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Oxidative damage in gill of *Mytilus edulis* from Merseyside, UK, and reversibility after depuration

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Oxidative damage to DNA and lipids was greater in gill of mussels of a polluted site compared to a cleaner one and it was reduced by subsequent depuration.

Abstract

Mussels were collected from the urban/industrialized site of New Brighton, Merseyside and the relatively non-industrial site of Llandudno, North Wales. All mussels were identified as *Mytilus edulis* by PCR amplification of *Mefp1*. DNA single strand breaks and 8-oxo-7,8-dihydro-2′-deoxyguanosine were measured in gill within 24 h of collection, using the COMET assay, both with and without formamidopyrimidine glycosylase. Gill lipid peroxidation was also measured within 24 h. No difference between sites was found for frank SSB and malonaldehyde levels, however 8-oxo-dG and 4-hydroxynonenal were significantly greater in New Brighton mussels compared to Llandudno mussels. After 1-month laboratory maintenance, lipid peroxidation and 8-oxo-dG levels were lower. In contrast, frank SSB were higher. This could reflect enhanced DNA repair excision, though we cannot exclude the possibility of other non-oxidative DNA damage. The results suggest that laboratory maintenance allows recovery from environmentally induced oxidative damage, which was more extensive at Merseyside.

Keywords: Mussel; Comet; Lipid peroxidation; 8-oxo-dG; SSB

1. Introduction

Bivalves (oysters, hard clams and mussels) are valuable organisms for environmental monitoring and, as such, have been used for many years for this purpose. