containing 0.5ml of treatment solution: (i) PBS; (ii) anticoagulant at pH 4.6 or (iii) anticoagulant at pH 7. The samples were examined under a light microscope after 10 minutes to determine the degree of cellular aggregation for each solution. Three samples of coelomic fluid were then collected from each of 6 *Arenicola marina* specimens into 0.5ml of each (chilled) treatment solution and the comet assay performed on each sample as described above.

2.4. Cell type sensitivities

Nereids generally have two distinct types of coelomocytes present during gametogenesis; amoebocytes and eleocytes [18, 26]. To determine their individual sensitivities to genotoxins these cells were separated using a combination of filtering

and a density gradient technique. Coelomic samples were collected from 5 *Nereis diversicolor* and 6 *Nereis virens* specimens into a small volume of PBS buffer using a 21 gauge hypodermic needle and syringe, and stored on ice. Approximately 1ml of coelomic fluid in PBS was collected for each specimen. To separate out the amoebocytes from the larger eleocytes, the coelomic fluid was first passed over a 30µm mesh. This retains most of the larger, 'sticky' eleocytes, which were then backwashed and retrieved into a small Petri dish before being split into 2 microcentrifuge tubes for the *in vitro* exposures. 500µl of the remaining coelomic fluid (that had passed through the mesh) was then pipetted onto a 40:60 v:v Lympho Separation Medium (ICN Biomedicals Ltd, Ohio) in PBS and centrifuged at 78 x g for 15 minutes to further separate any remaining eleocytes from the amoebocytes. 200 µl of separated amoebocytes were then pipetted off the top of the density gradient and split into 2 microcentrifuge tubes for the *in vitro* exposures. This cell separation technique was also attempted for coelomocytes in *Arenicola marina* but the different cell types present were found to be of similar density and therefore could not be separated using this technique.

In *Arenicola marina*, males can contain developing spermatids or spermatozoa in their coelomic cavities for a significant proportion of the year [27]. Experiments were conducted to determine whether sperm cells can be used in genotoxic assays and whether they show different sensitivity to genotoxic damage compared to coelomocytes. Mature *Arenicola marina* specimens were collected from Mothercombe (as described above) during the breeding season in December 2006. Mature males were induced to spawn through the injection of 8,11,14-eicosatrienoic acid (13µg g^{-1} of body mass) directly into the coelomic cavity of the male *Arenicola marina* specimens [28]. Spawning usually followed approximately 1 hour after

injection. Sperm was collected 'dry' as it was extruded from the nephromixia, to prevent it from becoming activated prior to use, and stored in micro-centrifuge tubes on ice until use. Sperm were then diluted to a density of 10^5 ml^{-1} for the *in vitro* exposure using FSW.

To determine the genotoxic responses of the different cell types, cells were exposed to the reference genotoxin methyl methanesulfonate (MMS) [CAS number 66-27-3] at a concentration of 52mgl^{-1} *in vitro* for 1 hour whilst kept on ice in microcentrifuge tubes (N.B. concentration chosen to approximate the upper values used by Cheung et al. [29]). Cells were then washed in chilled PBS three times using centrifugation at 78 x g (higher centrifugation at 7, 826 x g was used for the spermatozoa), and then used for the comet assay as described above. Sperm were left in the lysis solution for 2 hours as oppose to the 1 hour used for coelomocytes.

2.5. Species Comparisons

Natural levels of DNA damage and cellular sensitivities to the direct acting genotoxin MMS were compared in three polychaete species; the king ragworm *Nereis virens,* the harbour ragworm *N. diversicolor* and the lugworm *Arenicola marina*. The cultured *Nereis virens* were used for this experiment (from Seabait Ltd). Un-separated coelomocytes, collected straight from the coelomic cavity, were collected within 24hours of the specimens being collected from their field populations. Five individuals for each species were used. Coelomocytes were then split into two microcentrifuge tubes per individual and the coelomocytes were then incubated *in vitro* in (A) FSW or (B) 52mgL^{-1} MMS for 1 hour. DNA damage was then assessed using the comet assay.

2.6. Population Comparison

To investigate variability in background DNA damage between different natural populations, levels of DNA damage in *Nereis virens* specimens from the aquaculture supplier Seabait were compared to those in *N. virens* from two natural populations, one at Torpoint, Cornwall and one from Poole Harbour, Dorset. Torpoint is known to be heavily contaminated with PAH's and heavy metals (Environment Agency, 2007; for a review see Langston et al., [20]). Poole Harbour has been reported to have lower levels of PAHs [22]. Animals were collected as described above and stored at 12°C for 24 hours in seawater from the sampling site prior to use (to prevent any recovery between sampling and the assay). Coelomic samples were collected from ten specimens from each 'site' and the comet assay was performed as described above.

2.7. In vivo Dose and Time responses in Arenicola marina

Adult *Arenicola marina* specimens (collected outside of the breeding season so that their coelomic cavities were not full of large gametes) were exposed to two reference genotoxins: the direct acting genotoxin methyl methanesulfonate (MMS); and the metabolically activated genotoxin benzo[α]pyrene (B[a]P) [CAS number 50-32-8], for a period of three days to determine time and dose responses in this species. MMS readily dissolves in seawater; for B[a]P it was necessary to use dimethyl sulphoxide (DMSO) as a solvent carrier (final concentration of 0.01% DMSO in exposure beakers). Concentrations of 18, 32 and 52mgL^{-1} MMS and 0.1, 1.0, and 10mgL^{-1} B[a]P were used for the exposures, together with FSW and solvent controls (N.B. concentrations chosen to approximate the range used by Cheung et al. [29]). These concentrations were chosen to induce damage with a short exposure time for this methodological study. Exposures were conducted in replicate (5) in 2-litre glass beakers, with one specimen per beaker and 5 beakers per treatment. Aeration was

provided and animals were maintained at 12ºC and 12:12 light: dark photoperiod during the 14 day exposure period. Coelomic samples were taken at time intervals of 1 hour, 24 hours and three days. Coelomic fluid was carefully withdrawn from the posterior part of each *A. marina* specimen using a 1ml syringe fitted with a 21g needle containing 0.2ml anticoagulant (at pH7.3, see Section 2.3), and stored on ice prior to use. The comet assay was then conducted as described above.

2.8. Statistical Analysis

Statistical analysis was performed using the Statgraphics Ltd. and Minitab Ltd. software programmes. The occurrence of a dose-response relationship was tested for using linear and non-linear regression.

3. Results

Following all exposures, no loss of cell viability was observed in any of the treatments (Eosin Y assay cell viability >90%). Sperm viability was assessed by visually observing motility (although this was not quantified) and was not conspicuously affected by the exposures.

3.1. Use of anticoagulant in Arenicola marina

Initial attempts at using the comet assay in *Arenicola marina* were unsuccessful due to the immediate clumping of cells removed from the animal, even when removed into chilled PBS or physiological saline, making the slides almost impossible to score (pers. obs.). The use of anticoagulant during the collection of *A. marina* coelomocytes prevented the cells from clumping and therefore enabled accurate

scoring of the comet slides. Using crustacean anticoagulant at pH 4.6, however, caused a significant increase in DNA damage (measured as % Tail DNA, Figure 1) compared to using chilled PBS only (one-way ANOVA [Levene's test for homogeneity of variance: P = 0.168], $F_{2,15} = 9.02$, P = 0.0027). Adjusting the pH of the anticoagulant to pH 7.3, the pH of *A. marina* coelomic fluid, reduced this effect so that the average %Tail DNA was not significantly different for the samples collected into PBS alone and the variation in damage measured was lower (standard deviation [SD] in % DNA damage for anticoagulant at pH 7.3 = 1.96; for PBS = 6.38; for anticoagulant at pH 4.6 = 8.79).

