Short Communication ESPR

Title: Diamondoid naphthenic acids cause *in vivo* genetic damage in gills and haemocytes of marine mussels

Short title: Genotoxic diamondoid acids

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Abstract

Diamondoids are polycyclic saturated hydrocarbons that possess a cage-like carbon skeleton approaching that of diamond. These 'nano-diamonds' are used in a range of industries including nanotechnologies and biomedicine. Diamondoids were thought to be highly resistant to degradation but their presumed degradation acid products have now been found in oil sands process-affected waters (OSPW) and numerous crude oils. Recently, a diamondoid related structure, 3-noradamantane carboxylic acid, was reported to cause genetic damage in trout hepatocytes under *in vitro* conditions. This particular compound has never been reported in the environment but led us to hypothesise that other more environmentally-relevant diamondoid acids could also be genotoxic. We carried out *in vivo* exposures (3 d, semi-static) of marine mussels to two environmentally-relevant diamondoid acids, 1-adamantane carboxylic acid and 3,5-dimethyladamantane carboxylic acid plus 3-noradamantane carboxylic acid with genotoxic damage assessed using the Comet assay. An initial screening test confirmed that these acids displayed varying degrees of genotoxicity to haemocytes (increased DNA damage above that of controls) when exposed *in vivo* to a concentration of 30 µmol L^{-1}. In a further test focused on 1-adamantane carboxylic acid with varying concentrations (0.6, 6 and 30 µmol L^{-1}), significant ($P<0.05\%$) DNA damage was observed in different target cells (*viz.* gills and haemocytes) at 0.6 µmol L^{-1}. Such a level of induced genetic damage was similar to that observed following exposure to a known genotoxin, benzo(a)pyrene (exposure concentration: 0.8 µmol L^{-1}). These findings may have implications for a range of worldwide industries including oil extraction, nanotechnology and biomedicine.

Keywords: oil sands process-affected water; Comet assay; genotoxicity; Mytilus; naphthenic acids; Adamantane

Introduction

Globally, oil industries produce vast quantities of waste water that either enter the environment directly or is processed in some manner. Within such waters, one class of polar organic compounds, the 'naphthenic acids' (NA), was implicated in causing hormonal disruption in fish populations in the North Sea, UK (Knag et al. 2013; Thomas et al. 2009). These compounds have the general formula $C_nH_{2n+z}O_2$ (where n refers to the number of carbon atoms and z is zero or a negative even integer referring to the hydrogen deficiency). NAs have been implicated as the causative agents responsible for observed sublethal effects including hormonal disruption, embryonic developmental defects and genotoxicity, arising from exposure to OSPW e.g. (He et al. 2012; He et al. 2011; Kavanagh et al. 2011; Lacaze et al. 2014; Peters et al. 2007; Sansom et al. 2013) and references therein) but studies concerned with the toxicity of individual NAs are rare.

An interesting group of NAs that have been identified in OSPW and in highly-degraded crude oils are the diamondoid acids (Rowland et al. 2011a; Rowland et al. 2011c). Diamondoids are polycyclic saturated hydrocarbons that possess a cage-like carbon skeleton approaching that of diamond, sometimes termed 'nano-diamonds', and are used in a range of industries including nanotechnologies and biomedicine (Mansoori et al. 2012). These structures, including adamantane and diamantane, were thought to be highly resistant to degradation but the discovery of their associated acids suggests that they are subject to metabolic processes. Little is known about the toxicities of individual adamantine acids although Jones et al. (2011) reported *V. fischeri* bioluminescence inhibition EC_{50}s in the range 340 to 780 µmol L^{-1} and Scarlett et al. (2012) predicted relatively low toxicological effects for a range of human and environmental health endpoints based on sophisticated modelling software

(Admet™, Simulations Plus). The latter study also experimentally tested several adamantane acids using a panel of human cell-derived nuclear receptor reporter gene bioassays (CALUX® panel, Biodetection Systems, NL) for estrogenic, androgenic, peroxisome-proliferation, and aryl hydrocarbon receptor-mediated transactivation but no significant effects were observed. Recently however, Lacaze et al. (2014) reported that 3-noradamantane carboxylic acid (Fig. 1) produced a significant genotoxic effect on trout hepatocytes as measured by single cell gel electrophoresis (SCGE), commonly referred to as the Comet assay. Concentrations in the range 3 - 90 µmol L^{-1} caused DNA damage of *ca.* 20 - 27 %, (compared to 10% damage observed in negative controls) similar to that produced by exposure to 0.1% OSPW. Although similar in structure to adamantane carboxylic acids (Fig. 1), 3-noradamantane carboxylic acid has never, to our knowledge, been identified in OSPW or any environmental sample to date. We hypothesised that if the cage structure was at least in part responsible for the observed genotoxicity (Lacaze et al. 2014), then other diamondoid acids should also produce similar effects.

