Baseline and oxidative DNA damage in marine invertebrates

Running head: Oxidative DNA damage in marine invertebrates

Andrea Sahlmann[1,*], *Raoul Wolf*[1], *Tor Fredrik Holth*[1,2], *Josefin Titelman*[1], *Ketil Hylland*[1]

[1] Department of Biosciences
University of Oslo
P.O. box 1066 Blindern
N-0316 Oslo
Norway

[2] current address: Fylkesmannen i Vestfold
P.O. box 2076
N-3103 Tønsberg
Norway

[*] Corresponding author: a.s.sahlmann@ibv.uio.no

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Authors details:

Andrea Sahlmann
Department of Biosciences
University of Oslo
P.O. box 1066 Blindern
N-0316 Oslo
Norway
phone: +47-22854506
e-mail: a.s.sahlmann@ibv.uio.no

Raoul Wolf
Department of Biosciences
University of Oslo
P.O. box 1066 Blindern
N-0316 Oslo
Norway
phone: +47-22854506
raoul.wolf@ibv.uio.no

Josefin Titelman
Department of Biosciences
University of Oslo
P.O. box 1066 Blindern
N-0316 Oslo
phone: +47 22854501
e-mail: josefin.titelman@ibv.uio.no

Ketil Hylland
Department of Biosciences
University of Oslo
P.O. box 1066 Blindern
N-0316 Oslo
phone: +47 22857315
e-mail: ketil. hylland@ibv.uio.no

Tor Fredrik Holth
Fylkesmannen i Vestfold
P.O. box 2076
N-3103 Tønsberg
Norway
phone:+47 33371192
e-mail: fmvetfh@fylkesmannen.no

Abstract

Anthropogenic pollutants cause oxidative stress in marine organisms, directly or following generation of reactive oxygen species, potentially resulting in increased accumulation of DNA strand breaks. We quantified baseline levels of DNA strand breaks in marine species from four phyla and assessed their relative sensitivity to oxidative stress, as well as capacity to recover.

DNA strand breaks were quantified using a formamidopyrimidine DNA glycosylase (Fpg)-amended comet assay on circulating cells from blue mussel (*Mytilus edulis*), shore crab (*Carcinus maenas*), sea star (*Asterias rubens*) and vase tunicate (*Ciona intestinalis*). Lymphocytes from Atlantic cod (*Gadus morhua*) were used as a reference. In addition to immediate analysis, cells from all species were exposed *ex vivo* to two concentrations of H_2O_2 (25 and 250 μM) prior to being assayed.

Mean baseline DNA strand breaks were highest for cells from sea star (34%) followed by crab (25%) mussel (22%), tunicate (17%) and cod (14%). Circulating cells from invertebrates were much more sensitive to oxidative stress than were cod lymphocytes. DNA strand breaks exceeded 80% for sea star, crab and mussel cells following exposure to the lowest H_2O_2 concentration. There was no recovery for cells from any species following 1 hr in buffer.

This study provides an in-depth analysis of DNA integrity for ecologically important species representing four phyla. The results indicate that circulating cells from invertebrates are much more sensitive to oxidative stress than cells from fish, measured as DNA strand breaks. Future studies should address the extent to which DNA strand breaks have consequences for body maintenance costs in marine invertebrates.

Introduction

There has been an increasing focus on the genotoxic potential of anthropogenic pollutants over the past couple of decades (Bolognesi and Cirillo, 2014). Pollutants can affect DNA through several mechanisms (Bolognesi and Cirillo, 2014), including by causing oxidative stress. Intracellular production of radicals can overcome antioxidant defences, resulting in oxidative damage to other macromolecules such as lipid peroxidation, DNA strand breaks and alterations in critical cellular processes (Livingstone, 2003).

Knock-on effects of DNA damage include cell death, mutation, carcinogenesis and genotoxicity, with long-term consequences of which may include embryonal aberration (Barranger et al., 2014), reduced hatching rates, gamete development and reduced fitness (Lee et al., 2012; Linhartova et al., 2013; Matić et al., 2016). Measures of DNA damage provide an early warning signal of genotoxic exposure (Rybakovas et al., 2009). Fish and mussels have served as useful indicator species in ecotoxicological and genotoxicity studies (reviewed in Frenzilli et al., 2009; Lee and Steinert, 2003). Inter-species differences in vulnerability to toxicity have led to the inclusion of additional invertebrate species such as polychaetes and sea urchins (Lewis and Galloway, 2008; Pinsino and Matranga, 2015). There is however still limited understanding of DNA damage and repair in invertebrates. Mussels, sea stars, crabs and tunicates are present in most coastal ecosystems and representatives of those groups were selected as model organisms in this study.

Antioxidant activity and DNA repair mechanisms such as base excision repair play a major role in balancing out the continuous damage to DNA caused by radicals (Collins et al., 1997). The antioxidant system includes both antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase and antioxidants like

glutathione (Birben et al., 2012). Impaired antioxidant defence and DNA repair will increase base oxidation and DNA strand breaks (Azqueta et al., 2009). Persistent genotoxic damage depends on the balance between repair and replacement of damaged cells (El-Bibany et al., 2014). Differences in the replacement of damaged cells specifically, and cell proliferation rate in general may affect species relative sensitivity to accumulate DNA damage. For example, echinoderms had low levels of cell proliferation compared to vertebrates (Dixon et al., 2002; Hernroth et al., 2010; Holm et al., 2008).

