

# DNA strand breaks in marine invertebrates

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## Abbreviations

8-oxoG	Oxo-7, 8-dihydro-2'-deoxyguanosine
BaP	Benzo[a]pyrene
Cu(SO <sub>4</sub> <sup>2-</sup> )	Cu used for copper, Cu(SO <sub>4</sub> <sup>2-</sup> ) used for copper sulfate
CAT	Catalase
DSB	Double strand break
DBT	Dibenzothiophene
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
Fpg	Formamidopyrimidine glycosylase
GPx	Gluthathione peroxidase
GSH	Glutathione
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
MMS	methanesulfonate
MN	Micronucleus
OECD	The Organisation for Economic Co-operation and Development
OGG1	8-oxoguanine DNA glycosylase 1
ROS	Reactive oxygen species
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyl
PBS	Phosphate buffer saline
PHE	Phenanthrene
SCGE	Single cell gel electrophoresis
SSB	Single strand break
SOD	Superoxide dismutase

## Summary

In their natural environment, marine organisms are exposed to a wide range of chemical and physical factors that can damage their genetic material. Polycyclic aromatic hydrocarbons (PAHs) and trace metals (e.g. copper) are chemicals that are known to have such effects on marine organisms, either directly or indirectly. Arguably the most important mechanisms for toxicity are chemical interactions of contaminants, their metabolites or reactive intermediates with nucleic acids, as this can potentially cause genotoxicity. Damage to DNA can ultimately cause cell death, mutations and carcinogenesis, which may not only affect the individual, but can be transferred to offspring, with potential transgenerational consequences. A major reason for the genotoxicity of some contaminants is the generation of radical oxygen species (ROS), either directly or as a consequence of cellular processing or detoxification.

This thesis aimed to elucidate how environmental stressors in the form of oxidative stress, low-molecular-weight PAHs and metals, affect DNA strand breaks and recovery from such damage in marine invertebrates. The alkaline single cell gel electrophoresis or comet assay was the method used to quantify DNA strand breaks. Baseline levels of DNA strand breaks were determined in hemocytes and coelomocytes of blue mussel (*Mytilus edulis*), shore crab (*Carcinus maenas*), vase tunicate (*Ciona intestinalis*) and common starfish (*Asterias rubens*). The sensitivity to oxidative stress in the same cells and species was assessed following exposure to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Lymphocytes from Atlantic cod (*Gadus morhua*) were used as a reference. Then, the genotoxic potential of the low-molecular-weight PAHs phenanthrene and dibenzothiophene was examined *in vitro* in hemocytes from *M. edulis* and *C. maenas* and coelomocytes from *A. rubens*. Finally, *in vivo* copper genotoxicity and mortality was explored in the calanoid copepods *Acartia tonsa*, *Temora longicornis* and the harpacticoid copepod *Tigriopus brevicornis*.

The results demonstrated species-dependent differences in the sensitivity of circulating cells to oxidative stress. Invertebrate circulating cells were more susceptible to oxidative stress than cod lymphocytes (**paper I**). Oxidative stress responses in **paper II** indicate that coelomocytes from *Asterias rubens* were more susceptible than hemocytes from *Mytilus edulis* and *Carcinus maenas* (**paper I**). Low-molecular-weight PAHs exposure resulted in a weak genotoxic effect of phenanthrene to *Asterias rubens* coelomocytes and *Mytilus edulis* hemocytes, but not to *Carcinus maenas* hemocytes. Dibenzothiophene was not genotoxic to cells from any of the examined species (**paper II**). The three copepods *Acartia tonsa*, *Temora longicornis* and *Tigriopus brevicornis* differed significantly in their apparent genotoxic sensitivity to copper exposure, with

*T. brevicornis* being more robust than the other two species, but there were no clear links between Cu exposure and DNA strand breaks in any of the species (**paper III**).

This thesis exhibited the sensitivity of invertebrate hemocytes/coelomocytes as model systems in genotoxicity studies, including challenges and advantages in their applicability. The thesis highlights the potential of oxidative stress, low molecular weight PAHs and copper to cause DNA strand breaks in circulating cells and differences in sensitivity between species.

## List of Papers

This thesis is based on the following papers which will be referred to in the text as their Roman numerals.

**Paper I:** Andrea Sahlmann, Raoul Wolf, Tor Fredrik Holth, Josefin Titelman & Ketil Hylland, 2017. Baseline and oxidative DNA damage in marine invertebrates. *Journal of Toxicology and Environmental Health, Part A*, 80:16-18, 807-819, DOI:[10.1080/15287394.2017.1352179](https://doi.org/10.1080/15287394.2017.1352179)

**Paper II:** Andrea Sahlmann, Raoul Wolf, Ane Haarr, Tor Fredrik Holth, Tom Andersen, Josefin Titelman, Ketil Hylland, 2019. Genotoxicity of low-molecular-weight polycyclic aromatic hydrocarbons in marine invertebrate circulating cells. *Toxicology – in Vitro*, submitted.

**Paper III:** Andrea Sahlmann, Torben Lode, Jan Heuschele, Katrine Borgå, Josefin Titelman, Ketil Hylland, 2019. Genotoxic response and mortality of three marine copepod species exposed to waterborne copper. *Environmental Toxicology & Chemistry*, submitted.



## Introduction

In the past decades, there has been growing concern about the increasing number of environmental pollutants and their potential to interact with and damage the genes of marine organisms. The integrity and stability of the DNA molecule is essential, as it is the source of genetic information in each living cell (Martins and Costa, 2015). However, the DNA molecule is under constant attack from various physical and/or chemical agents present in the environment, both naturally and as a result of human activity. Chemical interference with DNA can result in the accumulation of lesions that can cause metabolic dysfunction, cell death (apoptosis) or fixation of mutations, which may lead to cancer. While human toxicology focuses on the fate of the individual, genetic ecotoxicology assesses the potential of environmental pollution to cause genotoxic effects on populations (Shugart and Theodorakis, 1998). DNA damaging agents may have long-lasting consequences, including embryonal aberrations (Barranger et al., 2014), reduced hatching rates and or overall reduced fitness (Lee et al., 2012; Linhartova et al., 2013), and may impact future generations if germ cells are affected (Lacaze, Geffard, et al., 2011).

Chemical interactions of genotoxicants with DNA include covalent binding to the DNA molecule, alteration of bases or DNA strand break formation through structural changes in DNA, addition or deletion, rearrangement or unequal chromosome division (Devaux et al., 1997; Lindahl and Wood, 1999). Adduct formation occurs when a genotoxicant, such as PAHs or their metabolites, binds directly to the DNA backbone and distorts the helix structure, preventing transcription, potentially leading to mutations (Friedberg et al., 2005). Another main source of damage is DNA modifications due to reactive oxygen species (ROS) such as superoxide anion radical ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ ) and hydrogen peroxide ( $H_2O_2$ ) (Fig. 1) (Dizdaroglu et al., 2002; Lesser, 2006). ROS are produced during normal cell metabolism involving oxygen (e.g. mitochondrial respiration) and are essential components of cell signalling pathways (reviewed by Yu et al., 2016). Exposure to environmental genotoxicants e.g. halogenated hydrocarbons (PCBs), PAHs (e.g. benzo(a)pyrene, phenanthrene) and trace metals e.g. cadmium (Cd), mercury (Hg) and copper (Cu) (Coteur et al., 2004; Giannapas et al., 2012; Zeeshan et al., 2016) may increase intracellular ROS to potentially toxic levels (Fig. 1). Cu and  $H_2O_2$  can damage DNA base associated sugar (Mitchelmore et al., 1998; Tkeshelashvili et al., 1991). The  $\cdot OH$  radical is considered as the ultimate radical to cause DNA damage, due to its high reactivity and the lack of enzymatical detoxification (Matros et al., 2015).

Intracellular ROS levels are usually kept in a physiological steady-state homeostasis through the complex action of non-enzymatic and enzymatic cellular mechanisms. This includes radical

scavengers (e.g. glutathione, vitamin C, vitamin E/tocopherol), antioxidant proteins such as metallothionein and antioxidant enzymes, such as superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase (Azqueta et al., 2009). Oxidative stress occurs when internal or external stress (e.g. pollutants) increases ROS to levels exceeding the antioxidant capacity (Lushchak, 2011), potentially causing peroxidation of lipids, oxidation of proteins and damage to DNA (Fig. 1), as well as disrupting the cellular redox balance, e.g. through changing the ratio of reduced to oxidized glutathione (Abele and Puntarulo, 2004).

DNA damage in cells will generally be recognized and repaired continuously by DNA and cellular repair enzymes. Several pathways of DNA repair mechanisms have been described that are more or less specific for different types of lesion within the DNA molecule (Lindahl and Wood, 1999; Collins and Gaivão, 2007). The range of DNA repair mechanisms include base excision repair (BER) and nucleotide excision repair (NER) (Fig. 1), double strand break repair, homologous recombination (HR), non-homologous end-joining and mismatch repair (Lindahl and Wood, 1999). DNA repair is a crucial step in maintaining error-free replication and limiting endogenous and exogenous induced damage. Small changes in DNA repair efficiency can alter the susceptibility of a species to a genotoxicant (El-Bibany et al., 2014). BER and NER repair are major mechanisms that are evolutionarily preserved across phyla (Taylor and Lehmann, 1998). The efficiency of BER and NER is lower in invertebrate and fish cells as compared to mammalian cells (Dixon et al., 2002; Kienzler et al., 2013).



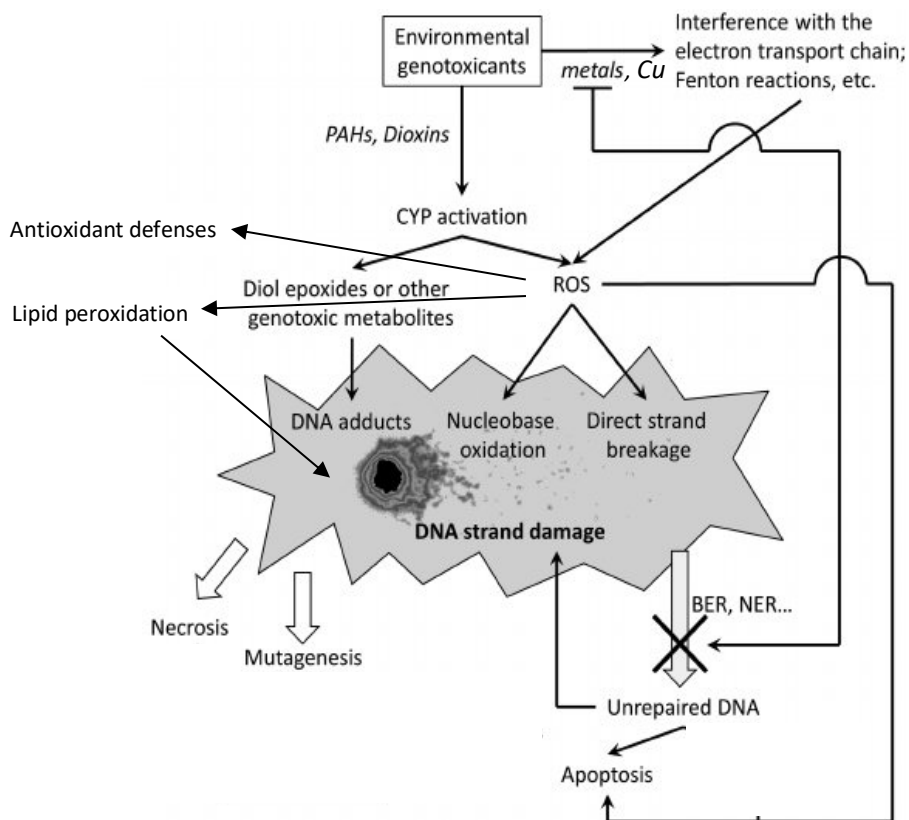


Fig. 1 Simplified diagram of the molecular mechanisms potentially linking DNA strand breaks with PAH and metal exposure. Adapted from Martins and Costa (2015).