3.2. Cell type sensitivities

The two different coelomocyte cell types examined in *Nereis diversicolor* and *N. virens* showed significantly different responses to the direct acting genotoxin methyl methanesulfonate (MMS) (Figure 2a & 2b). A 2-way ANOVA showed a significant effect of MMS exposure (*N. diversicolor*: $F_{1,26} = 61.77$, P <0.001; *N. virens*: $F_{1,31} = 575.52$, P < 0.001) and also an effect of cell type (*N. diversicolor*: $F_{2,26} = 5.58$, P < 0.01; *N. virens*: $F_{2,31} = 16.99$, P < 0.01) on the amount of DNA damage measured after the 1hour *in vitro* exposure to MMS. Eleocytes showed a significantly higher level of DNA damage in response to MMS than amoebocytes in both Nereid species, suggesting they are more sensitive to genotoxic damage.

DNA damage in *Arenicola marina* spermatozoa showed a significant linear dose response to MMS exposure (regression analysis ANOVA P < 0.001, $R^2 = 61.9$, correlation coefficient = 0.793, Figure 3). This response was significantly lower than that measured in coelomocytes from the same males (2-way ANOVA; for cell type

effect: $F_{1,39} = 17.75$, $P < 0.001$; for dose effect: $F_{3,39} = 43.74$, $P < 0.001$) after the 24h exposure period.

3.3. Species Comparisons

Comparing the responses of un-separated coelomocytes from *Arenicola marina*, *Nereis diversicolor* and *N. virens* to MMS exposure (Figure 4), shows a significant effect of both species and MMS exposure on the percentage of DNA damage measured (2-way ANOVA; for species effects: $F_{2,29} = 10.83$, $P < 0.001$; for MMS effects: $F_{1,29} = 35.02$, $P < 0.001$). *A. marina* had significantly higher natural levels of DNA damage than *N. diversicolor* and *N. virens* (one-way ANOVA, $F_{2,12} = 22.19$, $P < 0.001$), but also showed a greater relative response to MMS, (a 76.03% increase in damage compared to 67.15% for *N. diversicolor* and 34.28% for *N. virens*).

3.4. Population Comparison

A significant difference in percentage DNA damage was measured in *Nereis virens* specimens from the three sample populations studied, with animals from Torpoint showing significantly higher levels of DNA damage than animals from Poole Harbour or the cultured animals (Figure 5, Kruskall Wallis [due to unequal variances] $H = 19.75$, $DF = 2$, $P < 0.001$).

3.5. Dose and Time responses of Arenicola marina *to known genotoxins*

Figures 6a and 6b show the time and dose responses of *Arenicola marina*, measured as percentage DNA in the comet tail, to two reference genotoxins: methyl methanesulfonate (MMS) a direct acting mutagen; and benzo[α]pyrene (B[a]P) an indirect acting (and environmentally relevant) mutagen. N.B. no significant difference between FSW and solvent control were observed ($P = 0.973$) and so the solvent

control data was excluded from the analysis. Regression analysis reveals significant dose dependant logarithmic relationships for each time point for BaP ($P < 0.001$, $R^2 = 0.601$ for 1 h; $P < 0.01$, $R^2 = 0.414$ for 24 h and $P < 0.01$, $R^2 = 0.407$ for 3 day) and the 24h and 3 day exposures to MMS ($P < 0.001$, $R^2 = 0.81$ for 24 h and $P < 0.001$, $R^2 = 0.852$ for 3 day).. A significant linear relationship between dose and DNA damage was observed in cells exposed to MMS for 1 hour ($P < 0.01$, $R^2 = 0.411$). DNA damage increased with time in cells exposed to MMS (2-way ANOVA, for time effects $F_{2, 52} = 12.18$, $P<0.001$, for dose effect $F_{3,52} = 35.34$, $P < 0.001$). Cells exposed to B(a)P showed no clear increase in levels of DNA damage over time during the three day exposure period.

4. Discussion

This investigation has clearly demonstrated that different cell types within the same species and different species within the Polychaeta show significantly different responses to genotoxic insult. Since these cellular investigations used *in vitro* exposure to the direct acting mutagen MMS, to remove any confounding factors associated with route of uptake, bioaccumulation or biotransformation, these differences in cellular sensitivities must be due to differences in the cells capabilities for either defence or repair. Eleocytes were found to be more sensitive to genotoxic damage than amoebocytes in the two Nereid species investigated, and spermatozoa were less sensitive to genotoxic damage than coelomocytes in *Arenicola marina*. The DNA repair capacities of these different cell types had not previously been investigated. Whilst differences in genotoxic response between tissue types within a species have been reported for a number of aquatic species (e.g. in the green lipped

mussel *Perna viridis* [30]; in brown trout *Salmo trutta* [31] and in *Mytilus galloprovincialis* [32]), differing genotoxic responses within invertebrate haemocyte or coelomocyte cells of the same species have not been previously reported. This is also the first report of genotoxin-induced DNA damage in spermatozoa of a marine invertebrate. The only studies of between species differences in tolerance to environmental genotoxins in polychaetes have concentrated on differences in uptake, bioaccumulation and biotransformation of PAHs [33, 34]. Antioxidant defence and repair capabilities of free cells (i.e. coelomocytes or haemocytes) are not well understood for polychaetes or many other invertebrate species.

Significant relationships between DNA damage and the antioxidant status [35] and repair capacity of cells [36] have been shown to account for cellular and tissue specific differences in genotoxic responses within human cell lines [36, 37]. Any differences in defence or repair capabilities of polychaete coelomocytes are likely to be related to the different functional roles of these cells. Amoebocytes are mainly involved in the immune functioning of the polychaete [17] whilst eleocytes have a mainly nutritive role for the developing gametes [18]. Antioxidants are known to improve antibacterial function in human leukocytes and mouse macrophages [38-40] and so are likely to be present in greater quantities in a cell with an immune function. It follows, therefore, that amoebocytes, which are active in phagocytosis, would have a higher antioxidant status than eleocytes or spermatozoa, making them better protected against DNA damage. Eleocytes also differ from amoebocytes in that they are no longer actively dividing (Hoeger *unpublished data*), which might mean they lesser DNA repair capacities, also affecting their susceptibility to DNA damage.

Unlike the relatively loose structure of chromatin (DNA and nuclear proteins) in somatic cells, sperm chromatin is tightly compacted because of the unique associations between the DNA and sperm nuclear proteins. This nuclear compaction is considered important for protecting the sperm genome from external stresses such as oxidative damage or temperature elevation [41], supposedly making sperm less susceptible to DNA damage from environmental genotoxin exposure. Human and some mammalian biomonitoring studies using the comet assay have, however, revealed significant DNA damage in sperm of males exposed to phthalates [42] and polycyclic aromatic hydrocarbons such as benzo(a)pyrene [43]. This work has revealed *in vitro* exposures of spermatozoa from mature *Arenicola marina* results in significant damage to the sperm DNA, although the consequences of this damage require further investigation. Sperm did show lower levels of DNA damage than coelomocytes from the same males, suggesting that the tightly compacted nature of sperm chromatin may offer a certain level of protection. This difference may simply be due to differences in DNA repair capabilities, however, since DNA strand breaks will transiently be present during DNA repair [44] and sperm, unlike somatic cells, are generally considered to have few or no repair enzymes [45]. In spermatozoa, therefore, the alkaline comet assay will only measure strand breaks caused directly by the action of a chemical or alkaline induced breaks at alkali labile DNA adducts. Many studies have shown that the Comet assay is still sensitive enough to pick up significant chemical induced DNA damage in sperm [41-42, 46-47].