DNA damage in terms of strand breaks can easily be quantified with the comet assay, a method widely used in both human toxicology and ecotoxicology (Collins et al., 1997; Frenzilli et al., 2009). The comet assay also allows for quantification of DNA repair capacity by measuring removal of DNA strand breaks (Collins, 2004). In addition, by including a base excision repair enzyme such as formamidopyrimidine DNA glycosylase (Fpg), oxidised nucleotides can also be quantified (Collins and Azqueta, 2011). Although not inherently limited to a species or cell type, use of the comet assay in ecotoxicology has to a large extent been limited to studies using fish or mussels (de Lapuente et al., 2015). There is however a large potential in using the comet assay to assess genotoxicity in circulating cells from species from other taxonomic groups.

Genotoxicity studies using the comet assay require preparations with dissociated cells, either cells already in suspension or cells separated prior to performing the assay. The assay has however been most widely used with blood cells or sperm from humans and vertebrates (Collins and Azqueta, 2011) or hemocytes or coelomocytes from invertebrates - the equivalent to human blood cells (Dhawan et al., 2009). Hemocytes and coelomocytes are involved in essential functions such as nutrient and oxygen transport, immunity and wound healing (Matranga et al., 2005). Cell characteristics and

121 the abundance of different cell types vary between phyla, species and even among
122 individuals (Arizza and Parrinello, 2009; Carballal et al., 1997; Söderhäll and Smith,
123 1983). In addition, pollution and traumatic events may alter the relative cell abundance
124 (Hernroth et al., 2010; Matranga et al., 2006; Pinsino et al., 2007). Specific cells differ
125 in their sensitivity to DNA damaging agents. For example, circulating cells appear to
126 be less sensitive to DNA damaging agents than gill, digestive gland or sperm cells
127 (Frenzilli et al., 2009). Mammalian lymphocytes are particularly robust (Andreoli et al.,
128 1999; Collins et al., 1995), whereas circulating cells from invertebrates have more
129 variable sensitivity (Venier et al., 1997; Lewis and Galloway, 2008). However,
130 implications of changes in cell composition for species susceptibility to genotoxicity
131 remain poorly understood. To evaluate possible differences in the susceptibility of
132 circulating cells to DNA damage, it is crucial to have knowledge about natural baseline
133 levels in the species investigated.
134 This study aimed to determine genotoxic responses in coelomocytes and hemocytes of
135 *M. edulis*, *A. rubens*, *C. maenas* and *C. intestinalis* by quantifying 1) baseline levels of
136 DNA strand breaks, 2) species-specific responses to oxidative stress, and 3) recovery
137 of the cells following oxidative stress.

Methods

Reagents

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), Merck (Kenilworth, NJ, USA) and VWR (Radnor, PA, USA) unless stated otherwise. The DNA repair enzyme Fpg (20 ng mL^{-1}), was kindly provided by Gunnar Brunborg (Norwegian Health Institute, Oslo, Norway).

Animal collection

Animals were collected outside their natural breeding season. *Ciona intestinalis, Asterias rubens*, and *Carcinus maenas* were collected by divers in the outer Oslofjord in the vicinity of Drøbak (59.66° N, 10.59° E), Norway in March 2013, and maintained at Drøbak Aquarium until assessment. *Mytilus edulis* were collected from control site and kept in outdoor tanks with running seawater until use. The pollution load in outer Oslofjord has been studied for several years and may be characterised as moderate (Green et al., 2014). All species were maintained at their local environmental conditions or acclimated to new conditions.

Lymphocytes from Atlantic cod (*Gadus morhua*) were included as reference based on previous experience in our group on oxidative stress responses and DNA damage (Fredriksen, 2013). Cod were collected from outer Oslofjord and transported to Drøbak Biological Station, University of Oslo where they were held in outdoor tanks supplied with running seawater.

Cell sampling

Syringes (Sarstedt, 1 mL) were rinsed and preloaded with 0.2 mL ice-cold PBS (14.5 mM NaCl, 0.6 mM Na_2HPO_4, 0.4 mM KH_2HPO_4, 10 mM EDTA, pH = 7.4) before use.

163 pH and osmolality were adjusted to the respective tissue characteristics (*G. morhua:*
164 pH = 7.4 and 340 mOsm; invertebrates: pH = 7.4 and 700 mOsm).
165 *Gadus morhua* (n = 8) were sacrificed and 1 mL blood was withdrawn from the caudal
166 vein and white blood cells were isolated by discontinous Percoll gradient centrifugation
167 (with densities: 1.05, 1.06, and 1.07 g mL^{-1}) spun at 400 G for 40 min and cells
168 collected from the 1.05:1.06 g mL^{-1} interface.
169 0.8 mL circulating fluid was sampled of each invertebrate species (with n = 8 per
170 species). *C. maenas* was bled from an unsclerotized membrane of the pereiopods. *M.*
171 *edulis* hemolymph was withdrawn from the aductor muscle. Coelomic fluid of *A.*
172 *rubens* was sampled from the coelomic cavity and *C. intestinalis* coelomic fluid was
173 extracted from visceral cavity by inserting a syringe from posterior side.
174 Cell density was determined using a Bürker-Türk hemocytometer and cell viability was
175 assessed using the trypan blue exclusion test. Cell viability was > 90% for all
176 preparations.
177
178 **Comet assay**
179 Single strand DNA breaks were measured using the alkaline comet assay as described
180 by Singh et al. (1988) with modifications by Tice et al. (2000). Briefly, cells were
181 diluted in PBS and embedded in 0.75 wt% low melting point agarose in PBS before 25
182 µL of this mixture was transferred onto a hydrophilic GelBond film (Lonza, Basel,
183 Switzerland) (Gutzkow et al., 2013) with a final concentration of approximately 1.250
184 cells gel^{-1}. One gel per individual per species was applied to one film per treatment.
185 Films placed in boxes with 0, 25 and 250 µM H_2O_2 in PBS and embedded cells were
186 exposed for 15 min at 15 °C. After being rinsed in dH_2O, one films was placed in PBS
187 without EDTA for 1 h at 15 °C to allow for DNA repair. After exposure, films were