## Genotoxins in the marine environment

Any chemical that in itself or its metabolites is either electrophilic, thus reacting with nucleophilic DNA components, or generates ROS or other reactive intermediates, e.g. epoxides, diols or quinones, during biotransformation or detoxification processes, has genotoxic potential (Fig. 1) (Gates, 2009). While contaminant-related oxidative stress is of concern, increased oxidative stress and related DNA damage can also be produced following exposure to other sources of environmental stress, such as environmental hypoxia and hyperoxia, increased temperature, changed salinity or elevated UV radiation (Liu et al., 2007; Lushchak et al., 2001; Verlecar et al., 2007; Kammerlander et al., 2017).

Numerous chemicals may elicit genotoxicity, including pesticides and metals (Mai et al., 2012), biocides (Kwok and Leung, 2005), PAHs (Large et al., 2002), dioxins (Nigro et al., 2002), nanoparticles (Al-Subiai et al., 2012) and brominated flame retardants (Lee et al., 2012). Chemicals may also interact with other environmental factors. Decreased hatching rate and DNA

damage was associated with genotoxic and developmental effects of UV-exposed brominated flame retardant in grass shrimp (*Palaemonetes pugio*) embryos (Lee et al., 2012). Environmentally relevant concentrations of two pesticides, irgarol and metolachlor, were found to be embryotoxic and genotoxic to Pacific oyster (*Crassostera gigas*) larvae (Mai et al., 2012). DNA damage in mussel hemocytes was related to exposure to copper oxide and silver nanoparticles (Gomes et al., 2013).

Polycyclic aromatic hydrocarbons (PAHs) constitute a large group of substances composed of two or more aromatic rings (e.g. benzene rings). Heterocyclic PAHs contain oxygen, nitrogen and sulphur substitutes for one or more carbons in the ring structure. Important sources of PAHs to the marine environment include atmospheric fallout, oil spills, offshore activities, run-off from land, as well as industrial effluents and domestic sewage (Hylland et al., 2006). PAH pollution poses a significant risk to marine life and especially to coastal environments where anthropogenic pressure is high, and oil spills most often occur (Fig. 2). A large body of research studies report on the biological and genotoxic effect of particularly high molecular weight PAHs (more than three aromatic rings), such as benzo[a]pyrene (Hylland et al., 2006). Low molecular weight PAHs, such as phenanthrene and dibenzothiophene, contribute significantly to the environmental risk of produced water from offshore oil platforms and the water-soluble fraction of crude oil (Brinkmann et al., 2014; Radovic et al., 2012). The genotoxicity of low molecular weight PAHs, in particular to marine organisms, is however less studied than that of other PAHs (Wang et al., 2017).

Exposure to PAHs may result in carcinogenicity, mutagenicity and immune suppression (Sen 2013, Martins and Costa 2013, Hannam 2010). Biotransformation of PAHs may lead to detoxification but also re-toxication, as reactive metabolites such as epoxides, quinones and diols can be produced, which can enter redox cycling (Xue and Warshawsky, 2005). PAH metabolism occurs via the cytochrome P450-mediated mixed function oxidase (CYP450) system with oxidation or hydroxylation (Phase I) and subsequent conjugation with functional groups (phase II) to more soluble compounds (Rewitz et al., 2006). Conjugation of metabolites with glutathione may result in the consumption of GSH, which can decrease the antioxidant defence capacity (Hannam, Bamber, Galloway, et al., 2010) or the activity of glutathione *S*-transferase may generate reactive metabolites (Speisky et al., 2009). Cytochrome (CYP) enzyme activity is regularly moderated through the nuclear aryl hydrocarbon receptor (AhR) (Tompkins and Wallace, 2007). PAH-mediated ROS generation is another major mechanism through which these compounds may cause deleterious effects, including DNA damage (Giannapas et al., 2012).

Essential trace metals, such as chromium, cobalt, copper, iron, magnesium, selenium and zinc, are, by definition, crucial in nutrition and physiology (Coleman, 1992). Iron is essential for oxygen

transport in human and many marine organism blood cells. Zinc is a co-factor for over 100 enzymes, mainly in protein and amino acid metabolism (Tubek et al., 2008). Copper is similarly an important co-factor for many enzymes (Linder and Hazegh-Azam, 1996). Non-essential trace metals, such as plutonium, mercury and lead, are taken up by organisms but have no known physiological function (Singh et al., 2011).

Copper is essential for a large number of metabolic and antioxidant defence enzymes, such as the cytochrome oxidase enzyme complex in the mitochondrial respiratory chain and copper-zinc superoxide dismutase (Horn and Barrientos, 2008). The functional role of copper in enzymes is mainly electron transfer and binding oxygen molecules (Li et al., 1996). In some invertebrates, including many molluscs and malacostracan crustaceans, the iron-based blood pigment hemoglobin is replaced by the copper-complexed hemocyanin pigment (Markl 2013). Copper, like e.g. chromium, iron, manganese, is redox active, meaning it occurs in the divalent Cu(II) or monovalent form Cu(I) and is thus an important catalytic co-factors in redox reactions (Camakaris et al., 1999). This redox chemistry via a Fenton and Haber-Weiss-like reaction can result in the generation of ROS (Stohs and Bagchi, 1995; Letelier et al., 2005).

Organisms in coastal areas are exposed to higher trace metal concentrations than organisms in the open oceans (Thomas and Brooks, 2010). All aquatic organisms take up trace metals, whether essential or not (Rainbow, 2002). Different strategies for regulating internal concentrations of essential trace metals such as copper exist in all living organisms and includes active regulation and/or sequestration and storage by the metal-protein metallothionein or in metal-containing granules (Brix et al., 2001; Rainbow, 2007). Together with balanced antioxidation, e.g. through glutathione-complexation, this ensures a physiological homeostatic state of essential trace metals.

When surpassing a threshold bioavailability, trace metals are potentially toxic. Factors affecting toxicity include those influencing metal uptake and those affecting the organism's ability to handle and detoxify accumulated trace metals (Rainbow et al., 1990). Exposure to high concentrations of copper may cause membrane dysfunction (Viarengo et al., 1996), lipid peroxidation (Barata et al., 2005) and disruption of osmoregulatory mechanisms (Bjerregaard and Vislie, 1986). Copper binds to sulfhydryl groups of membrane proteins and enzymes, such as Na<sup>+</sup>/K<sup>+</sup>-ATPases, disrupting vital energy-metabolism (Li et al., 1996).

Copper can also be genotoxic, as documented from DNA strand break induction in e.g. bivalves, fish and polychaetes (Bopp et al., 2008; Caldwell et al., 2011; Santos et al., 2010; Xu et al., 2018). Increased incidences of micronuclei in the cladoceran *Daphnia magna* (Barka et al., 2016) and induction of DNA damage- and repair-related genes in fish gills (Oliveira et al., 2014) and

copepods (Rhee et al., 2012) were reported following copper exposure. This copper genotoxicity is often due to redox-related ROS formation leading to oxidative stress.

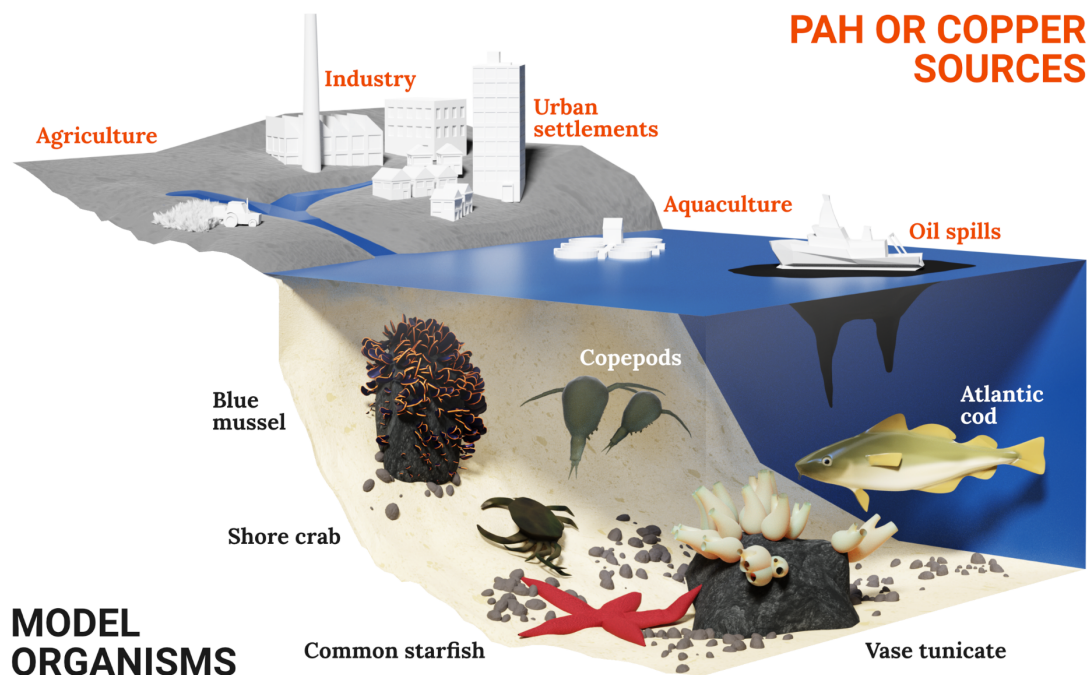


Fig. 2 Overview of model species and main pollutant sources of contaminants used in this thesis. Image: Jan Heuschele.

## Marine invertebrates and genotoxicity

Invertebrate species make up to 95% of the world's animals' diversity (Beatty and Blackwelder, 1974). Invertebrates make up key populations in all ecosystems, particularly marine ones, playing pivotal roles in the food chain and ecosystem functioning (Wilson, 1987).

Early studies in ecotoxicity documented that invertebrates are affected by genotoxic contaminants, based on the presence of similar types of DNA and chromosomal damage to those found in higher organisms, e.g. point mutation, adduct formation, strand breaks and chromosomal aberration (see early reviews by Dixon et al., 2002; Mitchelmore and Chipman, 1998). Nacci et al. (1992) reported increased DNA damage in the mussel *Mytilus edulis* and the oyster *Crassostrea virginica* from contaminated urban estuaries and in laboratory exposures to the model genotoxicant *n*-methyl-*n*-nitro-nitrosoguanidine (MMNG), which alkylates DNA bases and leads to transition mutations (Nacci et al., 1996). Other studies found micronucleus formation following benzo(a)pyrene-exposure in bivalve hemocytes and gill cells (Venier et al., 1997) and increased levels of DNA strand breaks following metal exposure (Cd and Cu) in the mussel *M. edulis* (Bolognesi et al., 1999).