The fact that the Comet assay also measures transient DNA breaks present during repair, and is therefore not necessarily measuring permanent DNA damage [16, 44] has lead to many questioning its relevance for use in environmental monitoring in terms of fitness effects on the organisms being monitored [48]. We are still missing

much of the information needed to link short-term effects of this type of DNA damage to any long term consequences for at the population level. A lack of repair enzymes in spermatozoa would not only make them more susceptible to accumulated DNA damage from continued, low dose exposures to environmental genotoxins, but would also mean that the Comet assay performed on spermatozoa is measuring permanent DNA damage. Monitoring of sperm may therefore provide more relevant information for environmental monitoring purposes. Since sperm directly contribute to the genetic make up of the next generation, measuring DNA damage in spermatozoa may also be of greater consequence in terms of long term effects, although this would need further investigation.

Significant differences in genotoxic response were also observed between the three polychaete species used in this investigation. *Nereis virens* appears to be the most resistant to genotoxic damage, supporting the findings of De Boeck and Kirsch-Volders [8], whilst *Arenicola marina* was the most sensitive showing the highest relative increase in DNA damage. Since these experiments used a direct acting mutagen, MMS, and cellular exposures were conducted *in vitro* to remove any confounding factors related to uptake or biotransformation, these differences in sensitivity must relate to differences in cellular defence or repair capabilities between the species. The animals used for this comparison were all from sites reported to be relatively free of pollutants (Environment Agency 2007), however the differences in responses measured here could be due to the animals being collected from different sites. Since the three species do not generally occur on the same shores a true comparison is not possible.

The population comparison revealed *Nereis virens* from Torpoint were able to survive with very high levels of DNA damage, in a site known to be highly contaminated with PAHs and other contaminants (Environment Agency data. 2007 [20, 21]). It also proved difficult to find a local population of *N. virens* in the south west of the U.K. that was known to be relatively free from pollution (using Environment Agency data), with populations tending to occur in the more contaminated estuaries (pers. obs.), further suggesting that these species have a high tolerance to PAH pollution. This study did reveal that differences in the levels of DNA damage in *Nereis virens* collected from different areas can be detected, however, so this species may still be able to provide useful information in terms of genotoxic contamination of sediments if used as part of an integrated environmental monitoring programme. The ability of *N. virens* to survive in the highly contaminated habitat at Torpoint, with the incredibly high natural levels of DNA damage measured for this population (which appears to be reproducing normally, Lewis unpublished data) is surprising. The mechanisms underlying this tolerance remain unclear.

The results of the *in vivo* experiment with *Arenicola marina* confirm that B[a]P is accumulated, biotransformed and subsequently results in DNA adduct formation in this species. *A. marina* responded differently over time to the two genotoxins used in the *in vivo* exposures. Animals exposed to the direct acting mutagen MMS showed an increase in DNA damage with time and dose of exposure. Animals exposed to B(a)P, however, showed a significant dose response but no clear increase in DNA damage with time of exposure over the three day exposure period. This difference in response is most likely related to the different mechanisms of damage caused by the two compounds. The alkylating agent MMS is a direct acting genotoxin, which reacts almost exclusively with the ring nitrogens of the purine bases,

particularly N-7 of guanine. The majority of these lesions are processed via the base excision repair system [49]. The highest levels of DNA damage were recorded after 1 hour B(a)P exposure in two out of the three exposure concentrations (at 1.0 and 10mgL^{-1}). These results agree with the findings of Siu et al. [30] who found the highest levels of DNA damage in the green lipped mussel, *Perna viridis*, after one day of B(a)P exposure, and decreasing levels of damage over the following 12 day exposure period. The observed time-dependent variations in the levels of DNA strand breaks can be explained by the DNA repair theory suggested by Ching et al. [50] who suggest that a DNA repair system may be activated after the exposed cell/tissue has accumulated sufficient toxicant above a threshold level. Below this level, the DNA repair activity may be facilitated by only a basal level of DNA repair enzymes.

5. Conclusions

This investigation has clearly demonstrated that different coelomic cell types within the same species and different species within the Polychaeta show significantly different responses to genotoxic insult. These differences in responses have implications both for species survival in polluted environments and for the interpretation of comet assay data collected as part of environmental monitoring studies. Of particular interest is the DNA damage observed in spermatozoa, which showed a lower, but linear response to genotoxic insult. A thorough understanding of the consequences of this sperm DNA damage in terms of transfer to the next generation will aid in understanding of the long term consequences of damage measured using the comet assay.

Acknowledgements

This research was funded via the EU 6th Framework directive, as part of the FACE-iT Project. The authors would like to thank Chris Pook and Carmen Morales for their help in the collection of animals, Jo Hagger for help with the comet assay and Trevor Worsey for technical support.

References

[1] M.H. Depledge. The ecotoxicological significance of genotoxicity in marine invertebrates, Mutat. Res. Fund. Mole. Mechan. Mutagen. 399 (1998) 109–122.

[2] D.R. Livingstone, J.K. Chipman, D.M. Lowe, C. Minier, C.L. Mitchelmore, M.N. Moore, L.D. Peters, R.K. Pipe. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates, Int. J. Environ. Pollut. 13 (2000) 56–91.

[3] M. Cornet. Detection of genotoxicity in the marine environment: A preliminary feasibility study using primary mussel tissue culture, Sci. Total Environ., 382 (2007) 22-29.

[4] D.R. Dixon, A.M. Pruski, L.R. Dixon, A.N. Jha. Marine invertebrate eco-genotoxicology: a methodological overview, Mutagenesis, 17 (2002) 495-507.

[5] Z. Jaksic, R. Batel, N. Bihari, M. Micic, R.K. Zahn. Adriatic coast as a microcosm for global genotoxic marine contamination—A long-term field study, Mar. Poll. Bull. 50 (2005) 1314-1327.

[6] A.T. Large, J.P. Shaw, L.D. Peters, A.D. McIntosh, L. Webster, A. Mally, J.K. Chipman. Different levels of mussel (*Mytilus edulis*) DNA strand breaks following chronic field and acute laboratory exposure to polycyclic aromatic hydrocarbons, Mar. Environ. Res. 54 (2002) 493–497.

[7] J. Rank, K. Jensen, P.H. Jespersen, Monitoring DNA damage in indigenous blue mussels (*Mytilus edulis*) sampled from coastal sites in Denmark, Mutat. Res. Genet. Toxicol. Environ. Mut. 585 (2005) 33-42.

[8] M. De Boeck, M. Kirsch-Volders. *Nereis virens* (Annelida, Polychaeta) is not an adequate sentinel species to assess the genotoxic risk comet assay) of PAH exposure to the environment, Environ. Mol. Mutagen. 30 (1998) 82-90.