188 immediately immersed and stored for four weeks in cold lysis buffer (2.5 M NaCl, 100
189 mM EDTA, 10 mM Tris, 1 vol% Triton X-100, and pH = 10) in the dark. DNA damage
190 levels are stable for up to three weeks when immersed in ice-cold lysis buffer (Collins,
191 2014; Hylland et al., 2016). Films were rinsed in dH_2O, placed in cold Collins' buffer
192 (40 mM HEPES, 100 mM KCl, 500 μM EDTA, pH = 7.6) for 60 min, and incubated in
193 fresh Collins' buffer containing BSA (40 mg mL^{-1}) and incubation with Fpg was for 1
194 h at 15 °C. DNA unwinding was performed in electrophoresis buffer (300 mM NaOH,
195 1 mM EDTA and 6 vol% concentrated HCl, pH > 13) for 20 min before electrophoresis
196 was run at 15 V for 25 min. The films were rinsed in neutralization buffer (400 mM
197 Tris and pH = 7.5) for 15 min at room temperature. After films were rinsed in dH_2O,
198 they were fixed in 96% ethanol for 1.5 h and air dried overnight in darkness. DNA was
199 stained with 1× SYBR Gold (Life Technologies, Carlsbad, CA, USA) in TE buffer (10
200 mM Tris, 1 mM EDTA, and pH = 8) for 20 min at room temperature in darkness prior
201 to scoring using a Leica DMR fluorescence microscope with an objective with 40×
202 magnification. Fluorescence was read at 520/610 nm excitation/emission. 50 cells gel^{-1}
203 were scored and DNA damage, in terms of DNA strand breaks, was quantified using
204 Comet Assay IV software (version 4.3; Perceptive Instruments; Bury St Edmunds,
205 UK).
206
207 **Statistics**
208 DNA damage data was analyzed and visualized using open-source statistical software
209 R (version 3.3.2; R Core Team 2016) and its add-on packages nlme (version 3.1-128;
210 Pinheiro et al., 2017) and multcomp (version 1.4-6; Hothorn et al., 2008).
211 Baseline values were analyzed using a linear mixed effects (LME) model with species
212 as fixed effect and individuals as random factor. Intra-species differences in DNA

213 damage after peroxide exposures were analyzed in paired *t*-tests following non-
214 significant Shapiro-Wilk and *F*-tests. Inter-species differences were analyzed in an
215 LME model with species and peroxide treatments as fixed effects, and individuals as
216 random factor. Recovery abilities were analyzed in an LME model with species as fixed
217 effect, and individuals as random factor. Differences in species responses to peroxide
218 exposure and subsequent recovery were analyzed in an LME model with species,
219 peroxide treatments, and recovery as fixed effects, and individuals as random factors.
220 For all LME models DNA damage was the response variable, and inter-species
221 differences were analyzed after significant Wald *F*-tests with marginal (type III) sum
222 of squares for the respective fixed effects and subsequent multiple comparisons of
223 means with Tukey contrasts and Holm's *P*-value adjustment for multiple comparisons
224 (Bretz et al., 2011). A level of significance of $P = 0.05$ was set for the rejection of the
225 null-hypothesis.

Results

Baseline DNA damage for all species increased as follows: *G. morhua* (14 %) < *C. intestinalis* (17 %) < *M. edulis* (22 %) < *C. maenas* (25 %) < *A. rubens* (34 %) (mean values; Fig. 1). There were no statistical differences in baseline DNA damage between cells from *M. edulis, C. maenas, C. intestinalis* and *G. morhua.* Cells from *A.rubens* had higher baseline levels than cells from *G. morhua* and *C. intestinalis* ($P < 0.01$ and $P < 0.05$, respectively).

Both peroxide treatments resulted in increased DNA damage compared to baseline values (invertebrates $P < 0.05$, *G. morhua* $P < 0.001$; Fig. 2). DNA damage increased 3–4 fold compared to baseline values in the four invertebrate species. Total DNA damage exceeded 80% in *M. edulis*, *A. rubens* and *C. maenas* following exposure to 25 μM H_2O_2. DNA damage increased 2–3 fold following peroxide exposure compared to baseline values for cells from *C. intestinalis* and *G. morhua*.

In *M. edulis* and *G. morhua*, DNA damage increased following exposure 250 μM H_2O_2 compared to 25 μM H_2O_2 ($P < 0.05$ and 0.01, respectively). There was no significant change in DNA in cells from *M. edulis, A. rubens, C. intestinalis* following exposure to 250 μM H_2O_2 and subsequent recovery, but increased in *C. maenas* and *G. morhua* ($P < 0.05$ and $P < 0.001$, respectively; Fig. 3). Significantly increased levels of DNA strand breaks following incubation with Fpg were observed in cells from *A. rubens* and *C. intestinalis* ($P < 0.05$), *C. maenas* and *G. morhua* ($P < 0.001$) (Fig. 4). In *A. rubens* and *C. maenas* oxidised lesions accounted for 3-5% additional strand breaks. *C. intestinalis* and *G. morhua* had 21% and 29% additional strand breaks following Fpg-amendment.