The preliminary study reported herein tested this hypothesis using an adamantane acid and an alkylated homologue. As living organisms have the capacity to repair genetic damage more efficiently, we performed *in vivo* exposures. Two diamondoid acids known to be present in OSPWs from multiple industries in the Athabasca region of Canada (Lengger et al. 2015; Rowland et al. 2011a; Rowland et al. 2012; Rowland et al. 2011c), numerous crude oils (unpublished data) and commercial mixtures of NA (Rowland et al. 2011b), namely 1-adamanatane carboxylic acid (1-Ad) and 3,5-dimethyladamantane carboxylic acid (DM-Ad), plus 3-noradamantane carboxylic acid (N-Ad) were assessed for their genotoxic potential using the widely-employed Comet assay as an accepted method of DNA damage

assessment (Collins 2014 and references therein; Jha 2008). As diamondoid acids are present in crude oils they are likely to be present in waste waters from oil platforms and pose the potential for genetic damage in the biota. We therefore utilised the marine mussel *Mytilus galloprovincialis* as a test species. Bivalves filter large quantities of water (*ca.* 2 - 3 L h^{-1}) and have commonly been used to assess various endpoints following exposure to oil and petroleum-derived products (e.g. Bayne et al. 1982; Booth et al. 2007; Di et al. 2011; Dixon et al. 2002; Donkin et al. 2003; Rowland et al. 2001; Scarlett et al. 2011).

Materials and Methods

For all exposures conducted, positive controls, benzo(a)pyrene (BaP, 0.8 μmol L^{-1}) for *in vivo* and hydrogen peroxide (1000 μM) for *in vitro* and negative controls (procedural blank, seawater, and acetone (0.001% v/v) were performed. Concentrations of BaP and hydrogen peroxide used in the study as positive controls were based on the previous validation studies carried out in our laboratory (Dallas et al. 2013; Di et al. 2011).

Preparation of *in vivo* test solutions

Sodium hydroxide, hydrochloric acid, HPLC-grade water, acetone, BaP (purity ≥96%), 1-adamantane carboxylic acid (purity ≥99%) and 3,5-dimethyladamantane-1-carboxylic acid (purity ≥97%) were purchased from Sigma (Gillingham, UK). 3-noradamantane carboxylic acid (≥98% purity) was supplied by Fluorochem (Hadfield, UK). Stock solutions of 30 mmol L^{-1} were prepared by dissolving the test compounds in HPLC-grade water and NaOH with pH >9 then lowering the pH dropwise with addition of HCl to achieve a final pH of 7.5 – 8.

The stock solutions were diluted in HPLC-grade water to give additional concentrations of 6 and 0.6 mmol L^{-1}. Working solutions (1.8 ml) were dissolved in 1.8 L seawater to produce test solutions of 30, 6 and 0.6 µmol L^{-1}. A procedural blank was created using similar volumes of NaOH and HCl with a final pH of 7.5 – 8. BaP was used as positive control since it is a known genotoxin (Tung et al. 2014). This was dissolved in acetone and spiked into seawater to give a concentration of 0.8 µmol L^{-1} (acetone 0.001%). An acetone (0.001%) negative control for the BaP exposure was also prepared. Water quality measurements, (O_2 saturation, salinity, pH and temperature) were taken before commencement of the tests and daily thereafter. Temperature was 15°C ± 0.5°C, salinity of 34 (± 1) psu, pH 8.1 ± (0.1) and O_2 saturation >95% throughout the tests.