Differences in physiology and life strategies of invertebrates may increase or decrease their sensitivity to genotoxicant exposure in comparison to fish or mammalian vertebrates. Phylogenetic differences in e.g. bioaccumulation (Amiard et al., 1987), biotransformation, antioxidative capacity (Gamble et al., 1995) and DNA repair ability may affect genotoxicant/contaminant susceptibility between fish and invertebrates, and also between invertebrates (Dixon et al., 2002; Frenzilli et al., 2009).

Fish maintain a stable internal osmolarity (osmoregulators), while most invertebrates maintain osmolarity close to that of the surrounding environment (osmoconformers). Although biotransformation of e.g. PAHs occurs in all species to some extent, the biotransformation activity and efficiency are lower in invertebrates (e.g. crustaceans and mussel) than in vertebrates (e.g. fish) (Livingstone, 1998; Solé and Livingstone, 2005), but also varies between invertebrates and between cells and tissues within species (Grundy, Ratcliffe, et al., 1996; Livingstone, 1998; McElroy et al., 2000). Many invertebrates have an open circulatory system with hemolymph containing hemocytes or coelomic fluid with coelomocytes as equivalents to vertebrate blood cells. Hemolymph/coelomic fluid is generally pumped into a body cavity, where the fluid surrounds tissues, before the fluid diffuses back to the circulatory system between cells. Following absorption via gills or skin, contaminants are transported to tissues by the hemolymph and coelomic fluid. Hence, hemocytes and coelomocytes are exposed to genotoxicants very soon after absorption (Hannam, Bamber, Galloway, et al., 2010; Pan et al., 2006).

There are inter-species differences in the vulnerability of different invertebrates to genotoxicant exposure (Han et al., 2016; Lewis and Galloway, 2008; El-Bibany et al., 2014). The number of invertebrate species used in genotoxicity studies has expanded in the last few years (Martins and Costa, 2015). The vast majority of studies have focused on bivalves, especially mussels, however species from other phyla used in genotoxicity studies include echinoderms (Reinardy and Bodnar, 2015; El-Bibany et al., 2014; Everaarts and Sarkar, 1996), crustaceans (Hook and Lee, 2004, **paper I-III**), polychaetes (Lewis and Galloway, 2008), cnidarians (Mitchelmore and Hyatt, 2004) and ascidians (Ballarin et al., 2008, **paper I**) and more (reviewed in Gajski et al., 2019).



## Objectives

The objective of this PhD thesis was to quantify natural and chemical-induced DNA strand breaks in pelagic and benthic marine invertebrates using *ex vivo* and *in vivo* exposures. H<sub>2</sub>O<sub>2</sub> was used as a model substance to induce oxidative stress to elucidate the relative sensitivity of each model species.

More specifically, the aims were to:

- Quantify baseline levels of DNA strand breaks in circulating cells from selected marine invertebrates and their relative susceptibility to *ex vivo* oxidative stress. **(papers I-II)**
- Clarify the genotoxicity, measured as DNA strand breaks, of the low-molecular-weight PAHs phenanthrene and dibenzothiophene to circulating cells from selected marine invertebrates following short-term, *ex vivo* exposure. **(paper II)**
- Clarify the genotoxicity, measured as strand breaks, of environmentally relevant concentrations of copper to three copepods. **(paper III)**
- Evaluate circulating cells from marine invertebrates as models for genotoxicity studies. **(papers I-II)**
- Evaluate the comet assay for studying genotoxicity *ex vivo* and *in vivo* in marine invertebrates. **(papers I-III)**





## Methods

### Study species

In **paper I** and **II** the blue mussel *Mytilus edulis*, the common starfish *Asterias rubens* and the shore crab *Carcinus maenas* were chosen models as species represent ecologically and functionally relevant taxonomic groups that are widely found in North Atlantic coastal ecosystems. All three are prominent species in tidal and subtidal habitats, with *M. edulis* representing an abundant filter-feeder and both *A. rubens* and *C. maenas* as important benthic predators. *M. edulis* bioaccumulates a wide range of marine pollutants, including PAHs and metals (Grundy, Moore, et al., 1996; Namiesnik et al., 2008; Rank et al., 2005). Mussel hemocytes are probably the best studied circulating cells regarding contaminant-related cellular and molecular mechanisms (Cajaraville and Pal, 1995; Pipe et al., 1997; Santarém et al., 1994). Previous studies have reported *A. rubens* and *C. maenas* to show pollution-induced DNA damage due to field contamination (Everaarts, 1997; Fossi et al., 1996; Maria et al., 2009) and laboratory exposure to numerous contaminants, including pharmaceuticals and model genotoxicants (Fossi et al., 2000; Aguirre-Martínez et al., 2013; Canty et al., 2009).

Ascidians, such as the solitary living vase tunicate *Ciona intestinalis*, can often be found in man-made environments, such as harbours and marinas, and have evolutionary a close relationship to vertebrates as the closest invertebrate relative (Delsuc et al., 2006). The urochordate *Ciona intestinalis* (**paper I**) has been used to study the role and activation of DNA damage and oxidative stress-related genes and enzymes (p53 and AP endonucleases, Hsp70) (Satoh, 2003). While DNA strand breaks were qualitatively investigated (comet assay) in an immunological study (Liu et al., 2006), pollution-related DNA damage has not been studied.

Copepods are possibly the most abundant organism on the planet (Huys and Boxshall, 1993) and are crucial in marine food webs through their functions as predators, prey and energy transfer vehicles (Ruppert et al., 2004). The copepods *Temora longicornis*, *Acartia tonsa*, and *Tigriopus brevicornis* (**paper III**) are key species of the zooplankton along the North Atlantic coast. *A. tonsa* and *T. brevicornis* are widely used model species in life-cycle and acute toxicity tests (OECD, 2006; Raisuddin et al., 2007). There are only few studies that studied pollutant-related DNA strand break induction in copepods (Barka et al., 2016; Charry et al., 2018; Ternjej et al., 2009). While acute copper toxicity has been extensively investigated in copepods previously, the potential of this essential metal to induce DNA strand breaks in copepods has not been explored so far.

## Exposure systems

Measuring the effect of genotoxic exposure to native species in a field (*in situ*) or semi-field (mesocosms or on-site processing) setting will give the most relevant information ecologically. This way, genotoxicant-induced DNA damage of realistic and complex exposure situations may be assessed in comparison to an uncontaminated environment or reference sites (Halldórsson et al., 2004; Rank et al., 2005; Amat, Pfohl-Leszkowicz, Burgeot, et al., 2004). Controlling for confounding factors or deciphering contaminant-specific mechanisms is, however, often challenging in a field setting (Borràs and Nadal, 2004).

Controlled *in vivo* exposure in the laboratory are useful to measure genotoxic effects in whole organisms, taking into account internal complex biological processes such as chemical uptake, biotransformation and excretion. In **paper III**, copepods were exposed *in vivo* to copper under static laboratory conditions to study copper-mediated DNA damage and copper-related mortality.

Reassessing the use of living organisms in toxicology by the scope of the three R approach (replacement, refinement and reduction), *in vitro* studies on cell cultures are useful alternatives by which to investigate cellular mechanisms of toxicity. Although not reflecting the complexity of the biochemical interactions occurring within a living organism, cellular mechanisms can be investigated more specifically when using a single cell type. Also, cell cultures are often more cost-effective (Rinkevich, 2005). Within this thesis, primary cells (**papers I-II**) freshly isolated from living organisms (*ex vivo*) were used, as they are often more sensitive, have a higher metabolic capacity and thus reflect the *in vivo* state better compared to immortalised cell lines (Rinkevich, 2005). Also, there are no available cell-lines for marine invertebrates (Cai and Zhang, 2014; Yoshino et al., 2013).

Isolated cells from tissue (e.g. gill and hepatocytes/digestive gland cells) are widely used model systems, with sensitivity to e.g. model genotoxicants ( $H_2O_2$ , BaP, MMS), organic contaminants (PAHs, PCBs), metals and other emerging contaminants in genotoxicity studies (Michel and Vincent-Hubert, 2012; Mitchelmore et al., 1998; Pérez-Cadahía et al., 2004; Vincent-Hubert et al., 2011). Plazar et al. (2007) exposed slices of rat and human tissues to genotoxicants. Cell isolation and cell dissociation procedures pose a source for artificial stress to circulating cells (Azqueta and Collins, 2013). Microcentrifuge tubes are commonly used to expose e.g. hemocytes to chemicals. The exposure chemical is usually removed by centrifugation with subsequent washing and resuspension of hemocytes for further analysis.

While applicable to human blood cells (Jackson et al., 2013), cryopreservation of invertebrate hemocytes is not feasible at this time, due to freezing-related loss of DNA integrity using current

protocols (Azqueta and Collins, 2013). Kwok et al. (2013) suggested possible cryopreservation of mussel hemocytes, but chemically exposed cells showed reduced DNA integrity due to the cryopreservation. However, the authors concluded that this did not impair the ability to identify differences between treatments. In my opinion, the measured DNA strand breaks using this approach could only be used qualitatively.

In **papers I-II**, freshly isolated invertebrate hemocytes/coelomocytes (*ex vivo*) from field sampled organisms were exposed to H<sub>2</sub>O<sub>2</sub> and the low molecular weight PAHs phenanthrene and dibenzothiophene. Hemocytes and coelomocytes are involved in essential functions such as nutrient and oxygen transport, immunity and wound healing (Matranga et al., 2005). Hemocytes are also useful model systems for studying genotoxicity - because hemocytes are easy to retrieve and already present as cell suspension (Frenzilli et al., 2009) - a prerequisite for assays such as the comet assay. After cell extraction, cells embedded in agarose gel were exposed to the selected genotoxicants before subsequent comet procedure (see the following paragraph), thereby reducing steps of cell handling, i.e. cell washing and centrifugation.

Osmotically adjusted phosphate buffered saline (PBS) was the common buffer for all cells in all studies (**papers I-III**). *A. rubens*, *M. edulis*, *C. maenas* and *C. intestinalis* are osmoconformers or poor osmoregulators. Thus, osmolality was measured and PBS modified for each species.

Extracting cells from *A. rubens* was most challenging and some cell aggregation was observed (**papers I-II**). Aggregated cells were separated by gently pipetting with a cut pipette tip. Embedding cells quickly after extraction and exposing them in-gel ensured fast processing and reduced the risk of cell aggregation, especially for cells from *A. rubens*.

In **paper II**, cells were exposed while embedded in gel. To ensure acceptable cell viability and baseline damage in embedded cells, different media (L-15 medium with/without fetal calf serum; PBS with/without EDTA) and treatment periods were tested. PBS with EDTA and a total treatment period of four hours, with two hours exposure and one hour and two hours recovery, were found to be most suitable.

*A. rubens*, *M. edulis*, *C. maenas* and *C. intestinalis* have different circulating cells: ranging from two cell types in mussels to up to eight different cell types in tunicate (see Table 1 and references therein).