Peroxide exposure caused higher DNA damage in *M. edulis* and *A. rubens* compared to *C. intestinalis* and *G. morhua* ($P < 0.001$; Fig. 2). *C. maenas* and *C. intestinalis* did not

differ in their exposure responses. Lymphocytes from *G. morhua* had lower DNA damage than the invertebrate cells ($P < 0.001$; Fig. 2). Following recovery, DNA damage increases in cells from *C.maenas* and *G. morhua* ($P < 0.05$ and 0.001, respectively; Fig. 3).

Discussion

We quantified species-specific differences in baseline DNA damage, in susceptibility to oxidative stress and DNA repair capacity of four common and widely distributed invertebrates from different phyla. The circulating cells from the invertebrates were more susceptible to DNA damage caused by oxidative stress than were cod lymphocytes. For cod, which was included as a reference species, both baseline DNA strand breaks and the responses following oxidative stress agreed with published data (Fredriksen, 2013), indicating consistent results from the assay.

To our knowledge this is the first time that DNA damage was determined quantitatively in an ascidian using the comet assay (but see Ballarin et al., 2008; Liu et al., 2006). A summary of published studies suggests that chordates, such as tunicate and fish, generally exhibit lower baseline DNA strand breaks than the species from the other phyla (Table 1, references therein). Such differences could be due to differences between species in cell or protein turnover or different constitutive levels of DNA repair (Collins et al., 2001; Siu et al., 2004; Siu et al., 2003).

Within taxa different levels of baseline DNA damage may reflect differences in animal state or methods as standardised comet assay protocols are lacking for most invertebrates (Dixon et al., 2002; Martins and Costa, 2015). Levels of DNA damage in coelomic epithelial and pyloric caeca cells of *A. rubens* (Everaarts, 1995; Hernroth et al., 2010) are similar to the levels found in this study (Table 1). Canty et al. (2009), however, observed lower levels of DNA damage in sea star coelomocytes.

DNA damage increased in circulating cells from all invertebrate species and treatments, with *C. intestinalis* cells apparently being the most robust. The damage observed for *M. edulis, A. rubens* and *C. maenas* cells exposed to the lowest H_2O_2 concentration was close to the upper limit of damage detection, as cells with totally fragmented DNA may

be overlooked or disregarded (Kumaravel et al., 2009, Lorenzo et al., 2013). Hence, the observed additional increases in DNA damage following exposure to a ten times higher concentration of H_2O_2 or Fpg-amendment were small. The results agree with previous observations of little additional increase of DNA damage in mussel gill cells exposed to higher concentrations than 100 μM H_2O_2 (Wilson et al., 1998). Although small, the increase in oxidised DNA damage (detected using Fpg-treatment) was significant for all invertebrate species except mussel, which was probably due to the high initial DNA damage in that species. Fpg-amendment thus appears to be a useful addition to the comet assay for invertebrates, as well as for vertebrates (Collins, 2014). Increased levels of oxidised lesions have previously been detected using a Fpg-amended comet assay for bivalve hemocytes and gill cells (Gielazyn et al., 2003; Michel and Vincent-Hubert, 2012). Similar concentrations of H_2O_2 as used in our study resulted in increased DNA damage in mussel digestive gland cells and hemocytes (Lee and Steinert, 2003; Mitchelmore et al., 1998).

Varying levels of relative sensitivity have been reported in invertebrates exposed to different contaminants (Table 2, references therein). Many species had higher sensitivity than blue mussel (Table 2). Looking at tissue sensitivity, gill cells were more sensitive than digestive gland and hemolymph cells in bivalves (Rigonato et al., 2005; Pereira et al., 2011). Based on the latter studies and studies in Table 2, it appears, that sensitivity is tissue-specific at a species level and can differ between species within a taxonomic group. Immediate exposure responses were similar in the three sea urchins species studied by El-Bibany et al. (2014; Table 2). However, the species differed in their ability to repair strand breaks indicated by slower recovery in one of the species. These differences suggest that repair capacity could have an important role in species sensitivity to genotoxic compounds (see below). The differences in sensitivity between

mussel hemocytes and fish lymphocytes in the present study are consistent with the results in (Mamaca et al., 2005), who observed a higher sensitivity of mussel hemocytes compared to fish erythrocytes. Contrasting our findings of equal sensitivity in mussel and sea star cells exposed to peroxide, Canty et al. (2009) observed that echinoderm coelomocytes were more sensitive to MMS exposure than bivalve hemocytes. Both MMS and peroxide are direct acting mutagens but through different mechanisms (MMS: alkylating; H_2O_2: oxidising), which may explain the contrasting findings.

Sea urchin coelomocytes appear to be relatively robust to several DNA-damaging agents (Reinardy et al., 2015; El-Bibany et al., 2014) and are less sensitive to H_2O_2 and UV exposure than cells from the sea hare (*Aplysia dactylomela*), a mollusc, and the carribean spiny lobster (*Panulirus argus*), a crustacean (Loram et al., 2012). Our results indicated similar sensitivity of circulating cells from *C. maenas*, *M. edulis* and *A. rubens* at low peroxide concentration. Further studies with lower levels of oxidative stress are required to find the thresholds of impact for those three species.