[9] J.A. Hagger, A.S. Fisher, S.J. Hill, M.H. Depledge, A.N. Jha. Genotoxic, cytotoxic and ontogenetic effects of tri-n-butyltin on the marine worm, *Platynereis dumerilii* (Polychaeta: Nereidae), Aquat. Toxicol. 57 (2002) 243-255.

[10] A.N. Jha, T.H. Hutchinson, J.M. Mackay, B.M. Elliott, D.R. Dixon. Evaluation of genotoxicity of municipal sewage effluent using the marine worm *Platynereis dumerilii* (Polychaete: Nereidae), Mutat. Res. Genet. Toxicol. Environ. Mut. 391 (1997) 179–188.

[11] J.T. Wilson, D.R. Dixon, L.T. Dixon. Numerical chromosomal aberrations in the early life-history stages of a marine tubeworm, *Pomatoceros lamarckii* (Polychaeta: Serpulidae), Aquat. Toxicol. 49 (2002) 163-175.

[12] L. Verschaeve, J. Gilles, J. Schoeters, R. Van Cleuvenbergen, R. De Fré. The single gel electrophoresis technique or comet test for monitoring dioxin pollution and effects, in: H. Fiedler, H. Frank, O. Hutzinger, W. Parzefall, A. Riss, (Eds.) Organohalogen Compounds 11. Austria: Federal Environment Agency (1993) 213-216.

[13] L. Verschaeve, J. Gilles. Single gel electrophoresis assay in the earthworm for detection of genotoxic compounds in soils, Bull. Environ. Contam. Toxicol. 54 (1995) 112-119.

[14] J. Salagovic, J. Gilles, L. Verschaeve, I Kalina. The comet assay for the detection of genotoxic damage in the earthworms: a promising tool for assessing the biological hazards of polluted sites, Folia Biol. 42 (1996) 17-21.

[15] S. Cotelle, J.F. Férard. Comet assay in genetic ecotoxicology: A review, Environ. Molecul. Mutagen. 34 (1999) 246-255.

[16] S. Brendler-Schwaab, A. Hartmann, S. Pfuhler, G. Speit. The *in vivo* comet assay: use and status in genotoxicity testing, Mutagenesis 20 (2005) 245-254.

[17] R.P Dales, D.R. Dixon. Polychaetes, in: N.A. Ratcliff, A.F. Rowley (Eds.), Invertebrate blood cells, Academic Press, New York, 1 (1981) 35-74.

[18] U. Hoeger. Hydrolytic enzymes in the coelomic cells of the polychaete *Nereis virens* during sexual-maturation, Mar. Biol. 110 (1991) 7-12.

[19] C. Lewis, P.J.W. Olive, M.G. Bentley, G.J. Watson. Does seasonal reproduction occur at the optimal temperature for fertilization in the polychaetes *Arenicola marina* L. and *Nereis virens* Sars? Invert. Reprod. Develop. 41 (2002) 61-71.

[20] W.J. Langston, B.S. Chesman, G.R. Burt, S.J. Hawkins, J.W. Readman, P Worsfold. Site characterisation of the South West European Marine Sites:

Plymouth Sound and Estuaries cSAC, SPA, Mar. Biol. Assoc. Occ. Pub. 9. (2003) MBA, UK.

[21] J.W. Readman, R.F.C Mantoura, M.M. Rhead. A record of polycyclic aromatic hydrocarbon (PAH) pollution obtained from accreting sediments of the Tamar estuary, U.K. - evidence for non-equilibrium behaviour of PAH, Sci. Total Environ. 66 (1987) 73-94.

[22] M.F. Kirby, M.A. Blackburn, J.E. Thain, M.J. Waldock. Assessment of water quality in estuarine and coastal waters of England and Wales using a contaminant concentration technique, Mar. Poll. Bull. (1998) 631-642.

[23] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider. A simple technique for quantitation of low-levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184-191.

[24] K. Soderhall, V.J. Smith. Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution, Develop. Comp. Immunol. 7 (1983).229-239.

[25] A.A. Pacey, J.C. Cosson, M.G. Bentley. The acquisition of forward motility in the spermatozoa of the polychaete *Arenicola marina,* J. Exp. Biol., 159 (1994) 259-280.

[26] U. Hoeger, I. Kunz. Metabolic enzymes in coelomic cells (eleocytes) of the polychaete *Nereis-virens* - sex-specific changes during sexual-maturation, Mar. Biol. 115 (1993) 653-660.

[27] D.I.D. Howie. The reproductive biology of the lugworm, *Arenicola marina* L., Fortschritte Zool. 29 (1984) 247-263.

[28] M.G. Bentley, A.A. Pacey. Physiological and environmental control of reproduction in polychaetes, Oceanogr. Mar. Biol. Ann. Rev. 30 (1992) 443-481.

[29] V.V. Cheung, A. Jha, R. Owen, M.H. Depledge, T.S. Galloway. Development of the *in vivo* chromosome aberration assay in oyster (*Crassostrea gigas*) embryo-larvae for genotoxicity assessment. Mar. Environ. Res.62 (2006) S278-S282.

[30] W.H.L. Siu, E. Mak, J. Cao, S.B. De Luca-Abbott, B.J. Richardson, P.K.S. Lam. Micronucleus induction in gill cells of green-lipped mussels (*Perna viridis*) exposed to mixtures of polycyclic aromatic hydrocarbons and chlorinated pesticides, Environ. Toxicol. Chem 23 (2004) 1317-1325.

[31] C.L. Mitchelmore, J.K Chipman. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring, Mutat. Res. Fundam. Molec. Mechan. Mutagen. 399 (1998) 135-147.

[32] P. Venier, S. Maron, S. Canova. Detection of micronuclei in gill cells and haemocytes of mussels exposed to benzo[a]pyrene, Mutat. Res. Genet. Toxicol. Environ. Mutat. 390 (1997) 33-44.

[33] L. Bach, A. Palmqvist, L.J. Rasmussen, V.E. Forbes. Differences in PAH tolerance between *Capitella* species: Underlying biochemical mechanisms, Aquat. Toxicol. 74 (2005) 307-319.

[34] I. Linke-Gamenick, B. Vismann, V.E. Forbes. Effects of fluoranthene and ambient oxygen levels on survival and metabolism in three sibling species of Capitella (Polychaeta), Mar. Ecol. Prog. Ser. 194 (2000) 169-177.

[35] S.G. Jarrett, J. Albon, M. Boulton. The contribution of DNA repair and antioxidants in determining cell type-specific resistance to oxidative stress, Free Rad. Res. 40 (2006) 1155-1165.

[36] B.S. Anatassov, P.D. Ninova, B.B. Anachkova, G.C. Russev. Relationship between DNA repair capacity and resistance to genotoxins in four human cell lines, Cancer Detec. Prevent. 27 (2003) 24-29.

[37] P.J. Coates, S.A. Lorimore, E.G. Wright. Cell and tissue responses to genotoxic stress, J. Pathol. 205 (2005) 221-235.

[38] C.P. Alvarado, P. Alvarez, L. Jimenez, M. De la Fuente. Improvement of leukocyte functions in young prematurely aging mice after a 5-week ingestion of a diet supplemented with biscuits enriched in antioxidants, Antiox. Redox. Signal. 7 (2005) 1203-1210.