Table 1: Circulating cell types of the species studied

Species	Cell types	Reference
<i>Asterias rubens</i>	Phagocytes, amoebocytes, vibratile cells, hemocytes	Pinsino et al. (2007)
<i>Mytilus edulis</i>	Hyalinocytes, granulocytes	Carballal et al. (1997)
<i>Carcinus maenas</i>	Hyalinocytes, semigranulocytes, granulocytes	Johansson et al. (2000)
<i>Ciona intestinalis</i>	Hemoblast, circulantin hyaline amebocytes, small and large granulocytes, morula cells, unique refractile granulocytes, compartment and signet ring cells	Arizza and Parrinello (2009)

Response variability to genotoxicants has been related to cell-specific susceptibility (Lewis and Galloway, 2008). Thus, separation of specific cell populations might be preferable in order to identify specific responses to contaminant exposure (Carballal et al., 1997; Johansson et al., 2000; Pinsino et al., 2007). Percoll discontinuous density gradient separation was useful to separate for subpopulation of mussel and crab hemocytes (**paper II**) and cod lymphocytes (**paper I**). Similarly to Hernroth et al. (2010) and Pinsino et al. (2007), a satisfactory and reproducible separation of the coelomocytes of *Asterias rubens* was not achieved. Therefore, whole coelomic fluid was used in both **papers I and II**.

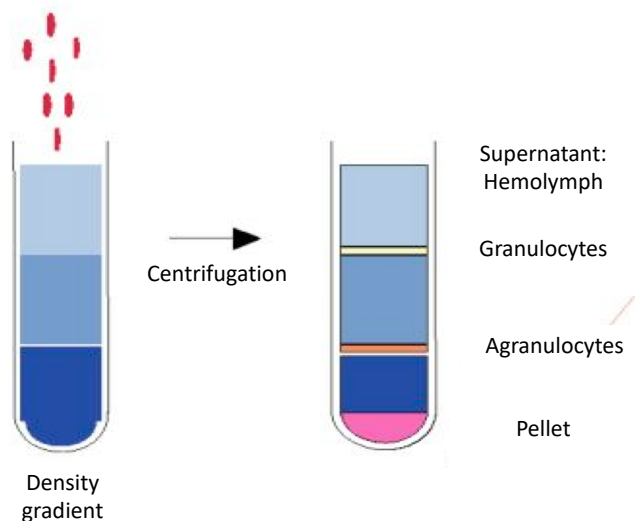


Fig 3. Overview of cell separation based on Percoll density gradient separation.

## Measurement of DNA damage

Most methods measuring DNA damage derive from mammalian systems and adaptation to aquatic organisms might be challenging (Martins and Costa, 2015). Changes on chromosome level following DNA breakage can be investigated by the sister chromatid- and micronucleus (MN) assay (Shugart, 2000). Sister chromatid exchange was used in embryo-larvae of *M. edulis* (Jha et al., 2000). Micronucleus formation is the consequence of chromosome fragments or whole chromosomes that are not incorporated into a daughter nucleus. In such cases, error-free cell division is impaired due to unrepaired DNA lesions (Fenech et al., 2011). Siu et al. (2004) used MN formation to detect Benzo[a]pyrene genotoxicity in hemocytes of the green-lipped mussel (*Perna viridis*). The formation of PAH-derived DNA adducts was investigated in mussel gill and digestive gland cells by the <sup>32</sup>P- postlabelling assay (Dolcetti et al., 2002). Nacci et al. (1992) and Bihari et al. (1992) used alkaline unwinding and alkaline elution assay to detect environmental pollution- as well as Benzo[a]pyrene and 4-Nitroquinoline 1-oxide-related strand breaks. Both, alkaline unwinding and alkaline elution measure DNA strand breaks based on the separation of double-stranded DNA, assessed by centrifugation/filtration or by the rate of denaturation and the incorporation of a fluorescent dye in double-stranded DNA (Bihari et al., 1992; Kohn et al., 1976). DNA damage was fluorometrically measured in mussel (*M. galloprovincialis*) gill and digestive gland cells and sea urchin coelomocytes by the Fast Micromethod (Jakšić and Batel, 2003; El-Bibany et al., 2014). Persistent organic pollutant-derived DNA strand breaks were detected by the agarose plug electrophoresis technique in the common eider (*Somateria molissima*) (Fenstad et al., 2014). Based on size-dependent separation, DNA fragments released from cells embedded in agarose plugs are electrophoretically separated by size by including a molecular size marker (Theodorakis et al., 1994; Krøkje et al., 2006).

### *Comet assay*

In the present thesis, the comet assay developed by Singh et al. (1988) and modified by Gutzkow et al. (2013) was used for the detection of DNA strand breaks (**papers I-III**). It has become one of the most popular tests for detecting strand breaks, because of its simplicity, sensitivity and as a quantitative method to study DNA strand breaks on a single-cell level (as in contrast to the above-mentioned assays). It is applicable to a wide range of eukaryotic cells (Frenzilli and Lyons, 2013; Langie et al., 2015; Lee and Steinert, 2003). The comet assay can detect a broad range of DNA damage, including single and double DNA strand breaks, alkali-labile sites, base damages and cross-links.

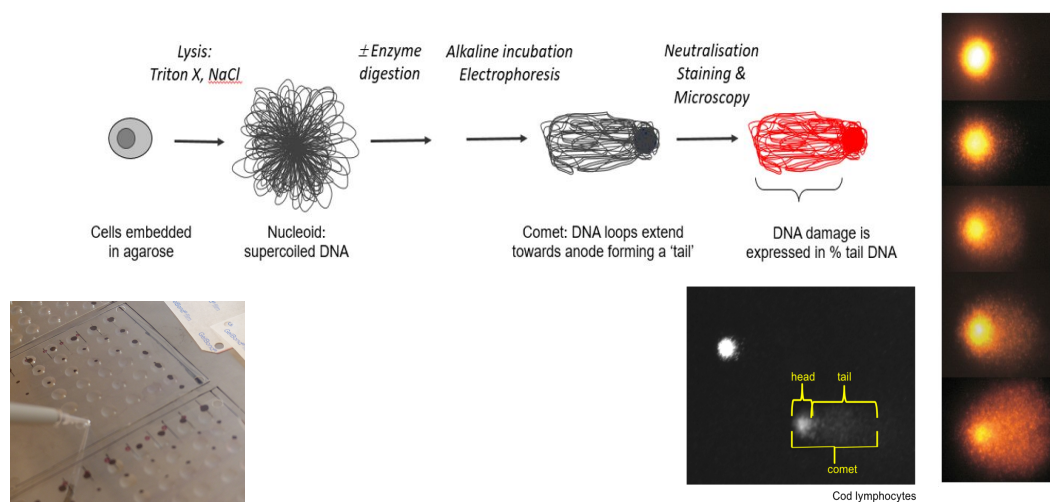


Fig. 4: Schematic overview of the main principle steps in the comet assay. Adapted from R&D Norgeno Tech.

The basic principles (Fig. 4) underlying the comet assay can be summarized in 6 steps. (1) a single cell suspension is derived and cells embedded in agarose on GelBond films or glass slides. (2) The cells are lysed in a solution containing Triton X-100 and high salts to remove cell compartments such as membranes but expose nucleoids in the gel. (3) Highly alkaline solution unwinds and denatures the remaining DNA, as well as alkaline labile sites, are hydrolysed to single strand breaks. (4) Electrophoresis is performed in a horizontal tank (preferably with circulation to assure constant current over the whole stage) using the same alkaline solution as electrophoresis buffer. Relaxed DNA loops migrate into the agarose gel due to electric forces, but only if breaks are present. (5) Gels are neutralised and fixed by dehydration in ethanol to allow for long-term storage (Collins, 2004; Hylland et al., 2016). (6) DNA is stained with a nucleic acid dye such as SYBRGold or ethidium bromide to allow image analysis of the comet-like structure of migrated DNA with a fluorescence microscope.

The sensitivity and specificity of the assay can be enhanced if the nucleoids are incubated with bacterial repair endonucleases, such as formamidopyrimidine glycosylase (Fpg), that recognize specific kinds of damage in the DNA (oxidized bases) and convert lesions to DNA breaks, increasing the amount of DNA in the comet tail (Dušinská and Collins, 1996). By using the Fpg-amended comet version in **paper I**, additional breaks related to oxidized bases were identified in hemocytes and coelomocytes in marine invertebrate species and fish lymphocytes.

Lysis conditions are a critical variable and may interfere with the strand breaks resulting from specific types of DNA modifications (certain DNA alkylation and base adducts). The lysis period should be a minimum of one hour and is recommended to be kept as constant as possible for all

slides within an experiment (OECD, 2016). Storage periods in lysis buffer of up to three weeks have been found to be acceptable for fish blood cells (Hylland et al., 2016). In this thesis storage in lysis buffer were 2 days (**paper III**), 4 days (**paper II**) or 3 weeks (**paper I**) before electrophoresis.

Cell extraction from very small species is challenging. Despite successful pilot tests, obtaining sufficient cells from copepods for comet analysis was challenging in **paper III**. In 27% of all samples, the recommended number of 50 cells per replicate could not be scored. To avoid replicate loss due to lower count numbers than the recommended 50 scores per cell per replicate, we included squared counts of each individual as a weighted random effect in the statistical models used in **paper III**.

## Measurement of DNA repair

The comet assay can also be used to study DNA repair by measuring the removal of strand breaks compared to controls within a given depurination time (Collins et al., 1995). In **papers I-III**, strand break removal of treatment-induced DNA damage was quantified following a recovery period.

The *in vitro* DNA repair assay is based on the comet assay principle but modified as such a cell-free extract is prepared and incubated with a DNA substrate containing specific lesions. It is the nature of the substrate that defines the particular repair pathway being followed. Using agents that induce specific damage, e.g. the photosensitizer Ro 19-8023 that predominantly induces 8-oxoGua or MMS, that induces alkylation damage, are used to further increase specificity (Azqueta and Collins, 2013; Collins et al., 2001; Gaivão et al., 2009).

DNA repair in terms of adduct removal at various intervals during depurination has also been measured by <sup>32</sup>P-postlabelling methods (Akcha et al., 2000). DNA repair is often studied by assessing changes in gene expressions of DNA repair-related genes (e.g. *xpc* for NER; *ogg1*, *parp1* and *parp2* for BER or *rad51* for HR) by PCR-based assays (Hunter et al., 2010; Reinardy and Bodnar, 2015; Rhee et al., 2012; Won and Lee, 2014). The underlying principle is, that DNA damage interferes with the progression of the DNA polymerase, resulting in a decrease of the amount of PCR product. The amount of PCR product can thus be inversely correlated with DNA damage (Hunter et al., 2010; Reinardy and Bodnar, 2015; Rhee et al., 2012; Won and Lee, 2014).

### *DNA repair measured by oligo-repair chip: pilot study*

An innovative and state of the art method to measure DNA repair was used in a pilot study during a three-month stay abroad at the Institute of Nucleic Acid (today CIBEST), CEA Grenoble. Similar to in the *in vitro* DNA repair assay, the multiplex oligonucleotide cleavage assay developed by

(Sauvaigo et al., 2004), enables the simultaneous measurement of cleavage activities and efficiency of DNA repair enzymes present in a sample on a set of modified oligonucleotides containing specific DNA lesions (Fig 5). While this microarray chip is mainly applied in functional diagnostics in human cancer medicine, it could also imply a great potential for ecotoxicology.

A three months *in vivo* flow-through exposure of Atlantic cod (*Gadus morhua*) to the emerging contaminant perfluorooctanoic acid (PFOA) and the model PAH fluoranthene (FLU) and a combined mixture was conducted. Both compounds are indicative or known genotoxicants (Palmqvist 2003, Kawamoto 2010). Unlike most other persistent and bioaccumulative organic pollutants, PFOA is water-soluble, does not bind well to soil or sediments, and bioaccumulates in serum rather than in fat. DNA strand breaks (comet assay) and DNA repair enzyme activity (multiplex oligonucleotide cleavage assay) were measured in fish gill cells and lymphocytes.