It is not obvious why cod lymphocytes were less sensitive to oxidative stress than circulating cells from the invertebrate species. One possible explanation is that vertebrate lymphocytes have inherently low levels of DNA damage and are robust against developing DNA strand breaks (Collins et al., 1995; Collins and Azqueta, 2011; Andreoli et al., 1999). Secondly, the partial pressure of oxygen is higher in fish blood than in circulating fluid of the invertebrate species (Abele and Puntarulo, 2004) necessitating a more efficient cellular defence against oxidative stress. Finally, fish lymphocytes have higher general metabolism than circulating cells in invertebrates (Ekau et al., 2010) Higher metabolism will often lead to higher intracellular generation of radicals. Fish cells will therefore require more efficient cellular mechanisms against oxidative stress (see below).

330 Since exposure of the cells were done *in vitro*, factors associated with uptake,
331 bioaccumulation or biotransformation are irrelevant, so the observed differences in
332 sensitivity will relate directly to cellular defence and repair capability (Lewis and
333 Galloway, 2008). Any differences in antioxidant defence contribute to the observed
334 differences in DNA damage levels and species-specific sensitivity.
335 Antioxidant enzyme activities differ for *M. edulis*, *C. maenas* and *A. rubens*, as mussel
336 and sea star had much higher enzymes activities in their digestive gland, respiratory
337 tissue and muscle tissues compared to crab (Gamble et al., 1995). Phylogenetic
338 differences in antioxidant enzymes activities exist and they are generally lower in
339 invertebrates compared to vertebrates (Livingstone et al., 1992). Glutathione
340 peroxidase (GPx), a central enzyme in H_2O_2 detoxification in mammalian cells
341 (Gamble et al., 1995) has generally 1–2 fold lower activities in invertebrates than
342 vertebrates (Livingstone et al., 1992). Enzyme isoforms of GPx exist in chordates that
343 are not found in molluscs, arthropods and echinoderms that could result in phylogenetic
344 differences in the biochemical properties and enzyme efficiency (Bae et al., 2009;
345 Margis et al., 2008). Two other relevant enzymes, superoxide dismutase and catalase,
346 are similarly or more active in invertebrates compared to vertebrates (Livingstone,
347 2001).
348 While cod blood was separated for white blood cells, invertebrate samples constituted
349 whole hemolymph and coelomic fluid. Their circulating fluids comprise a mixture of
350 morphologically distinct cells; ranging from two cell types in mussels to up to eight
351 different cell types in the tunicate (Arizza and Parrinello, 2009; Carballal et al., 1997;
352 Johansson et al., 2000; Pinsino et al., 2007). In all species, however, one cell type is
353 generally numerically dominant. The subpopulations of cells have distinct functions
354 (Hibino et al., 2006; Matranga et al., 2006) and differ probably also in their ability to

sense and repair DNA damage (Loram et al., 2012). Cell-specific sensitivities to DNA damaging agents are present in mussel hemolymph (Venier et al., 1997) and polychaete coelomic fluid (Lewis and Galloway, 2008). Also, cell composition or the number of individual cell types may change as a result from pollution or traumatic events (Hernroth et al., 2010; Matranga et al., 2006; Pinsino et al., 2007). Peroxide exposure increased the numbers of sea urchin amoebocytes (El-Bibany et al., 2014). Further studies may shed light on the susceptibility of individual circulating cell types or implications of cell composition and abundance to DNA damage or repair capacity.

Following 1 hr recovery, DNA damage increased in all species consistent with increased damage in mussel gill cells exposed to peroxide (Dixon et al., 2002). Likely, this is due to accumulation of cellular damage subsequently resulting in DNA strand breaks (Marnett, 2002) as well as primary DNA repair steps (Rastogi et al., 2010; Wilson and Bohr, 2007). The latter have been attributed to increased DNA strand breaks in mussel cells, sea urchin coelomocytes and grass shrimp embryos (Dixon et al., 2002; El-Bibany et al., 2014; Hook et al., 2004).

DNA repair capability is a particularly important determinant of susceptibility (Depledge, 1998). Small differences in the efficiency of DNA repair can result in up to 10-fold differences in susceptibility to genotoxicants (Hemminki et al., 1991). The significant increase of strand breaks in cod lymphocytes could reflect more efficient DNA repair compared to invertebrates. Indeed, fish cells repaired strand breaks efficiently within one to four hours (Nacci et al., 1996). Rainbow trout larvae had high rate of excision repair (Mitchell et al., 2009). Substantial or complete recovery from DNA damage occurred only within a few hours or days in sea urchins and bivalves (Akcha et al., 2000; El-Bibany et al., 2014; Pruski and Dixon, 2003). Sensitivity and repair capacity are also related to developmental stages. Sea urchin larvae were more

sensitive and had lower repair capacity than adult coelomocytes exposed to MMS, H_2O_2 and UVC (Reinardy et al., 2015).

Additionally, species differ in their rate of replacing damaged cells. Echinoderms have low levels of cell proliferation than vertebrates (Dixon et al., 2002; Hernroth et al., 2010; Holm et al., 2008). Different cell proliferation rates may affect species sensitivity to DNA damage due to damage accumulation.