Collection and maintenance of mussels

Mussels (*M. galloprovincialis*, shell length ca. 50 mm, sexually mature) were collected from Trebarwith Sands on the north coast of Cornwall, UK (N 50° 38.850', W 004° 45.680') and transported to the laboratory at Plymouth University within 2h. Under UK law, no specific permissions were required for these locations/activities as no endangered or protected species were involved. Following shell cleaning in clean seawater to remove barnacles and other epibionts, mussels were maintained in a 15°C temperature-controlled room. Mussels were fed daily with the alga *Isochrysis galbana* in accordance with manufacturer's recommendations (Reed Mariculture, Campbell, Ca, USA) with regular water changes. Prior to any exposures, from the collected stock mussels, individuals of similar size (50 ± 5 mm) were selected for the exposures and assigned randomly to Pyrex glass beakers containing 1.8L filtered (2 µm) natural seawater i.e. one mussel per beaker.

Exposure tests

An initial set of exposures were performed using test solutions of 30 µmol L^{-1} of all three adamantane acids (*n* = 6). This was the concentration of N-Ad reported by Lacaze et al. (2014) to cause significant damage in trout hepatocytes. Positive and negative controls were employed as described above. Having established that the genotoxic response was similar for all three NA, a second set of tests were performed using 1-Ad only with concentrations of 30, 6 and 0.6 µmol L^{-1} (*n* = 6) plus positive controls, procedural blanks (as above) and seawater negative controls. For both sets of tests individual mussels were placed in 1.8 L of test solutions and the exposure period was 3 d semi-static with daily water exchanges and dosing of test solutions. Mussels were fed daily (as above) following daily water exchanges (100 % replenishment). Beakers were coded and their positions were randomly allocated. Following the end of the exposures, haemolymph was extracted (and for secondary tests, gill tissue excised) from individual mussels and assigned new coding such that the cell preparation and Comet assay was performed without knowledge of the treatment received (i.e. blind scoring). Prior to performing the comet assay, cell viability was determined using Eosin Y staining (Canty et al. 2009), viability was deemed >95 %.

Comet assay

Determination of DNA damage such as induction of DNA strand breaks and alkali labile sites using haemocytes and gill cells of mussels were determined using the Comet Assay as described elsewhere (Jha 2008; Dallas et al. 2013). Further details are provided in

supplementary information. The replicate microgels on the slides were each stained with ethidium bromide and scored under an epifluoresence microscope (Leica, DMR) using the Komet 5.0 image-analysis software (Kinetic Imaging, Liverpool, UK). Slides were coded and randomised and 50 cells were scored per replicate. Although the software provided a range of parameters, DNA damage is reported here as % tail DNA as is considered to be the most reliable parameter and also allows for inter-laboratory comparison (Kumaravel and Jha 2006).

Statistical analysis

Statistical analyses of results were performed using Statgraphics® centurion XV, Statpoint Inc. (Warrenton, Virginia, USA). Prior to analysis of variance (ANOVA), data was tested for normality using Cochran's test and log-transformed as necessary. Where there was a significant of means, the data were further analyzed by post-hoc Tukey's HSD tests to determine significant differences between treatments and controls.

Results and Discussion

The noradamantane core structure is not a true diamondoid but has a similar three-dimensional cage structure (Fig.1). Diamondoids are widely used in many industrial and biomedical processes (Mansoori et al. 2012). Our study tested the hypothesis that carboxylic acids of true diamondoid structures would produce similar genotox damage as that previously reported for N-Ad (Lacaze et al. 2014). We chose to test a parent and an alkylated structure, both of which are known to be present

in OSPW, commercial mixtures of NA and crude oils (Rowland et al. 2011a; Rowland et al. 2012; Rowland et al. 2011b; Rowland et al. 2011c).