[39] Y. Arita, J.A. Kazzaz, A. Joseph, H. Koo, Y. Li, J.M. Davis. Antioxidants improve antibacterial function in hyperoxia-exposed macrophages Free Rad. Biol. Med. 42 (2007) 1517-1523.

[40] D.M.P. Morrow, T. Entezari-Zaher, J. Romashko, A.O. Azghani, M. Javdan, L. Ulloa, E.J. Miller, L.L. Mantell. Antioxidants preserve macrophage phagocytosis of *Pseudomonas aeruginosa* during hyperoxia, Free Rad. Biol. Med. 42 (2007) 1338-1349.

[41] A. Zini, J. Libman. Sperm DNA damage: clinical significance in the era of assisted reproduction, Canad. Med. Assoc. J. 175 (2006) 495-500.

[42] S.M. Duty, N.P. Singh, M.J. Silva, D.B. Barr, J.W. Brock, L. Ryan, R.F. Herrick, R. Christiani, D.C Hauser. The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay**,** Environ. Health Perspect. 111 (2003) 1164-1169.

[43] L. Gaspari, S.S. Chang, R.M. Santella, S. Garte, P. Pedotti, E. Taioli. Polycyclic aromatic hydrocarbon-DNA adducts in human sperm as a marker of DNA damage and infertility, Mutat. Res. Genet. Toxicol. Environ. Mutat. 535 (2003) 155–160.

[44] A.R. Collins, V.L. Dobson, M. Dušinská, G. Kennedy, R. Štětine. The comet assay: what can it really tell us? Mutat. Res. Fund. Molec. Mechan. Mutagen. 375 (1997) 183-193.

[45] J. Kopeika, E. Kopeika, T. Zhang, D.M. Rawson, W.V. Holt. Effect of DNA repair inhibitor (3-aminobenzamide) on genetic stability of loach (*Misgurnus fossilis*) embryos derived from cryopreserved sperm, Theriogenology 61 (2004) 1661-1673.

[46] G.B. Boe-Hanson, I.D. Morris, A.K. Ersbøll, T. Greve, P. Christensen. DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay, Theriogenology 63 (2005) 1789-1802.

[47] I.D. Morris, S. Llott, L. Dixon, D.R. Brison. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development, Hum. Reprod. 17 (2002) 990- 998.

[48] J.E. Hose. Large-scale genotoxicity assessments in the marine environment. Environ. Health Perspect. 102 (1994) Suppl. 12. 29-32.

[49] R. Mirzayans, M. Liuzzi, M.C. Patersen. Methyl methanesulfonate-induced DNA damage and its repair in cultured human-fibroblasts - normal rates of induction and removal of alkali-labile sites in xeroderma pigmentosum (group-a) cells, Carcinogenesis 9 (1988) 2257-2263.

[50] E.W.K. Ching, W.H.L. Siu, P.K.S. Lam, L. Xu, Y. Zhang, B.J. Richardson, R.S.S. Wu. DNA adduct formation and DNA strand breaks in green-lipped mussels (*Perna viridis*) exposed to benzo[a]pyrene: dose and time-dependent relationships, Mar. Poll. Bull. 42 (2001) 603-610.

Figure Legends:

Figure 1: The effects of using anticoagulant to prevent cell clumping in *Arenicola marina* on baseline DNA damage measurements made using the comet assay (n = 6). [One-way ANOVA (Levene's test for homogeneity of variance: P = 0.168) df = 2, F = 9.02, P = 0.0027.] N.B. * = a significant difference in DNA damage compared to PBS control.

Figure 2: Comparing the responses of different types of coelomocyte to the direct acting genotoxin methyl methanesulfonate (MMS) in (A) *Nereis diversicolor* and (B) *Nereis virens*. * = a significant difference in DNA damage in eleocytes compared to other cell types in that treatment.

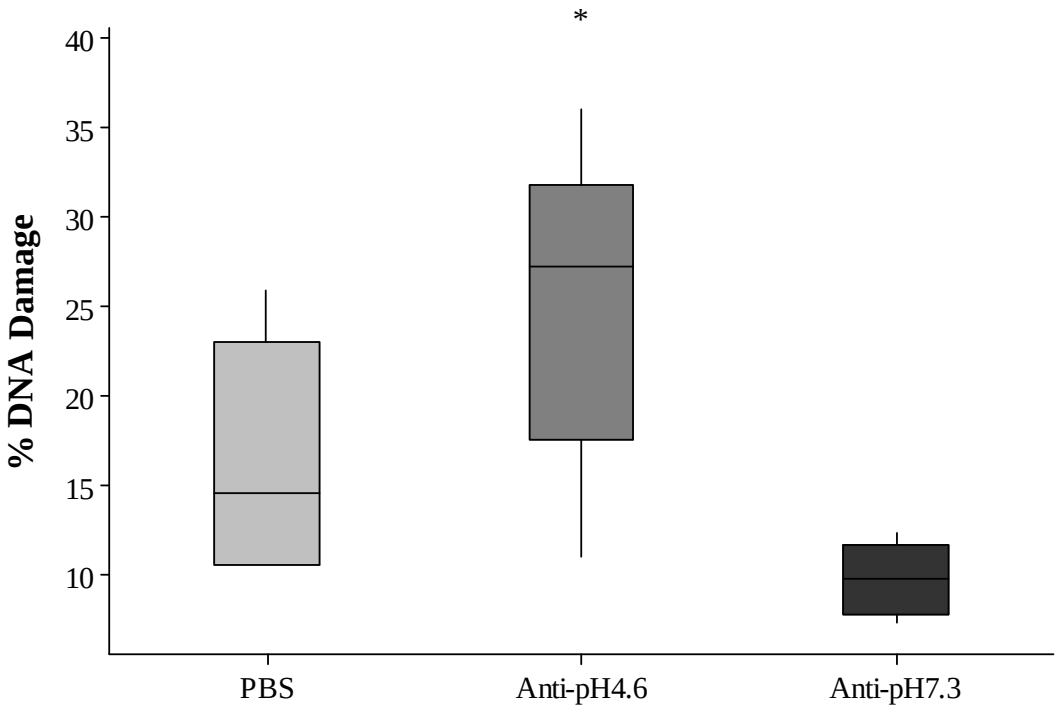
Figure 3: Genotoxic responses in coelomocytes and spermatozoa in *Arenicola marina* after 24 hour *in vitro* exposures to the direct acting genotoxin methyl methanesulfonate (MMS). * = a significant difference in DNA damage compared to the FSW control.

Figure 4: DNA damage in 'natural' populations of three species of polychaete: *Nereis virens*; *Nereis diversicolor* and *Arenicola marina*, and their different responses (shown as percentage increase in DNA damage) to *in vitro* exposure to the direct acting genotoxin, methyl methanesulfonate (MMS). N.B Farmed *Nereis virens* were used to mimic a natural population from a clean site, to remove the confounding affects of contamination at Torpoint and Poole. * = a significant difference in DNA damage compared to the FSW control for that species.