Conclusions

Baseline DNA damage of circulating cells of four invertebrate species from different phyla ranged from 14% to 34% strand breaks. *Ex vivo* 25 μM peroxide exposure for 15 mins caused more than 80% strand breaks in circulating cells from mussel, crab and sea star, and 61% strand breaks in tunicate hemocytes, whereas the treatment gave rise to 29% strand breaks in cod lymphocytes. In conclusion, the invertebrate circulating cells were much more sensitive to oxidative stress than were cells from fish. Recovery capacity of species should be addressed more in future studies as it will lead to a better understanding of the overall susceptibility of marine invertebrates to genotoxic stress.

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692

693 ## Tables

694 **Table 1**. Overview of baseline DNA damage from reference sites or laboratory control reported as mean tail intensity (TI) and other end points:

695 olive tail moment (OTM) or damage (%) using comet assay if not stated differently.

Phyla	Species	% DNA TI	other	Tissue	Method	Reference
Mollusca	*Mytilus edulis*	22%	3.5 OTM	hemocytes		present study
			2.61 OTM	hemocytes		Rank and Jensen, 2003
		25%	5 OTM	hemocytes		Halldórsson et al., 2004
		21%		digestive gland		Large et al., 2002
		14%		hemocytes		AlAmri et al., 2012
			2–4 OTM	gill cells		Rank et al., 2005
			3–4 OTM	gill cells		Rank et al., 2007
			1–2 OTM	hemocytes		Dallas et al., 2013
	Mytilus galloprovincialis	2.5%		hemocytes		Klobučar et al., 2008

		8%		hemocytes		Gomes et al., 2013
		6–10%	5.27 OTM	hemocytes		Almeida et al., 2011
	Cerastoderma edule		1–2 OTM	hemocytes		Dallas et al., 2013
	Tapes semidecussatus	24%		hemocytes		Hartl et al., 2006
Echinodermata	*Asterias rubens*	34%		coelomocytes		present study
		14%		coelomic epithelia cells		Hernroth et al., 2010
			15–25% damage	pyloric caeca	alkaline unwinding elution	Everaarts, 1995
			12–30% damage	pyloric caeca	alkaline unwinding elution	Everaarts and Sarkar, 1996
Arthropoda	*Carcinus maenas*	25%		coelomocytes		present study

	Paleomonetes pugio	~ 24%		embryo cells		Hook and Lee, 2004
	Charybdis japonica	42%			alkaline unwinding elution	Pan and Zhang, 2006
Chordata	*Ciona intestinalis*	17%		hemocytes		present study
	Brotyllus schlosserii		damaged	hemocytes	visual	Ballarin et al., 2008
	Brotyllus schlosserii		damaged		visual	Liu et al., 2006
	Gadus morhua	14%		lymphocytes		present study
				lymphocytes		Fredriksen, 2013
	Limanda limanda	2%		erythrocytes		Hylland et al., 2016
	Melanogrammus aeglefinus	7–50%		erythrocytes		Hylland et al., 2016
	Scophthalmus maximus	11%		erythrocytes		Hartl et al., 2006

696

697

698 **Table 2**: Studies on species sensitivity exposed to various compounds. The studies results were used to calculate a fold increase of exposure vs.
699 control (exposure/control-1) to show the relative sensitivity of the species by comet assay, sister chromatid exchange (SCE) and chromosomal
700 aberration (CA). All exposure concentrations in the respective studies were converted to micro molar (µM). Letters indicate [a] in vivo, [b] in vitro
701 and [c] in situ studies. n.d. = not defined.

Species	Chemical	Concentration	Fold increase	Method	Tissue/cells	Reference
Echinodermata						
Asterias rubens	MMS*	0.16-0.51 µM	3 at 0.16 µM 100% mortality >0.16 µM †	comet assay [a]	coelomocytes	Canty et al., 2009
	CP*	0.07-0.21 µM	1-2	comet assay [a]	coelomocytes	Canty et al., 2009
Lytechinus variegatus	H_2O_2	0-100 µM	0-7	fast micromethod [b]	coelomocytes	El-Bibany et al., 2014
	UVC	0–9999 J/m^2	0-6	fast micromethod [b]	coelomocytes	El-Bibany et al., 2014

Echinometra lucunter lucunter	H_2O_2	0-100 μM	0-8	fast micromethod[b]	coelomocytes	El-Bibany et al., 2014
	UVC	0–9999 J/m^2	0-2	fast micromethod[b]	coelomocytes	El-Bibany et al., 2014
Trippneustes ventricosus	H_2O_2	0-100 μM	0-6.5	fast micromethod[b]	coelomocytes	El-Bibany et al., 2014
	UVC	0–9999 J/m^2	0-7	fast micromethod[b]	coelomocytes	El-Bibany et al., 2014
Mollusca						
Mytilus edulis	MMS	0.16-0.51 μM	6-11	comet assay [a]	hemolymph	Canty et al., 2009
	CP	0.07-0.21 μM	3-6	comet assay [a]	hemolymph	Canty et al., 2009
	MMS	1000 μM	9-15	sister chromatid exchange, chromosomal aberration [a]	embryo-larvae	Jha et al., 2002

	TBTO*	5.4×10^{-4}– 9.4×10^{-3} µM	1- 6	sister chromatid exchange, chromosomal aberration [a]	embryo-larvae	Jha et al., 2002
	H_2O_2	100 µM	0	comet assay [b]	hemolymph	Cheung et al., 2006
	polluted sites	n.d.	1.5-3.5	comet assay [c]	hemolymph	Pereira et al., 2011
	polluted sites	n.d.	0-1	comet assay [c]	hemocytes	Dallas et al., 2013
Cerastoderma edule	H_2O_2	100 µM	0.4	comet assay [b]	hemolymph	Cheung et al., 2006
	polluted sites	n.d.	4-7	comet assay [c]	hemolymph	Pereira et al., 2011
	polluted sites	n.d.	0-1	comet assay [c]	hemocytes	Dallas et al., 2013