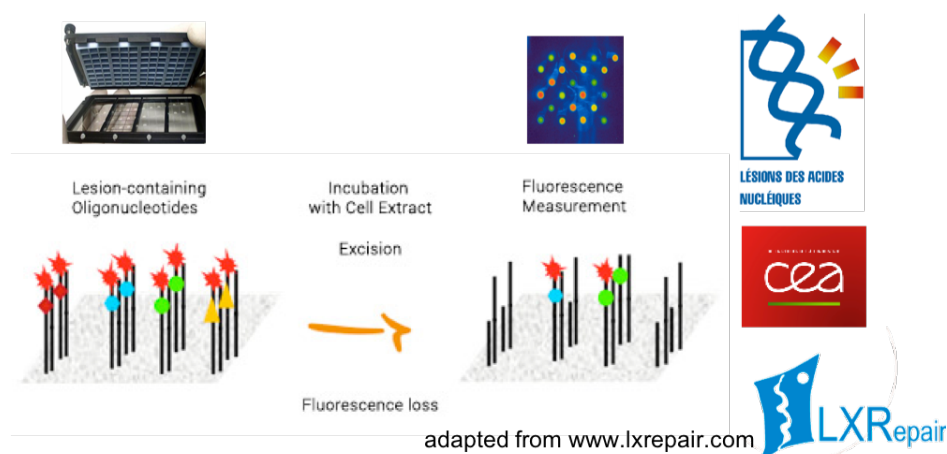


Fig 5. Schematic overview of the principle steps of the multiplex oligonucleotide cleavage assay

The basic principles of the multiplex oligonucleotide cleavage assay can be described in two major steps. 1) Oligonucleotides containing 8 common DNA lesions (e.g. oxidized bases, base mismatches, abasic sites, deaminated bases and alkylated bases) are complexed with a fluorescent probe and fixated on microscope glass slides. The fluorescence for each spot is maximal before any nuclear extract is added. 2) An excision reaction is initiated by incubating the nuclear extract on the slides. The residual fluorescence can be quantified and is proportional to the excision capacity of the nuclear extract. Thereby, specific lesions can be recognized, allowing to draw conclusions on specific repair enzymes activated by certain agents.



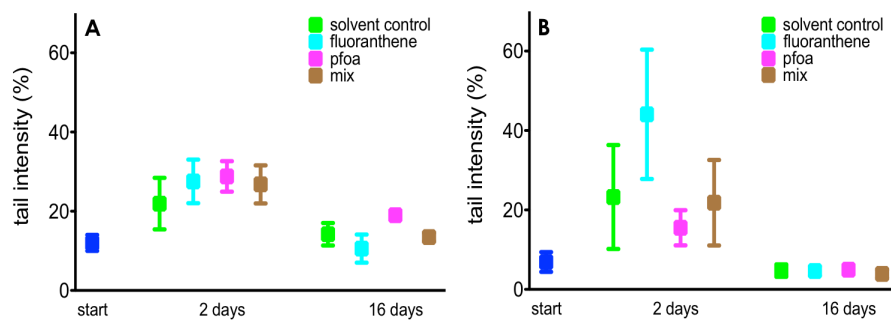


Fig 6. DNA strand breaks levels following an *in vivo* exposure to fluoranthene (FLU), perfluorooctanoic (PFOA) and a combined mixture in A) cod gill cells and B) cod lymphocytes. The blue point represents the basal DNA strand break levels for both tissues, respectively before the experiment.

Both, single compounds and the mixture induced DNA strand breaks in gill cells and lymphocytes after 2 days. Strand breaks were similar to the solvent control and returned to baseline levels within 16 days (Fig. 6). DNA repair enzyme activity was more diverse in cod lymphocytes with increased activity for some lesions, while others enzymes showed decreased activity as compared to gill cells, which had overall decreased repair enzyme activity (Tab. 2). The experimental data were, however, not used for publication due to experimental confinements concerning the carrier solvent during exposure. Also, data normalization of DNA repair activity could not be performed adequately, because determination of the sample protein content was confined.

Table 2: Qualitative DNA repair enzyme activity response in gill cells and lymphocytes from the Atlantic cod (*Gadhus morhua*) for specific lesions/DNA damage following exposure to fluoranthene (FLU), perfluorooctanoic (PFOA) and a combined mixture. Red colour indicates decreased activity, green colour indicates increased activity and white colour indicates no effect. na = not available.

DNA Damage	Gill cells			Lymphocytes		
	PFOA	Flu	mixture	PFOA	Flu	mixture
Alkylation	[Red]			na	na	na
Deamination	[Red]			[Green]	[Red]	[Green]
Deamination	[Red]	[White]	[Red]	[Green]		[Red]
Depurination (AP-site eqv.)				na	na	na
Oxidized purine	[Red]			[Green]		
Mispair	[Red]			na	na	na
Oxidized pyrimidine	[Red]				[Green]	[Red]
Mispair	[Red]			na	na	na

## Results and Discussion

### What is a natural baseline of DNA strand breaks?

Understanding the effect of genotoxicants on DNA strand breaks requires knowledge about the natural baseline (Würgler and Kramer 1992, Machalla 2006). Phylogenetic-dependent variability in baseline levels in invertebrates may mirror the diversity in taxa and may, therefore, appear more diverse than in mammalian cells (Andreoli et al., 1999; Collins et al., 1995) (Fig 7).

Based on early comet assay studies using human lymphocytes, a baseline level of DNA strand breaks of <10% is considered “normal” (Collins et al., 1995; Collins et al., 1996; Collins, 2004; Møller, 2006). This level appears unrepresentative for marine invertebrates (Fig. 7). Baseline levels in **papers I-III** averaged 25–30% in the circulating cells for most of the invertebrates used here. Other studies using different DNA strand break detection techniques further support relatively high baseline levels in invertebrates (Everaarts, 1995; Nacci et al., 1992; Steinert, 1996). Further, fish and tunicate (chordates) had lower baseline levels than starfish (echinoderm) (**paper I**). This is consistent with invertebrates generally exhibiting higher basal levels than vertebrates (Mitchelmore and Chipman, 1998; referenecs Table 1, **paper I**).

The variation of baseline levels among invertebrates may have several explanations, such as differences in DNA packaging and background alkali-labile sites (Hook and Lee, 2004; Machella et al., 2006; Mitchelmore and Chipman, 1998; Pérez-Cadahía et al., 2004). Lower cell proliferation and differences in DNA repair capacity may further explain higher levels in invertebrates (Dixon et al., 2002). Additionally, the different cell types can have different levels of DNA strand breaks, due to variation in repair efficiency, metabolic activity and antioxidant concentration (Lewis and Galloway, 2008). The presence of different cell types in the analysis may contribute to the variability within a species.

Several biotic and abiotic factors may influence baseline strand breaks levels, such as animal state (Hartl et al., 2004), gender and season (Almeida et al., 2011). Oxidative stress and DNA strand breaks may increase during warmer months (Almeida et al., 2011; Michel et al., 2013). Organisms of **paper I** and **II** were sampled in winter. Thus, possible confounding seasonal and physiological factors, e.g. reproduction and moulting activities, were thought to be minimal. In contrast to previous studies (Almeida et al., 2011), no sex differences were found for mussel, crab, copepod (**papers I-III**). However, high intra-species variability, low sample size and concurrent low statistical power hampered the analysis of sex differences.

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, **paper I**), but Canty et al. (2009) observed lower levels of DNA damage in starfish coelomocytes. These contrasting results may imply, that, within taxa, different levels of baseline DNA damage may reflect variation in animal state. It may also reflect methodological differences.

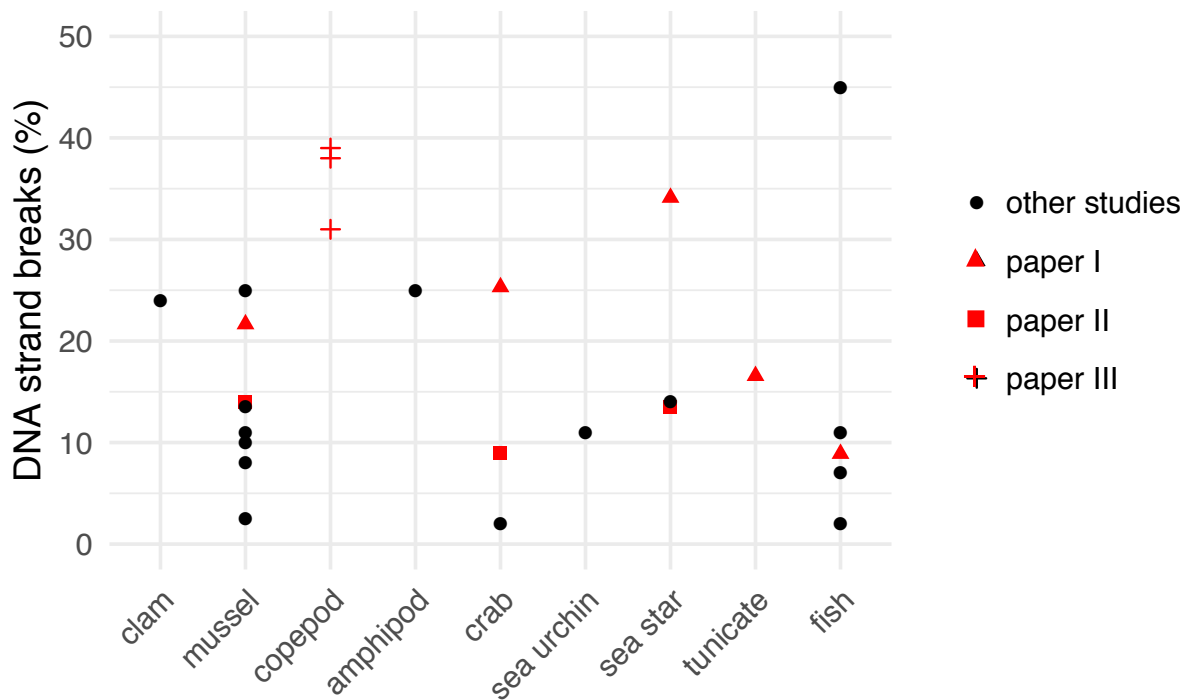


Fig 7. Baseline levels of species used in **papers I-III** with comet assay-derived baseline levels of other species as reported in Table 1 in **paper I**. New additions: amphipod (Cruzeiro et al., 2019), sea urchin (Taban et al., 2004). Data from **papers I-III**.

### Species susceptibility to genotoxicants

Determination of relative sensitivity of biota following exposures to contaminants is important for environmental protection. The comparative studies in the present thesis contribute with valuable information on inter-species susceptibility to common pollutants and genotoxic stressors. The susceptibility of hemocytes and coelomocytes of the studied species varied with the respective exposure contaminant used in **papers I-III**: H<sub>2</sub>O<sub>2</sub>, the low molecular PAHs phenanthrene and dibenzothiophene and copper (Cu).

## Oxidative stress

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a major model genotoxicant as it causes strand breaks and base oxidation (Benhusein et al., 2010) and was used as an oxidative stressor (**paper I**) and positive control (**papers II-III**) in this thesis.