A preliminary exposure revealed that a similar degree of genetic damage of *ca.* 15% was observed in mussel haemocytes (Fig. 2) following exposure to all three acids and BaP (0.8 µmol L^{-1}). The level of damage observed for the positive control (i.e. BaP) was consistent with previous studies (e.g. Banni et al. 2010; Di et al. 2011; Kwok et al. 2013; Mitchelmore et al. 1998). The degree of damage observed in mussel haemocytes was a little less than that observed by Lacaze et al. (2014) for *in vitro* exposures to N-Ad in fish hepatocytes and was not significantly different to controls at the 5 % probability level but was at the more precautionary 10 % level (Fig. 2). We further hypothesised that gill tissue cells would display a greater sensitivity than haemocytes as gills are the primary target tissue in aquatic filter feeding organisms. For the secondary tests we repeated the 30 µmol L^{-1} exposure to 1-Ad and assessed the damage in both haemolymph and gill tissue cells plus two lower concentrations (semi-log scale; 6 and 0.6 µmol L^{-1}). The amount of damage in the haemocytes following exposure to 30 µmol L^{-1}1-Ad (Fig. 3b) was the same as found in the initial test (Fig. 2). Increased damage (approx. double) was observed in the gill tissue cells compared to haemocytes but this was not significantly different ($P > 0.05$) from the respective controls (Fig 3). Greater genetic damage was observed in both cell types following exposure to the lower concentrations tested (Fig 3), with *ca*. 42 % mean DNA damage for gills (compared to *ca.* 26% in controls; Fig. 3a) and *ca* 21% DNA damage in haemocytes (compared to *ca* 6 % in controls; Fig. 3b). In gill cells, significant damage of around 40 % ($P < 0.05$) was found after exposure to both 6 and 0.6 µmol L^{-1} i.e. low and mid concentrations, respectively (Fig 3a). An increase in DNA damage was also observed in the gill tissue of mussels exposed to 0.8 µmol L^{-1} BaP similar to that produced by 0.6 µmol L^{-1} 1-Ad (Fig. 3). The results produced by Lacaze et al. (2014) suggest

oxidative stress as a mechanism of genotoxic damage caused by N-Ad and therefore this may be true for the diamondoid acids tested herein but tests for oxidative stress were not performed. Hence, the similarly in effect concentrations between BaP and the diamondoid acids does not imply similar mode of action. There was little difference between the genetic damage caused by the 6 and 0.6 µmol L^{-1} exposures (Fig. 3a and 3b) suggesting that the mussels' DNA repair mechanism was able to prevent any further damage. Such nonmonotonic responses and low-dose effects are reported to be common in studies of natural hormones and endocrine disrupting compounds (reviewed by Vandenberg et al. 2012). Mechanisms for these concentration-specific effects include signalling via single versus multiple steroid receptors due to non-selectivity at higher doses, receptor down-regulation at high doses versus up-regulation at low doses, differences in the receptors present in various tissues, tissue-specific components of the endocrine-relevant transcriptional apparatus and cytotoxicity at high doses (Vandenberg et al. 2012 and references therin). Cytotoxicity is unlikely to be a factor as the *in vitro* membrane integrity EC_{10} concentration was determined to be 290 µM 1-Ad (unpublished data) and, to date, specific receptor-mediated transactivation for estrogenic, androgenic, peroxisome-proliferation, or aryl hydrocarbon was found not to occur (Scarlett et al. 2012). Evidence has shown that DNA repair mechanisms can affect the response of invertebrates such as mussels when exposed to organic contaminants, since DNA breaks produced by these compounds may be repaired by base excision repair pathway (Villela et al. 2006). Furthermore, the lower DNA damage observed in the high 1-Ad treatment could be explained by the exclusion of the apoptotic cells in the Comet assay cell count (Hook and Lee 2004). Interestingly, Gagné et al (2013; 2012) reported that gene expression for DNA stand breaks in trout hepatocytes was associated with exposure to OSPW. Whether such

repair mechanisms would be capable of ameliorating the effects of exposure to genotoxic components in aquatic systems receiving inputs from oil industry waste waters or natural oil seepages is not known. Much greater DNA damage (92 % ± 6 %) was observed using hydrogen peroxide as an *in vitro* positive control, whereby embedded cells on slides (either gills or haemocytes) were exposed to a dose of 1000 μM for 30 min. The rationale for an *in vitro* positive control was two-fold, firstly to demonstrate the assay worked and secondly, to demonstrate damage with the effects of the *in vivo* DNA repair mechanisms *in absentia*. A no-observable-effect concentration (NOEC) was not established so we can only report a lowest-observable- effect-concentration (LOEC) of 0.6 μmol L^{-1} 1-Ad. The practice of reporting NOECs and LOECs have been criticised and considered outdated (Landis and Chapman 2011); further evidence such as EC values (e.g. EC_{10}) would be useful but could not be derived in this preliminary study.