Paphia malabarica	EMS*	0.14-0.45 μM	1.5-4.5	comet assay[a]	hemolymph	Kumar et al., 2014
Metrix casta	γ-radiation	2-10 Gy	3-8	comet assay[a]	hemolymph	Kumar et al., 2014
Annelida						
Platynereis dumerilii	MMS	1000 μM	9-22	sister chromatid exchange, chromosomal aberration[a]	embryo-larvae	Jha et al., 2002
	TBTO	5.4×10^{-4}-9.4×10^{-3} μM	1-5	sister chromatid exchange, chromosomal aberration[a]	embryo-larvae	Jha et al., 2002
Arenicola marina	MMS	0.16-0.47 μM	0.8	comet assay [b]	coelomocytes	Lewis and Galloway 2008
Nereis diversicolor	MMS	0.16-0.47 μM	0.7	comet assay[b]	coelomocytes	Lewis and Galloway 2008

Nereis virens	MMS	0.16-0.47 μM	0.3	comet assay[b]	coelomocytes	Lewis and Galloway 2008

† at day 5 of exposure

* MMS = methyl methanesulfonate, CP = cyclophosphamide, TBTO = tributyltin oxide, EMS = ethyl methanesulfonate

Figure legends

Fig. 1. Baseline DNA damage in *M. edulis*, *A. rubens*, *C. maenas*, *C. intestinalis* and *G. morhua*. Letters indicate differences between species ($P < 0.05$; LME model with Wald *F*-test on the fixed effects with *post-hoc* Tukey's multiple comparison). Box plots present median, first and third quartile.

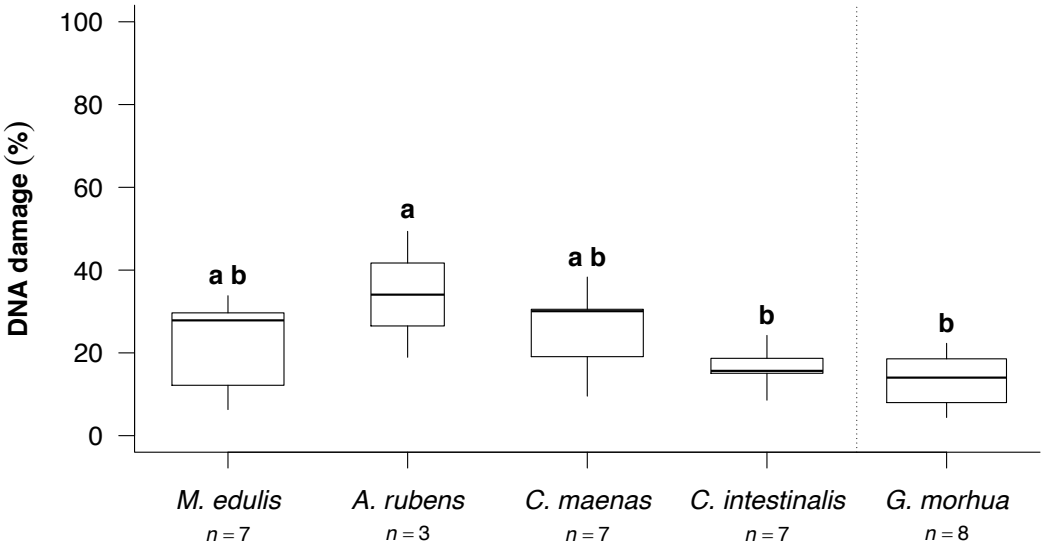
Fig. 2. Hydrogen peroxide induced DNA strand breaks in cells from *M. edulis*, *A. rubens*, *C. maenas*, *C. intestinalis* and *G. morhua*. Letters indicate differences between species ($P < 0.05$; LME model with Wald *F*-test on the fixed effects with *post-hoc* Tukey's multiple comparison). Box plots present median, first and third quartile.

Fig. 3. DNA strand breaks following H_2O_2 exposure and subsequent recovery in cells from *M. edulis, A. rubens*, *C. maenas*, *C. intestinalis* and *G. morhua*. Letters indicate differences between species ($P < 0.05$; LME model with Wald *F*-test on the fixed effects with *post-hoc* Tukey's multiple comparison). Box plots present median, first and third quartile.

Fig. 4. Total DNA strand breaks in cells from *M. edulis*, *A. rubens*, *C. maenas*, *C. intestinalis* and *G. morhua*, measured using the FPG-amended comet assay. Letters indicate differences between species ($P < 0.05$; LME model with Wald *F*-test on the fixed effects with *post-hoc* Tukey's multiple comparison). Box plots present median, first and third quartile.