While coelomocytes of *A. rubens* were equally affected as cells from *M. edulis* and *C. maenas* in **paper I**, the lower H<sub>2</sub>O<sub>2</sub> concentrations used in **paper II** as compared to **paper I** suggest *A. rubens* is more affected than *M. edulis* and *C. maenas*. Similar sensitivity-differences were found between *A. rubens* and *M. edulis* when exposed to both direct and indirect-acting genotoxicants (Canty et al., 2009). A comparison of studies in Table 2 in **paper I** further suggest sea urchin coelomocytes (*Lyechinus variegatus*, *Echinometra lucunter lucunter* and *Trippneustes ventricosus*) (El-Bibany et al., 2014) and hemocytes from the common cockle (*Cratosderma edule*) (Cheung et al., 2006) appeared more sensitive to H<sub>2</sub>O<sub>2</sub> exposure than *Mytilus* hemocytes. The relative lower oxidative-susceptibility of mussel and crab circulating cells observed in this thesis might reflect these animals' ability to handle rapid changes in oxygen tension and concurrent ROS production in the intertidal (Gamble et al., 1995). Starfish are stenoxic and their higher susceptibility might reflect a narrower tolerance to oxidative insult. However, all invertebrate cells were more sensitive than fish lymphocytes (Fig. 8 and **paper I**) or erythrocytes (Mamaca et al., 2005).

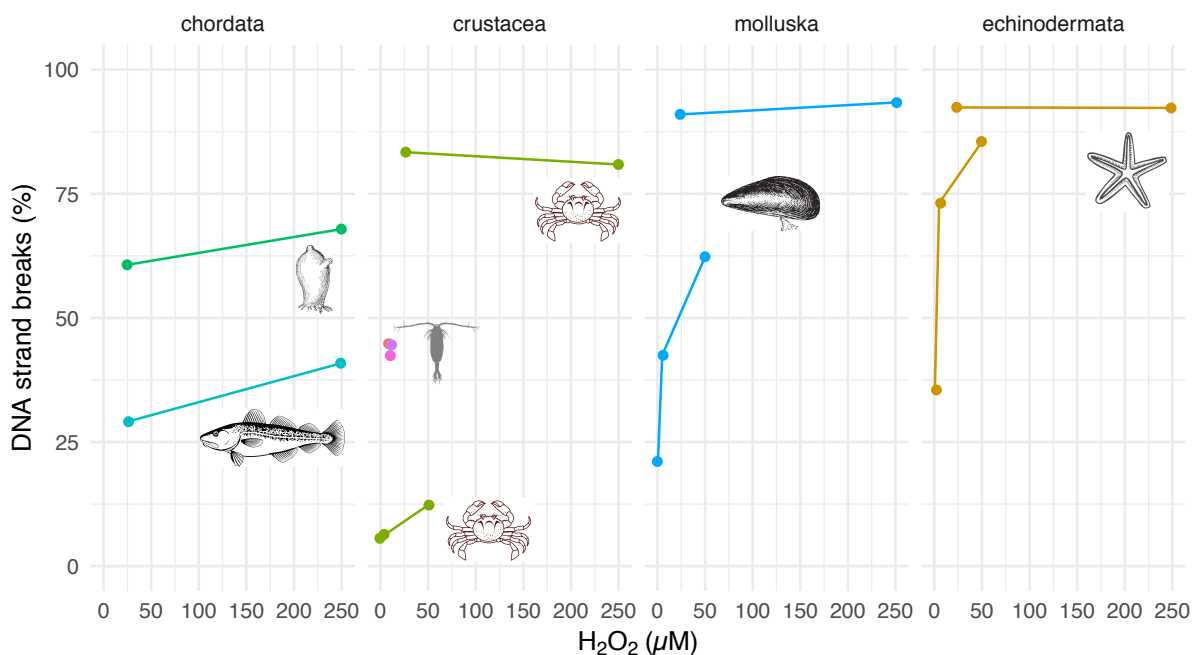


Fig 8. Compilation of H<sub>2</sub>O<sub>2</sub>-induced DNA strand break levels from **papers I-III**. Species Atlantic cod (*Gadhus morhua*), vase tunicate (*Ciona intestinalis*), shore crab (*Carcinus maenas*), copepods:

*Acartia tonsa*, *Temora longicornis*, *Tigriopus brevicornis*, blue mussel (*Mytilus edulis*) and common starfish (*Asterias rubens*) depicted next to according data line. Data from **papers I-III**.

### *Specific contaminants*

Low molecular weight PAH exposure (**paper II**) showed weak genotoxicity of phenanthrene in mussel hemocytes and starfish coelomocytes and dibenzothiophene did not increase strand breaks in circulating cells of any of the species (Fig 9). Increased antioxidation (Martins et al., 2013) or inhibition of CYP-biotransformation (Willett et al., 1998) could keep DNA strand break levels low. On the other hand, exposure to 2–5 ring PAHs induced DNA strand breaks in rainbow trout hepatocytes despite CYP1A inhibition (Yazdani, 2014). This suggests that low molecular weight PAHs might not necessarily need CYP-dependent metabolic activation to exert genotoxic effects. Also, phenanthrene induced DNA strand breaks during the same exposure period as used in **paper II** in hemocytes of the Mediterranean mussel *M. galloprovincialis* and erythrocytes of the olive flounder *Paralychtis olivaceus* (Dailianis et al., 2014; Woo et al., 2006).

PAH-mediated DNA adducts are not necessarily detectable by the comet assay *per se*, but if not repaired they will eventually lead to DNA strand breaks due to impaired DNA replication through helix distortion and prevention of polymerase (Friedberg et al., 2005). By weakening the N-glycosidic bond DNA adducts can also lead to the formation of alkali-labile sites (after spontaneous depurination in alkaline conditions), which in turn can cause DNA strand breaks (Sage and Haseltine, 1984; Akcha et al., 2003).

Exposure duration of the present study may be considered short for biotransformation processes, and maybe not sufficient to generate adduct-derived strand breaks. Hook and Lee (2004) observed a peak in DNA strand breaks at 12h of benzo(a)pyrene exposure in grass shrimp (*Palaemonetes pugio*) embryos. On the other hand, the results in **paper II** and those by Woo et al. (2006) indicate phenanthrene-dependent reactive metabolite generation. Thus, related strand break induction may occur already following short-term *ex vivo/in vitro* exposure and in cells with lower bioactivity as gills or digestive gland (Vincent-Hubert et al., 2011; Rank et al., 2005).

The few available studies on dibenzothiophene and the present results (**papers I-II**) may indicate that dibenzothiophene acts on DNA through other mechanisms, e.g. on the chromosomal level through clastogenic effects, rather than DNA strand breaks induction (Amat, Pfohl-Leszkowicz, and Castegnaro, 2004; Brinkmann et al., 2014; Leighton, 1989). Though, hemocytes might not necessarily represent the most sensitive system regarding PAH-related DNA damage and might be less affected than cells from e.g. digestive gland or hepatopancreas (Vincent-Hubert et al., 2011; Rank et al., 2005).

Glutathione (GSH) is involved in detoxification of reactive intermediates during phase II biotransformation, but the reduced form is also a biomarker for oxidative stress, as the most important cellular antioxidant. Phenanthrene-mediated GSH depletion and increased lipid peroxidation were observed in clam (*Pecten maximus*) and mussel (*M. edulis*) hemocytes (Giannapas et al., 2012; Hannam, Bamber, Galloway, et al., 2010). Thus, oxidative stress represents a mechanism through which low molecular weight PAHs can cause genotoxicity in invertebrate circulating cells. Both, GSH level and lipid peroxidation are often positively correlated with DNA strand breaks (Bhagat et al., 2016; Livingstone, 2003).

Similarly, to H<sub>2</sub>O<sub>2</sub>-responses, *Asteria* coelomocytes were most responsive to phenanthrene exposure as compared to *Mytilus* and *Carcinus* cells (Fig. 9) (paper II).

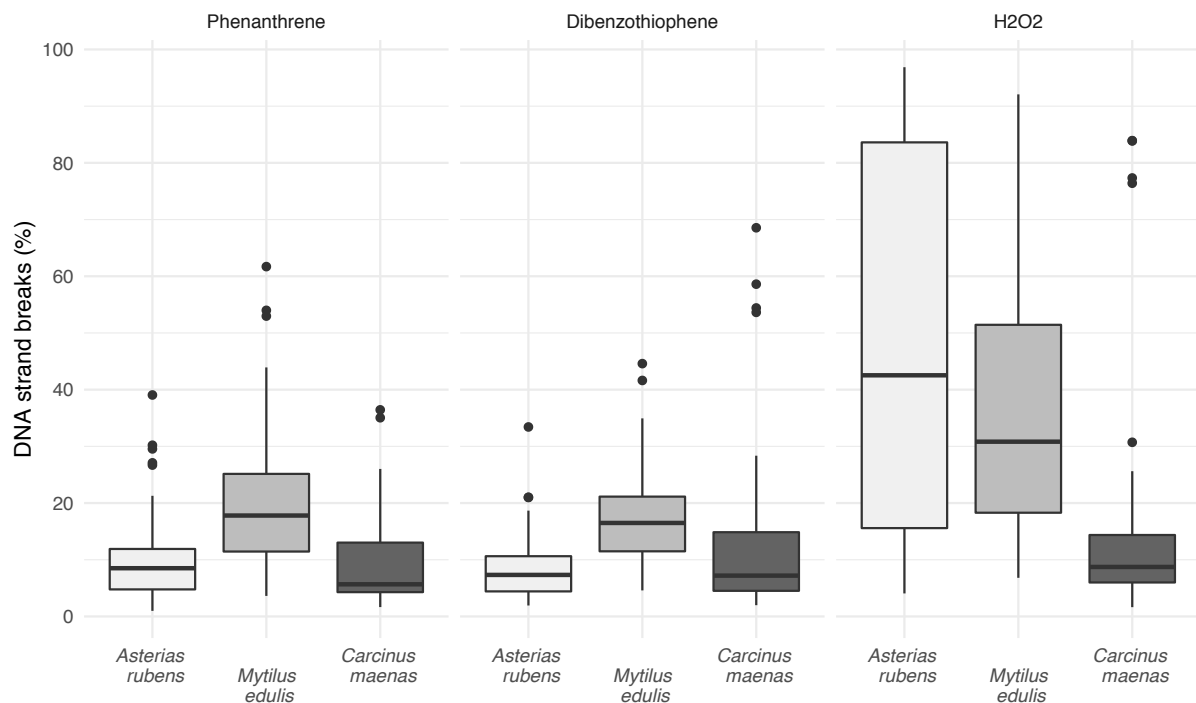


Fig 9. DNA strand breaks (%) following exposure to phenanthrene, dibenzothiophene and H<sub>2</sub>O<sub>2</sub> in circulating cells of *Asterias rubens*, *Mytilus edulis* and *Carcinus maenas*. The graph visualizes the general response pattern between species and treatments to picture the susceptibility pattern (no statistical analysis). Data are presented as median, 10 and 90 percentiles. Full detailed responses for each concentration, species and compound see figure 1 in paper II. Data from paper II.