Although present evidence suggests that the potential for genetic damage exists, the widespread significance of such results may be difficult to interpret. Diamondoid acids represent a substantial fraction of the acid-extractable organic fraction of OSPW (Reinardy et al. 2013; Scarlett et al. 2013; West et al. 2013) so collectively could easily be at >mg L^{-1} concentrations but distributions of individual adamantane acids can vary both within and between storage ponds from different industries (Lengger et al. 2015). At present, OSPW is contained within storage ponds although there is increasing evidence for possible leaching into the environment (Frank et al. 2014 and references therein). To date and to our knowledge, individual diamondoid acids have not been quantified in environmental systems.

Diamondoid acids are not just present in OSPW but are also present in many crude oils that have been substantially biodegraded (unpublished data). As the world's supply of "sweet" oil shrinks, greater reliance on biodegraded oil will likely occur and the NA content in produced waters increase. As with all toxicants, the potential for harm diminishes with sufficient dilution, especially if they have a low tendency to bioaccumulate or biomagnify. Bioconcentration factors for diamondoid acids have not been reported but predictive models suggest that they are very low (<10) (Scarlett et al. 2012). The presence of adamantane diacids in OSPW, especially aged ponds, (Lengger et al. 2013) suggests that further degradation of adamantane acids occur in the environment. However, at present it is not known how long diamondoid acids may persist in the environment or if their further breakdown products also cause genetic damage. Given that a similar degree of genetic damage was observed in two very different species, cell types and exposure route, i.e. *O. mykiss* hepatocytes *in vitro* (Lacaze et al. 2014), and *M. galloprovincialis* haemolymph and gill tissue *in vivo* herein, it would appear that the potential exists that diamondoid acids will most likely cause similar damage across a broad range of species.

Conclusions

This preliminary study confirmed that a diamondoid-like acid (3-noradamantane carboxylic acid), previously found to be genotoxic *in vitro* in trout hepatocytes also produced effects *in vivo* in mussel haemocytes. The results also demonstrated that metabolites of true diamondoid structures, an adamantane acid (1-Ad) and an alkylated homologue, displayed varying degrees of genotoxicity in mussel haemocytes. Greater DNA damage was caused in gill cells with both 6 and 0.6 µmol L^{-1} 1-Ad causing significant damage of ca 40% ($P < 0.05$), similar to that observed for a known genotoxic BaP

with a concentration of 0.8 µmol L^{-1}. Although further research would be required to establish the level of risk to the environment diamondoid acids may pose, this preliminary study may have implications for a range of worldwide industries including oil extraction, nanotechnology and biomedicine.

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Figure legends

Fig. 1 Chemical structures of selected diamondoid acids. Adamantane is the smallest true diamondoid but noradamantane is a close structural analogue containing one less CH_2 link.

Fig. 2 DNA damage in haemocytes (mean ± 1 SE) resulting from exposure to 30 µmol L^{-1} diamondoid acids. Letters represent significant differences ($P < 0.10$) from controls ($n = 6$). Different letters denote significant differences between treatments; same letters denote no significant difference e.g. a or b; treatments with multiple letters e.g. ab denote similarities with both groups a and b.

Fig. 3 DNA damage in (A) isolated gill cells and (B) haemocytes (mean ± 1 SE) resulting from exposure to 0.60 (low), 6 mid) and 30 (high) µmol L^{-1} 1-adamantane carboxylic acid (1-Ad). Letters represent significant differences ($P < 0.05$) from controls ($n = 6$). Different letters denote significant differences between treatments; same letters denote no significant difference e.g. a or b; treatments with multiple letters e.g. ab denote similarities with both groups a and b.

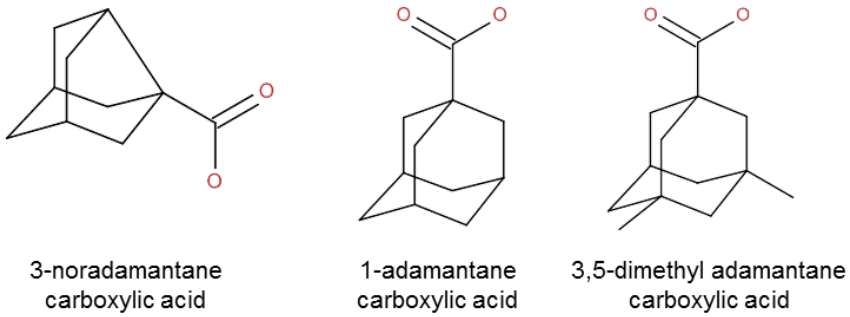

3-noradamantane carboxylic acid
1-adamantane carboxylic acid
3,5-dimethyl adamantane carboxylic acid

Fig. 1

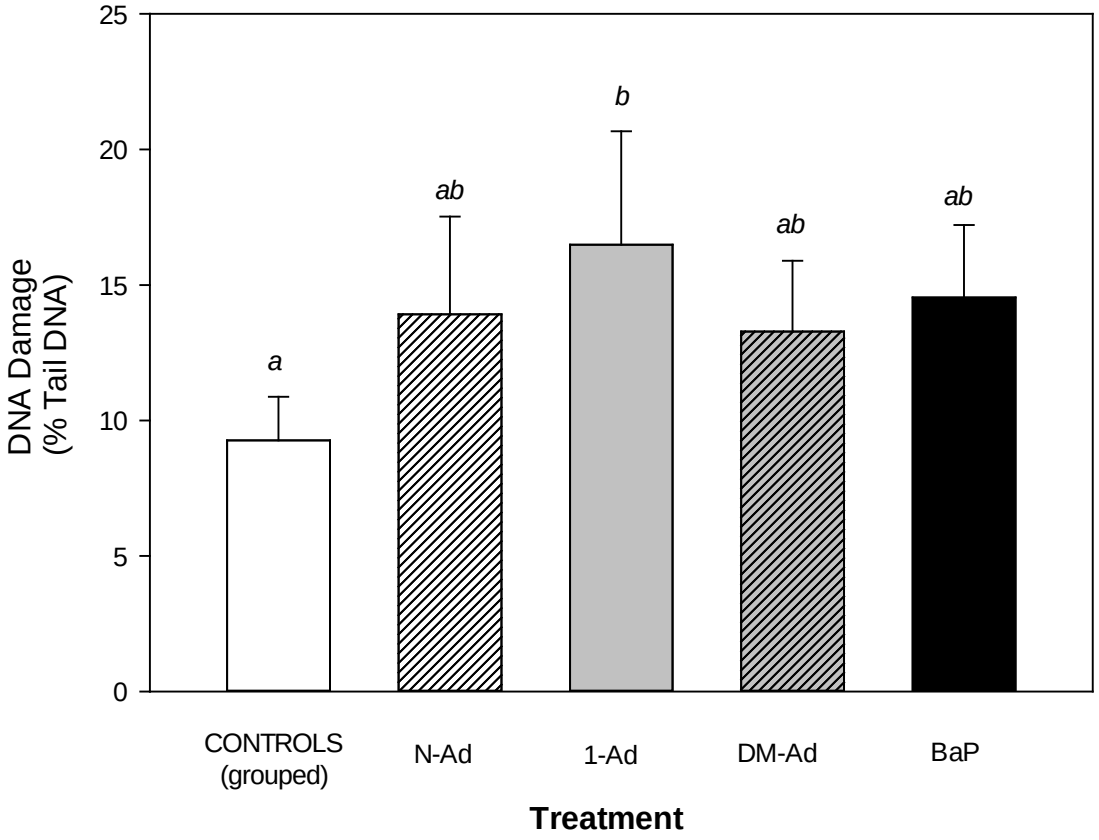
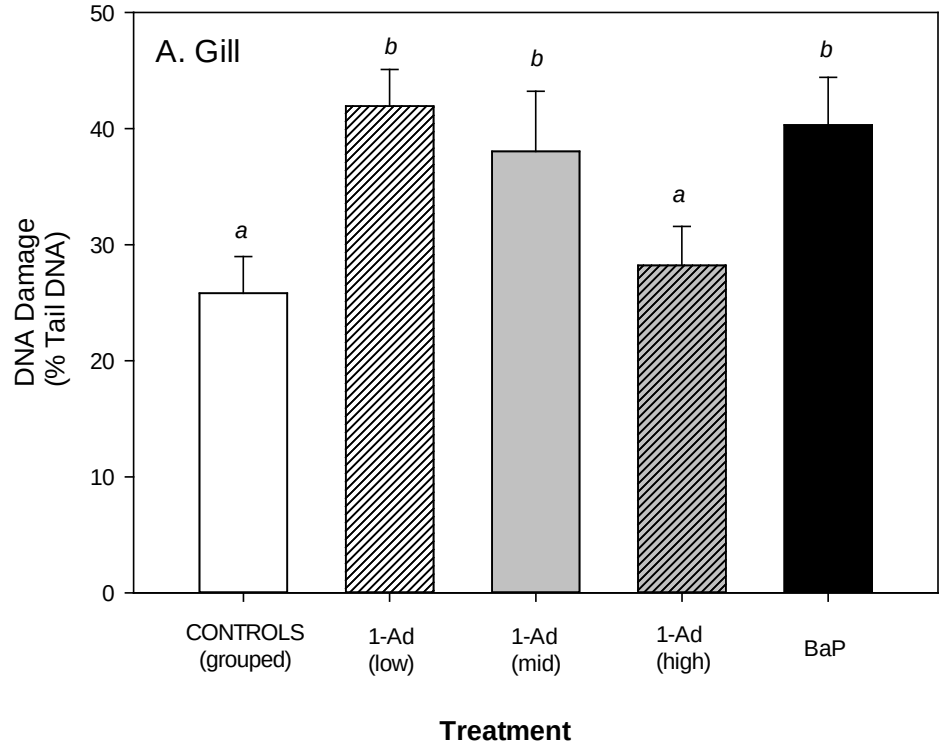