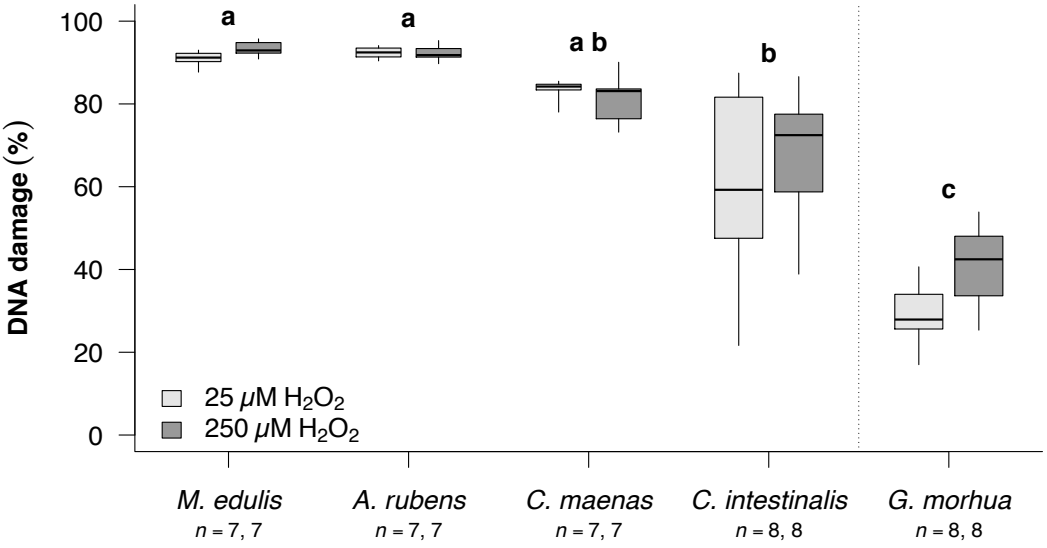
726 **Figures**

727 **Fig. 1.**



728

729 **Fig. 2.**

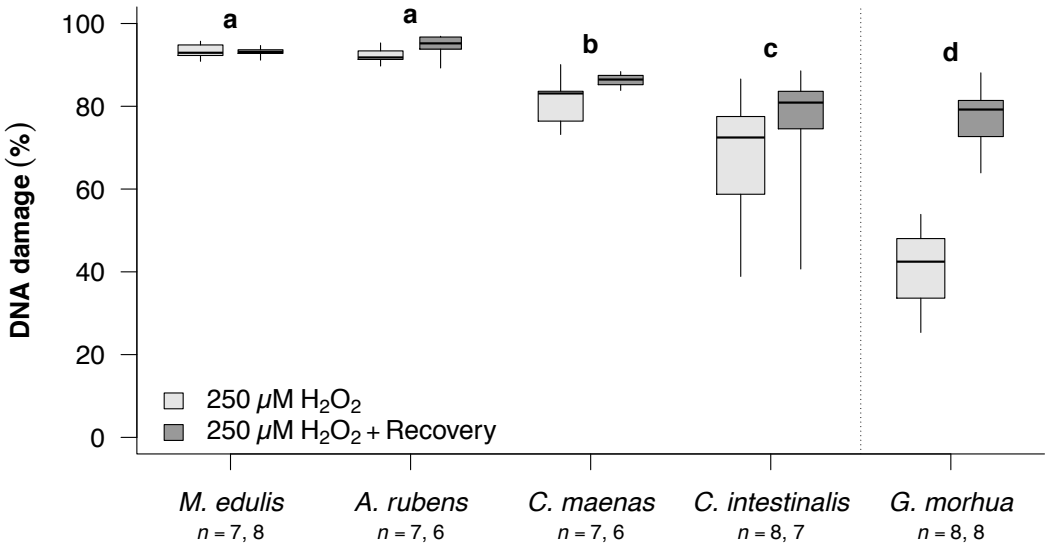


730

731

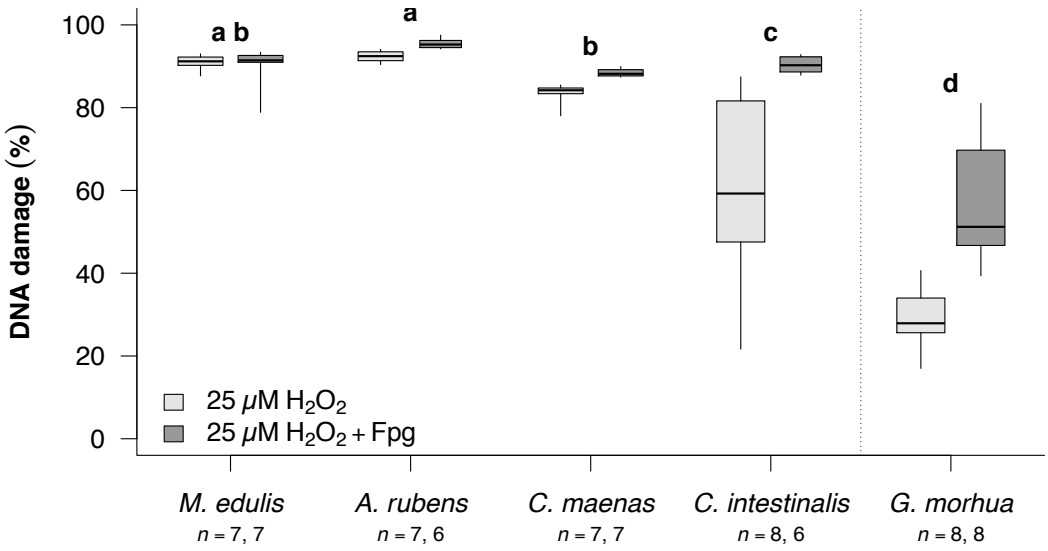
732 **Fig. 3.**

733



734

735 **Fig. 4.**



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