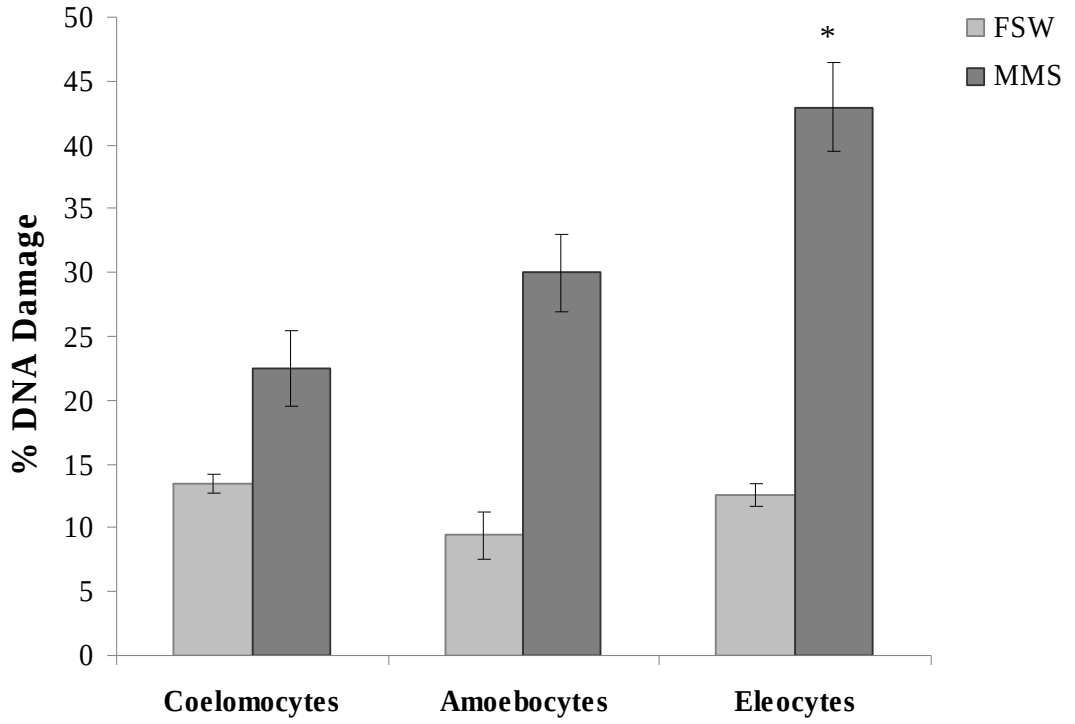
Figure 5: (a) Locations of the sampling sites for *Nereis virens*; (b) Comparison of natural levels of DNA damage in *Nereis virens* from Torpoint, Cornwall and Poole, Dorset (UK) with farmed worms (from Seabait Ltd UK based in Northumberland). N = 10, Kruskall Wallis: $H = 19.75$, $P < 0.001$. N.B. * = a significant difference in DNA damage compared to farmed animals.

Figure 6: DNA damage in *Arenicola marina* measured using the comet assay after *in vivo* exposure to the two genotoxins: (a) BaP and (b) MMS for 1 hour, 24 hours and 3 days.

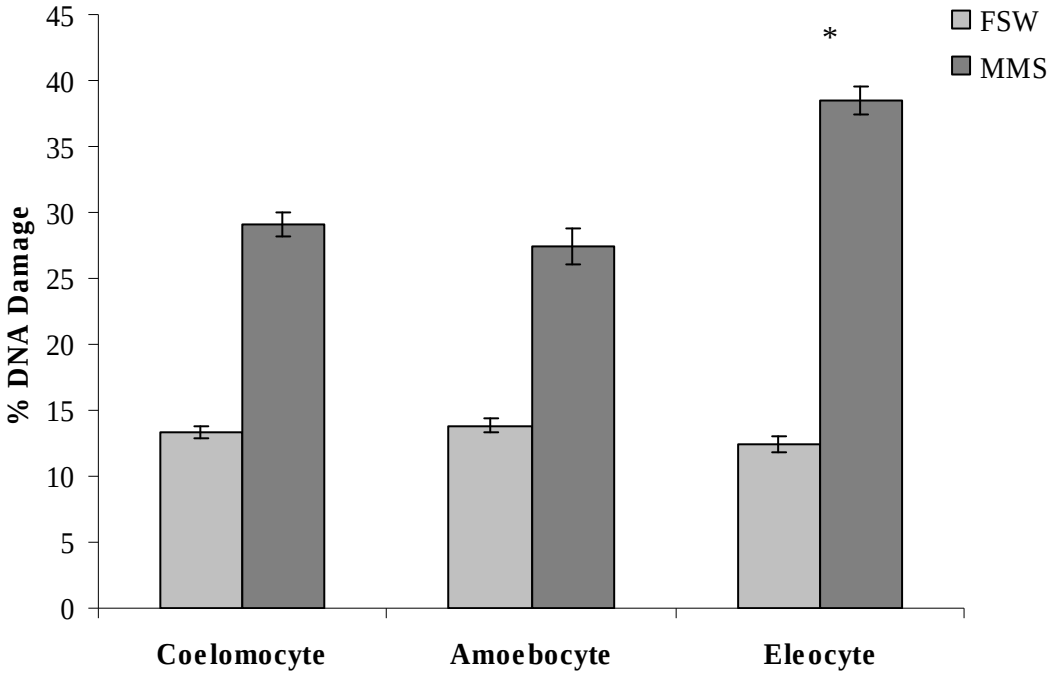
Figures:

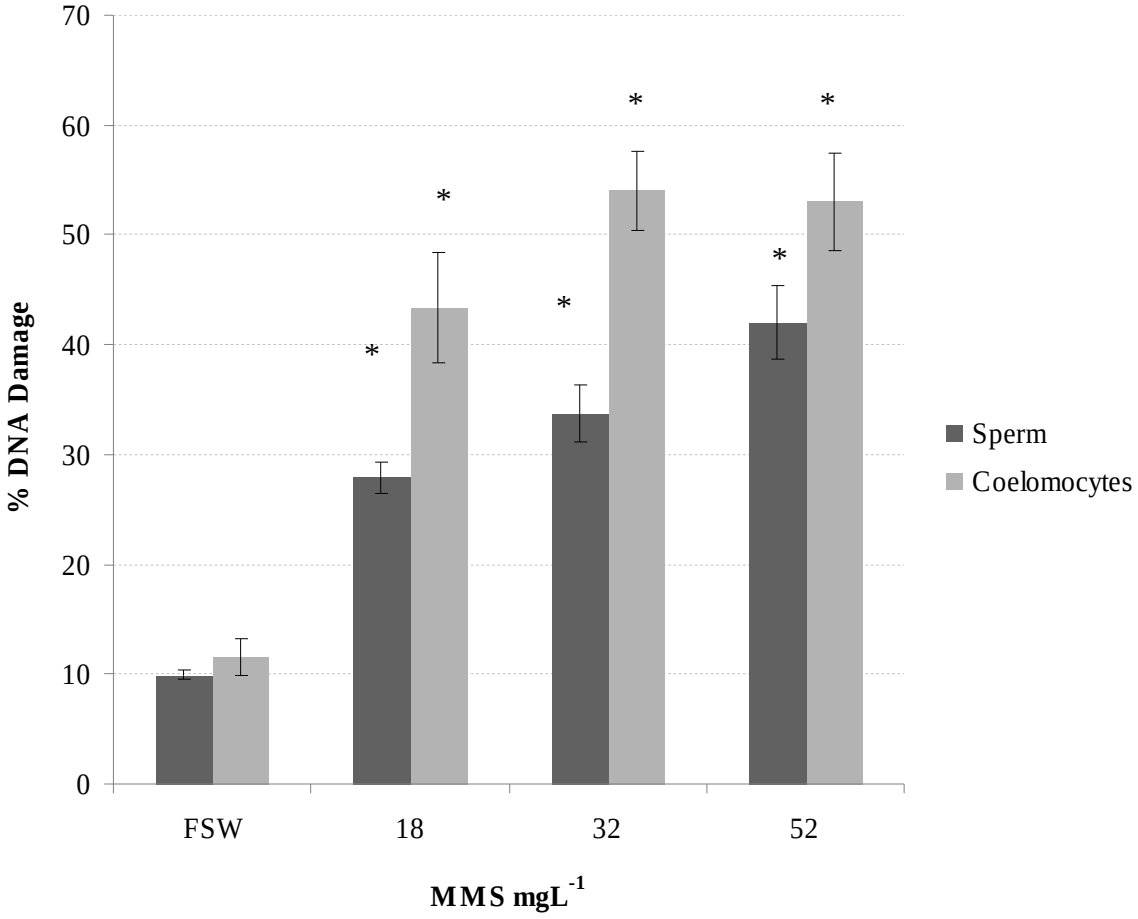


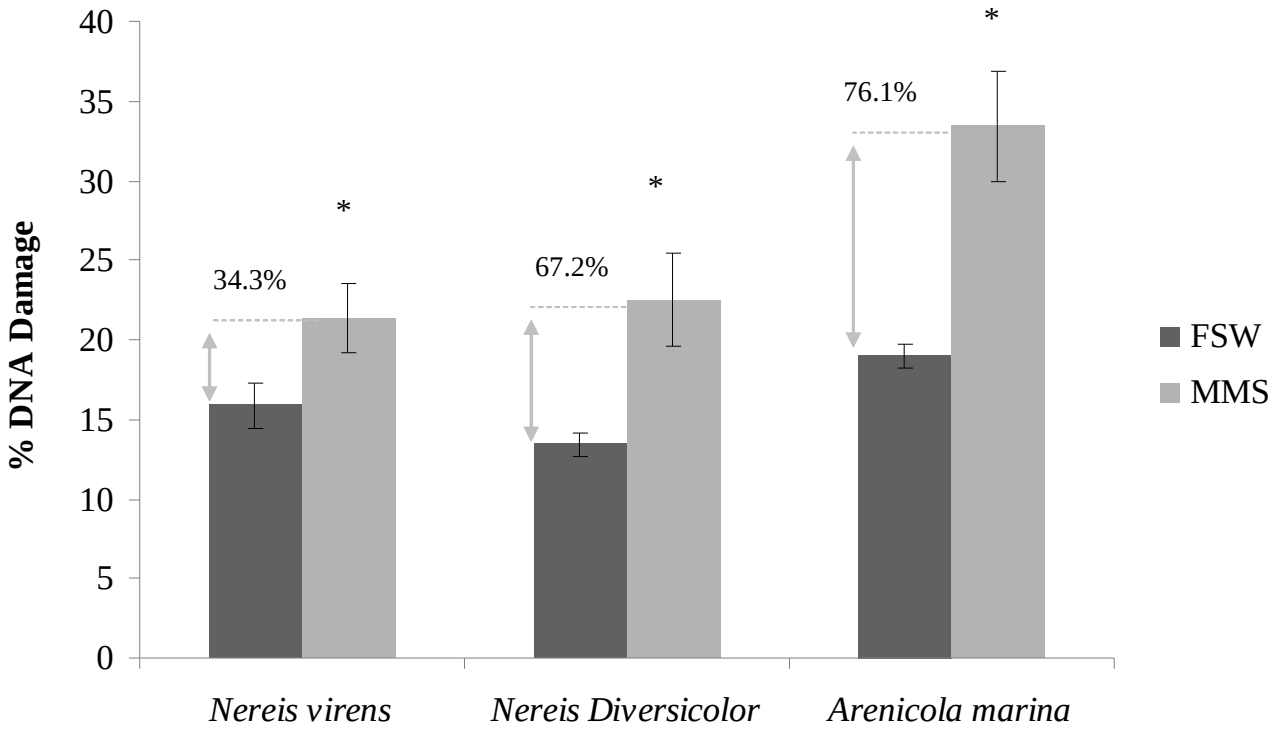
(A)


50
45
40
35
30
25
20
15
10
5
0
% DNA Damage
*
FSW
MMS
Coelomocytes
Amoebocytes
Eleocytes

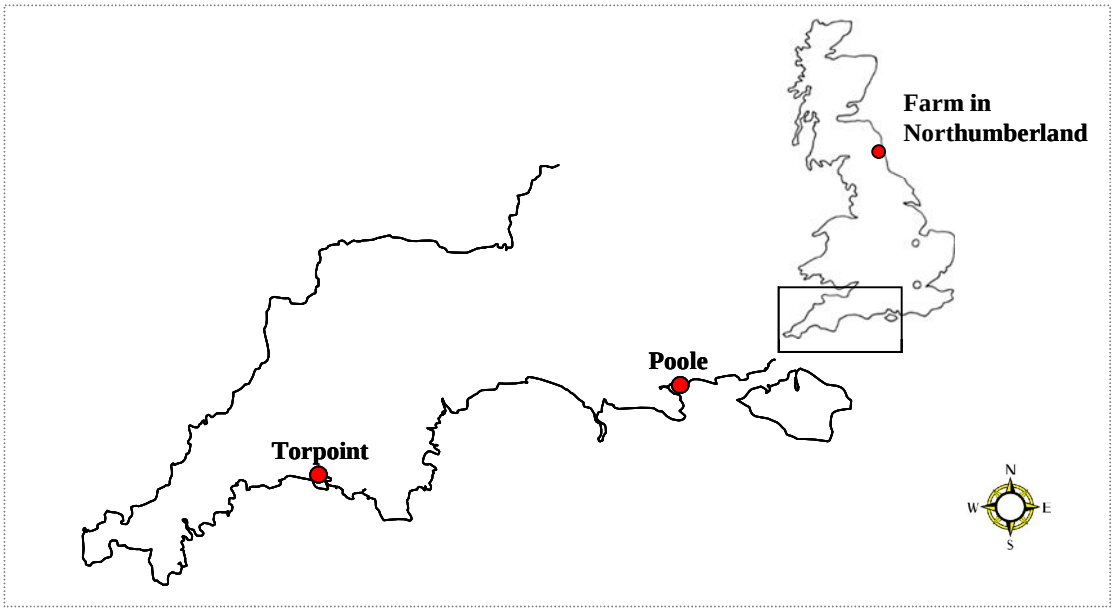
(B)


45
40
35
30
25
20
15
10
5
0
% DNA Damage
*
FSW
MMS
Coelomocyte
Amoebocyte
Eleocyte