The environmentally relevant Cu concentrations used in paper III did not increase DNA strand breaks *in vivo* in any of the three copepods at any concentration or time point (Fig 10). Conversely, DNA damage, such as increased frequency of micronuclei in the freshwater cladoceran *Daphnia*

(Barka et al., 2016), apoptosis in the shrimp *Litopenaeus vannamei* (Guo et al., 2017), mutagenicity in (Barka et al., 2016) were associated with Cu exposure. Our results are in contrast to other studies detecting Cu-induced DNA strand breaks (comet assay) in marine organisms, i.e. the clam *Corbicula fluminea* (Bonnail et al., 2016), the oysters *Crassostrea gigas* (Mai et al., 2012), the mussel *Mytilus edulis* (Al-Subiai et al.) and the polychaete *Nereis virens* (Caldwell et al., 2011). However, the effect concentrations were higher or exposure duration longer in these studies than in **paper III** (see also Table 2, **paper III**). DNA strand breaks induction was observed in copepods following exposure e.g. to persistent organic pollutants (Han et al., 2015; Shi et al., 2017; Won and Lee, 2014), crude oil (Han et al., 2014) and cadmium (Pavlaki et al., 2016). **Paper III** suggests the three copepods to be relatively robust to Cu genotoxicity. This may be related to processes that limit the uptake of Cu, internal distribution and complexation or that concentrations used in our study were within the range that could be buffered by antioxidant defence mechanisms in the copepods. Detoxification and DNA repair gene expression was increased following Cu exposure ( $10 \mu\text{g Cu L}^{-1}$ ) due to Cu-mediated ROS (Ki et al., 2009). However, higher exposure concentrations ( $>1$  and  $2 \text{ mg Cu L}^{-1}$ ) were needed to affect antioxidant enzyme activity in the same species (Lee et al., 2008; Rhee et al., 2013; Kim et al., 2014).

One of the major findings in **paper III** is the differences in Cu sensitivity between the three copepods, with strong effects on mortality in *A. tonsa*. Following post-exposure 50% and 100% mortality was detected in Cu-treated *A. tonsa*, while no mortality in the control group (Fig 10). It is not clear if the mortality is genotoxicity-related and/or a result of cellular damage accumulation, due to e.g. lipid peroxidation or overwhelming the antioxidation capacity of GSH. Also, Cu reduces ion concentration ( $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Mg}^{2+}$ ) (Pinho et al., 2007), disturbs osmoregulation (Lee et al., 2010) and inhibits the  $\text{Na}^+/\text{Ca}^{2+}$  ATPase (Bambang et al., 1995). All of which may eventually be lethal due to e.g. insufficient gas exchange and energy metabolism (Grosell et al., 2007; Wilson and Taylor, 1993).

*T. brevicornis* was particularly robust to Cu exposure. Non-enzymatic metal detoxification, such as metallothionein complexation or storage of metals in metal granules, might be more efficient in *T. brevicornis* as compared to the other two species (Barka, 2007; Barka et al., 2001). Although *Tigrioups* spp. have been widely used in Cu exposure studies, knowledge on e.g. cytotoxicity and genotoxicity of Cu is scarce (Rhee et al., 2013). The resistance to exposure conditions (**paper III**) corresponds well with reported tolerance of *T. brevicornis* to pollution stress and harsh environmental conditions (Medina et al., 2008; Seo et al., 2006).



While previous comet assay studies used copepods in pools of up to 120 individuals (Goswami et al., 2014; Guecheva et al., 2001; Pavlaki et al., 2016; Ternjej et al., 2009; Zeeshan et al., 2016), we demonstrate that the comet assay can be used to quantify genotoxicity in individual copepods.

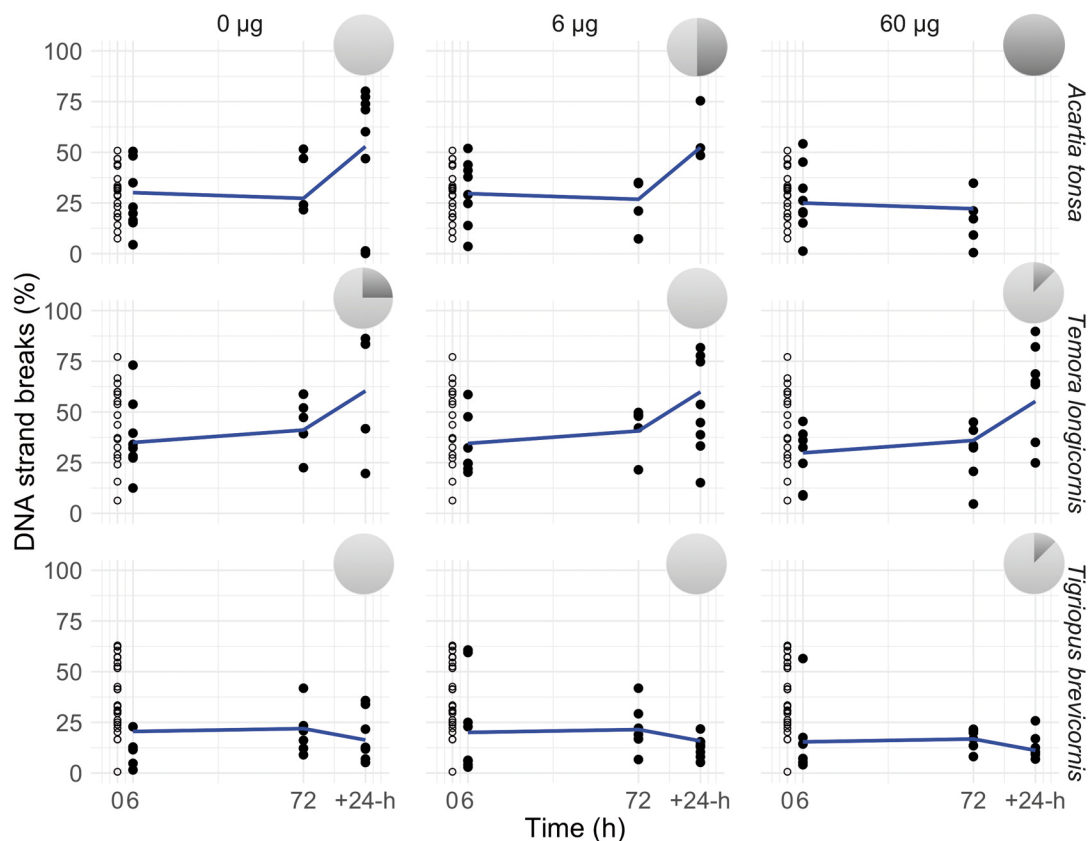


Fig 10. DNA strand breaks in *Acartia tonsa*, *Temora longicornis* and *Tigriopus brevicornis* following exposure to 0, 6 and 60 µg L<sup>-1</sup> Cu for 6 h and 72 h and subsequent 24 h post-exposure period (+24-h). Baseline levels (open circles) are included graphically as time 0 h. Pie charts of % survival (light grey)/mortality (dark grey) for 24 h post-exposure period (+24-h). From **paper III**.

### Recovery

Small differences in the efficiency of DNA repair may have a considerable impact on the overall species susceptibility (Depledge, 1998). Thus, recovery phase ranging from 1 to 2 h (**papers I-II**) to 24 h (**paper III**) were included in the three experiments. During this period, cells or individuals were allowed to recover and repair induced strand breaks in genotoxicant-free conditions. However, DNA strand breaks did not return to control levels in any of the experiments. In contrast, human blood cells seem to have efficient repair systems; oxidative stress-induced strand breaks, which are mainly thought to be single strand breaks (SSB) and oxidized bases, were repaired within 10-30 min and within 30 min to a few hours, respectively (Collins et al., 2001). Rainbow

trout larvae and blood cells from flounder showed high rate of excision repair, comparably to human blood cells (Nacci et al., 1996; Mitchell et al., 2009).

Our results, following previous studies, indicate that invertebrate cells require more time for significant or complete recovery of DNA integrity (Akcha et al., 2000; El-Bibany et al., 2014; Hook and Lee, 2004; Pruski and Dixon, 2003). Sea urchins and bivalves recovered only within a few hours or days (Akcha et al., 2000; El-Bibany et al., 2014; Pruski and Dixon, 2003). However, the increase in strand breaks observed in all experiments (**papers I-III**) could both reflect the accumulation of cellular damage and/or depletion of antioxidant defence capacity, subsequently expressed as strand breaks (Marnett, 2002) and intermediate strand breaks due to primary DNA repair steps (Rastogi et al., 2010; Wilson and Bohr, 2007). The initial repair steps of BER and NER includes nuclease activity that recognises and removes damaged bases or nucleotides, thereby introducing alkali-labile sites or single strand breaks, which might then be expressed in the comet assay.

Differences in susceptibility and repair capacity have also been related to ontogeny, with early life-stages often being more susceptible than later stages (Hook and Lee, 2004; Medina et al., 2008; Reinardy and Bodnar, 2015; Verriopoulos and Moraitou-Apostolopoulou, 1982). Sea urchin larvae were more sensitive with lower repair capacity than adult coelomocytes exposed to MMS, H<sub>2</sub>O<sub>2</sub>, and UVC (Reinardy and Bodnar, 2015). *Tigriopus brevicornis* nauplii were able to develop to adults under Cu exposure (20 µg Cu L<sup>-1</sup>). While development time was reduced due to Cu-exposure (Lode et al., 2018), adult copepods were similarly unaffected in their DNA strand break levels as *T. brevicornis* in **paper III** (comet assay data shown in supplement 4 in **paper III**).

## Hemocytes compared to other tissues

Choosing an appropriate and both ecologically and physiologically relevant exposure tissue is important when studying the ecotoxicity of a compound. Gill and intestinal cells are important targets for compounds as these cells are in relatively direct contact with chemicals during the filter process or intestinal uptake. Digestive gland cells are important for biotransformation and detoxification. Hemocytes and coelomocytes are also exposed from an early stage. Given the open vascular system in many invertebrates, hemocytes/coelomocytes are likely exposed more or less simultaneously to a toxicant entering the organism (Dixon et al., 2002; Canty et al., 2009). The multifunctional role of hemocytes lends further credibility to their use as models in studies of DNA damage (Venier et al., 1997).

Results from **paper I** and **II** comply with the general conception of invertebrate circulating cells being more sensitive to DNA damage than are vertebrate cells (Mamaca et al., 2005; Nacci et al., 1996). Hemolymph and coelomic fluids were easy to collect from all species (**papers I-II**). The non-destructive extraction of hemolymph complies with the three R-concept, which would even allow for repeated sampling in a time-course study and using the same hemolymph sample for different assays (Mamaca et al., 2005; Nacci et al., 1996). Digestive gland and gills from *M. edulis* (Large et al., 2002) are commonly used in the comet assay, and the assay can also be used with e.g. sperm as in a study with amphipods (*Gammarus fossarum*) (Lacaze, Devaux, et al., 2011). Using solid tissues with the comet assay requires mechanical or enzymatical (e.g. collagenase, trypsin) dissociation (see p. 10). Since cell dissociation is not required for hemocytes, handling damage is minimal (Hartmann et al., 2003; Rigonato et al., 2005). Furthermore, Rank and Jensen (2003) and Rigonato et al. (2005) agreed on the preference of mollusc hemocytes as model system over digestive gland or gill cells, despite similar or higher sensitivity of the latter cells. Their conclusion was mainly based on the reproducibility and reliability of results obtained from hemocytes, along with the ease of cell extraction, as increased cell manipulation was related to higher baseline levels of DNA strand breaks in gill cells. These advantages have the added benefit that using hemocytes shortens the time for comet slide preparation and facilitates multiple sample processing (Siu et al., 2004).