DNA Damage (% Tail DNA)
25
20
15
10
5
0
a
ab
b
ab
ab
CONTROLS (grouped)
N-Ad
1-Ad
DM-Ad
BaP
Treatment

Fig. 2


A. Gill
DNA Damage
(% Tail DNA)
50
40
30
20
10
0
a
b
b
a
b
CONTROLS
(grouped)
1-Ad
(low)
1-Ad
(mid)
1-Ad
(high)
BaP
Treatment

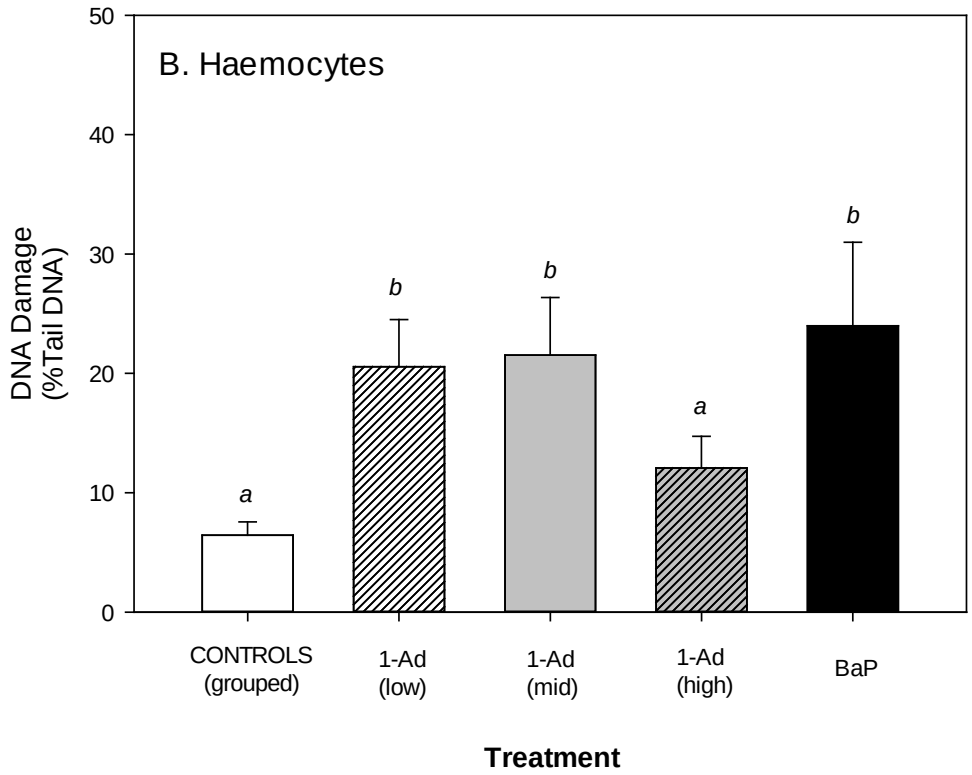

B. Haemocytes
DNA Damage
(%Tail DNA)
50
40
30
20
10
0
a
b
b
a
b
CONTROLS
(grouped)
1-Ad
(low)
1-Ad
(mid)
1-Ad
(high)
BaP
Treatment

Fig. 3