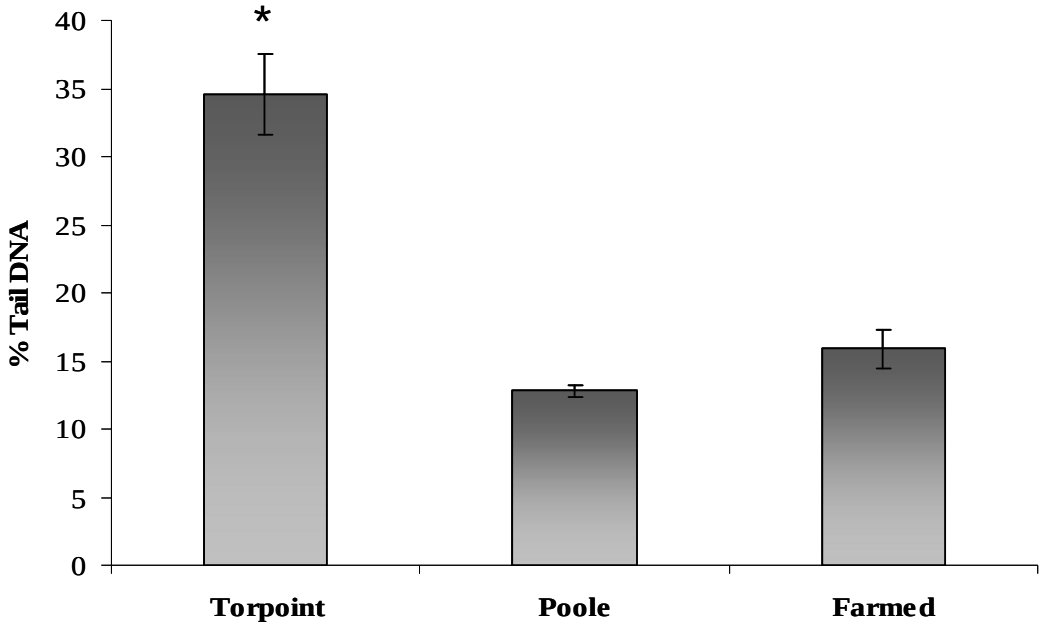

70
60
50
40
30
20
10
0
% DNA Damage
*
*
*
*
*
*
FSW
18
32
52
MMS mgL-1
Sperm
Coelomocytes


40
35
30
25
20
15
10
5
0
% DNA Damage
34.3%
67.2%
76.1%
*
*
*
FSW
MMS
Nereis virens
Nereis Diversicolor
Arenicola marina

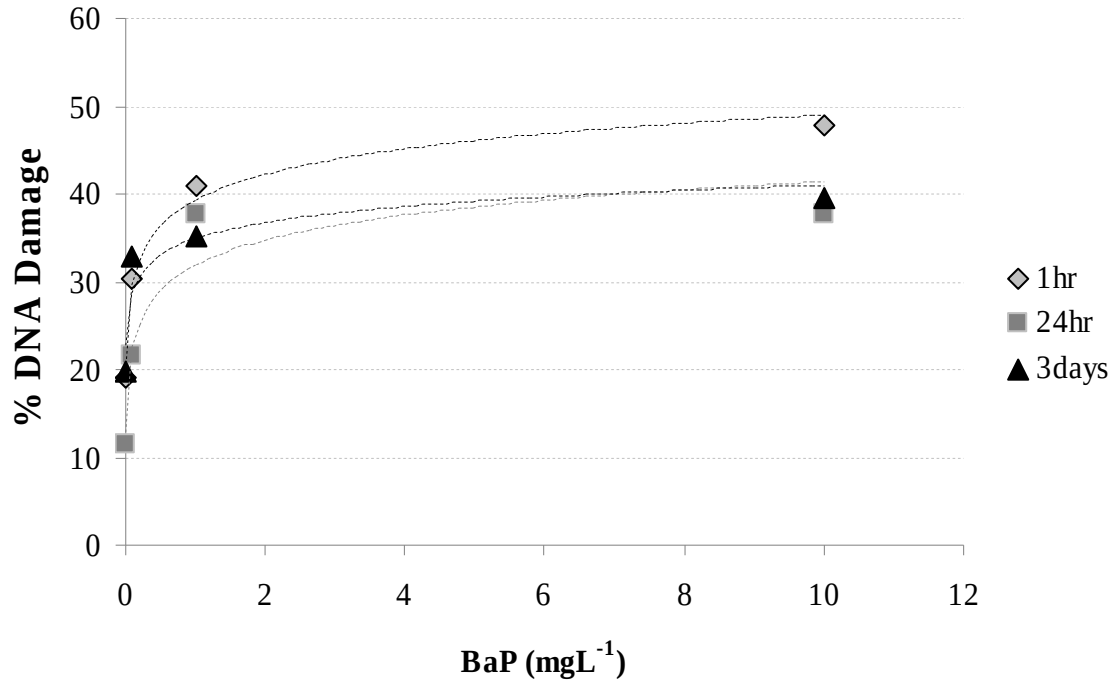
(a)


Farm in
Northumberland
Poole
Torpoint
N
W
E
S

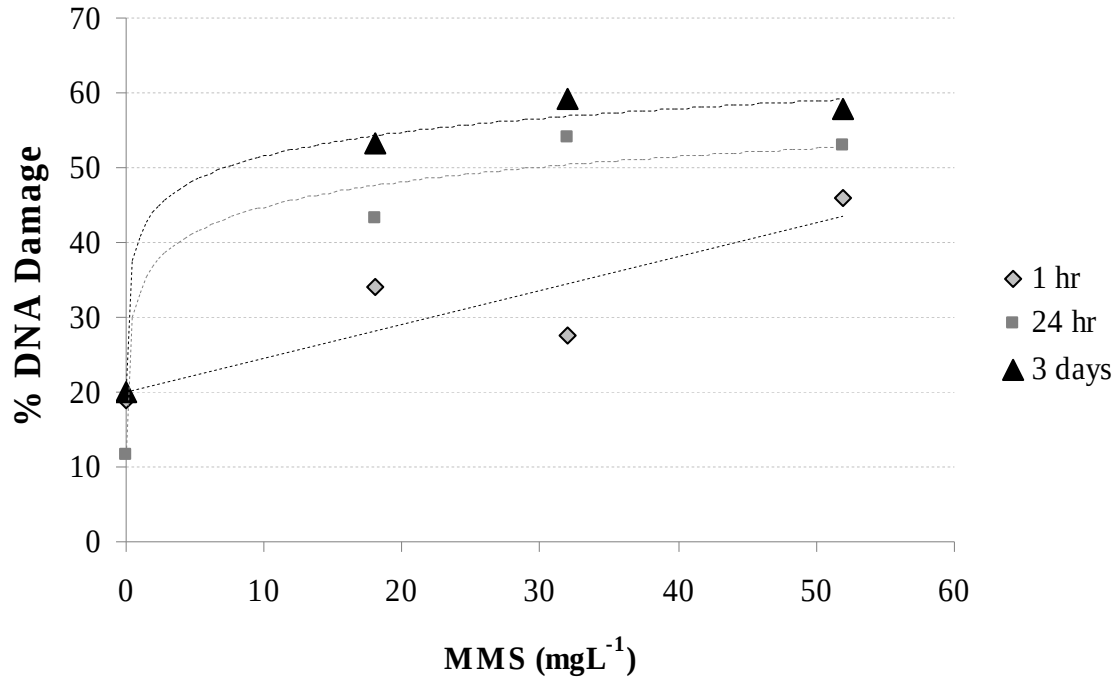
(b)


*
40
35
30
25
20
15
10
5
0
% Tail DNA
Torpoint
Poole
Farmed

(a)


60
50
40
30
20
10
0
% DNA Damage
0
2
4
6
8
10
12
BaP (mgL-1)
1hr
24hr
3days

(b)


70
60
50
40
30
20
10
0
% DNA Damage
0
10
20
30
40
50
60
MMS (mgL-1)
1 hr
24 hr
3 days