Cells might differ in the ability to sense and repair DNA damage (Lewis and Galloway, 2008; Dolcetti and Venier, 2002). Polychaete elocytes were found to be more sensitive than amoebocytes (Lewis and Galloway, 2008). Thus, the presence of various cell types in invertebrate hemolymph may pose a bigger challenge than other tissue cells, as they may be a source for the high inter- and intra-individual variability (Lewis and Galloway, 2008). However, Percoll separation did not appear to effectively reduce the variability in comet data (**paper II**), suggesting more factors underlying cell-specific variability.

Further, interspecies differences of hemocyte susceptibility seen in **papers I-III** comply with previous studies: for example, sea urchin hemocytes were relatively robust to several DNA-damaging agents (El-Bibany et al., 2014; Reinardy and Bodnar, 2015) including H<sub>2</sub>O<sub>2</sub> and UV compared to cells from the sea hare (*Aplysia dactylomela*) or Caribbean spiny lobster (*Panulirus argus*) (Loram et al., 2012).

## Comet assay compared to other methods

In this thesis, the comet assay detected DNA strand breaks in all invertebrate cells tested (**papers I-III**). Although weak or no genotoxicity was detected in **paper II** and **III**, “proof-of-principle”

was provided by significant strand break induction by H<sub>2</sub>O<sub>2</sub>. The comet assay clearly revealed differences in species susceptibility and is as such useful for measuring genotoxicity. Applying the comet assay quantitatively in *C. intestinalis* circulating cells and in individual copepods was done for the first time in **paper I** and **III**. Results from this thesis further support the versatility of the comet assay (Dhawan et al., 2009).

The <sup>32</sup>P-postlabelling, the micronucleus- and the comet assay are among the most applied and validated methods for studying genotoxicity in aquatic organisms (Bolognesi and Cirillo, 2014; Harvey et al., 1997). The low amount of DNA per cell, the numerous and small chromosomes and low mitotic activity of invertebrate cells (Dixon et al., 2002) may pose challenges for the applicability of the sister chromatid assay (Bolognesi and Hayashi, 2011). The <sup>32</sup>P-postlabelling assay is highly sensitive and has been used in ecotoxicology (Dolcetti and Venier, 2002; Harvey et al., 1997; Holth et al., 2009). The protocol is, however, complex and time-consuming, thus limiting its use in large scale biomonitoring (Bolognesi and Cirillo, 2014; Dolcetti et al., 2002). Similarly, while highly sensitive, in the alkaline elution/unwinding assays the number of samples are limited due to relatively complicated and time-consuming procedures (Schröder et al., 2006). While the glass format-based comet assay may also limit the number of samples, multiple samples (of up to 18 samples) were processed simultaneously in **papers I-III** by using hydrophilic polyester films (GelBond films) (Gutzkow et al. (2013), thereby reducing handling time and electrophoresis runs.

The comet assay does not detect a specific type of damage, like the <sup>32</sup>P-postlabelling assay, unless lesion-specific enzymes are included. Also, differentiation between accumulated damage and repair-related strand break intermediates during recovery (**papers I-III**) is generally not possible without the respective enzymes.

Bulk DNA samples are applied in the alkaline elution/unwinding methods, agarose plug electrophoresis and <sup>32</sup>P-labelling assay, while the comet and micronucleus assay provide DNA damage information on single cell level. The comet assay uniquely uses living cells, that are embedded in agarose, while cells are fixated i.e. by cryopreservation or glutaraldehyde prior to procedure for the other above-mentioned assays.

Induction of MN is a well-established tool for genotoxicity assessment of chemical compounds which has been standardized with OECD guidelines in mammalian cells (OECD, 2010, 2016) and which has been extensively applied in marine organisms to determine contaminant induced chromosomal damage (Barka et al., 2016). The comet assay is often compared to or used concurrently with the MN. Comet assay and MN results often correlate well (Klobučar et al., 2003; Klobučar et al., 2008), while equal or sometimes higher sensitivity was observed for the comet

assay. In mammalian systems, the comet assay (*in vitro*) provided an earlier significant response to genotoxicants (EMS, MMS, BaP-diol epoxide) than MN or alkaline elution (Andreoli et al., 1999; Leroy, 1996; Mouchet et al., 2005). In contrast, MN was found to be more sensitive in fish collected from polluted sites with unknown exposure profile in one study (Amado et al., 2006) and organic contaminants (including PAHs) (Bombail et al., 2001). Higher sensitivity or earlier significant results might be related to the nature of the damage. While the comet assay detects primary repairable, therefore reversible, DNA lesions (alkali-labile sites, single strand DNA breakages), the MN assay detects persistent DNA lesions (aneugenic and clastogenic) effects (Bombail et al., 2001; Hartmann et al., 2003). Good correlation was also found for comet assay and other cellular biomarkers, such as lipid peroxidation, GSH content and catalase activity (Martins et al., 2013; Pisanelli et al., 2009).

Marine invertebrate cells are short-lived (Rinkevich, 2005) and hemocytes are not thought to proliferate in the hemolymph (Cheng and Bivalves, 1981). Thus, the comet assay represents a suitable choice regarding *in vitro* studies on primary cell cultures (**papers I-II**) since e.g. MN requires cell division.

Several studies report DNA strand breaks related to PAH exposure *in vivo* as well as *in vitro* (Bhagat et al., 2016; Dailianis et al., 2014; Woo et al., 2006). However, the comet assay might not be the most sensitive assay for this kind of lesion (**paper II**). Similarly, Baussant et al. (2009) could not match PAH accumulation with a modest increase in DNA strand breaks *in vivo* in clams. However, DNA strand breaks were detected in benzo[a]pyrene-exposed hemocytes of the green-lipped mussel (*Perna viridis*) (Siu et al., 2004) and in the fish cell line RTL-W1 exposed to sediment extracts containing PAHs (Boettcher et al., 2010).

## Are genotoxicants a problem for marine invertebrates?

Chemicals with the potential to damage DNA are omnipresent in the marine environment; yet our understanding of the long-term consequences of DNA damage for populations remains limited (Lewis and Galloway, 2009). As with other environmental stressors, documentation of a causal relationship between genotoxic exposure and adverse effects on populations and ecosystem level is challenging (Shugart and Theodorakis, 1998). One of the first causative relationships of pollution-induced genotoxicity was the association between sediment pollutants and hepatic tumours in English sole (*Parophrys vetulus*) in the Puget Sound, USA (Stein et al., 1990), followed by hepatic DNA adducts observed in the Atlantic tomcod and the winter flounder environmentally exposed to PAHs (Stein et al., 1994). PAH-induced DNA damage was present in bivalves from natural populations more than a decade after the infamous Exxon Valdez oil spill (Thomas et al.,

2007). Developmental and thus ecological relevant consequences of genotoxicants include reduced hatching rates and gamete development, e.g. reported in grass shrimp (Lee et al., 2012), which may eventually lead to reduced genetic diversity (Bickham et al., 2000) and reduced fitness (Linhartova et al., 2013; Matić et al., 2016).

Functional hemocytes play vital roles in gas exchange, osmoregulation, nutrition, wound healing and immune defence (Pipe and Coles, 1995). Pollution-related oxidative stress may result in immunosuppression and reduced hemocyte viability (Giannapas et al., 2012) as well as GSH depletion and increased lipid peroxidation (Hannam, Bamber, Moody, et al., 2010; Hannam, Bamber, Galloway, et al., 2010) in turn causing reduced bivalve health and survival (Giannapas et al., 2012). While sensitivity of circulating cells to oxidative stress was high (**papers I-II**), linking the *in vitro* effects of the H<sub>2</sub>O<sub>2</sub> treatments in **paper I** and **II** to individual health is challenging as effects on higher organisational level were not examined. Boettcher et al. (2010) highlights the potential ecological relevance of *in vitro* studies, by showing that *in situ* MN formation in erythrocytes of European barbel (*Barbus barbus*) correlated with *in vitro* DNA strand breaks (comet assay) and MN in rainbow trout hepatocytes, induced by polluted sediment extract.

The weak genotoxicity observed in **paper II** for phenanthrene is presumably not sufficient to cause effects on individual health. Even minor or few changes in DNA integrity may, however, disturb the function of hemocytes or other tissues if vital genes are affected (Jha, 2008). Ecologically adverse effects i.e. reduced swimming and feeding activity, were reported in sea urchin larvae exposed to polar oil compounds (Arnberg et al., 2018). Such effects may eventually impact the growth and survival of the individual with possible consequences for population dynamics.

The Cu-dependent mortality of *A. tonsa* in **paper III** suggest that the environmentally relevant Cu concentrations chosen may impact populations of *A. tonsa*. Whether or not the increased mortality is linked to DNA integrity warrants further studies.

Ecologically relevant effects have been shown as reproductive consequences following parental genotoxicant exposure in the polychaete *Arenicola marina* and the mussel *M. edulis* (Lewis and Galloway, 2009). Transmission of such damage to offspring resulted in embryonal aberration and impaired development in the Pacific oyster (*Crassostrea gigas*) (Barranger et al., 2014).

## Conclusions and perspectives

Baseline levels of DNA strand breaks were similar in non-chordates circulating cells and were higher than for chordates (tunicate, fish). Circulating cells from non-chordate invertebrates had higher baseline damage than the 10% considered normal for human or vertebrate cells. Possible phylogenetic differences in the maintenance of DNA integrity merits more research.

While tunicate and fish cells were similar, circulating cells of the other invertebrate were highly susceptible to oxidative stress and more susceptible than fish. Circulating cells from marine invertebrates may, therefore, be useful model systems in which to study cellular consequences of oxidative stress in future studies.

Short-term *in vitro* exposure to the low molecular weight PAH phenanthrene caused weak genotoxicity in *Asterias rubens* and *Mytilus edulis* circulating cells, but not in *Carcinus maenas*. Dibenzothiophene did not cause increased levels of DNA strand breaks in any of the three species. Coelomocytes from *Asterias rubens* were more sensitive than *Mytilus* and *Carcinus* hemocytes to oxidative stress and phenanthrene exposure.

Time-dependent, but no concentration-dependent effects on DNA integrity was observed following exposure of copepods to environmentally relevant Cu concentrations. Strand breaks increased in *Acartia tonsa* and *Temora longicornis* in 24h-recovery period following exposure to the highest concentration applied. Whether the acute Cu-toxicity in *Acartia tonsa* is linked to genotoxicity needs further research. Significant differences in Cu-susceptibility between copepod species were observed. *Tigriopus brevicornis* appeared to be unaffected by all treatments, thus appeared particularly robust to Cu-exposure.

Invertebrate circulating cells and the comet assay were found to be sensitive to oxidative stress, because the positive control induced significant changes to control in each study. Secondly, the comet assay was sensitive to detect differences in species susceptibility in all experiments.

The applicability of the comet assay to quantify DNA strand breaks in the circulating cells from the tunicate *Ciona intestinalis* and individual copepods was demonstrated.

In conclusion, the results of this study showed the sensitivity of invertebrate hemocytes/coelomocytes varied between contaminants and between species. Oxidative stress was a clear inducer of DNA strand breaks, while future studies should investigate the potential genotoxicity of low molecular weight PAHs in *in vivo* systems.

The present thesis contributes basic knowledge on species responses to common contaminants/stressors, which may help in the evaluation of the choice of a sensitive model species.

Cu toxicity is predicted to increase in the next century due to ocean acidification effects on free cupric ion availability. The potential inducibility of DNA strand breaks by copper in other marine invertebrates should be considered, since to my knowledge, only few studies are available in the literature and DNA damage and repair related genes and enzyme induction have been documented in various invertebrates.



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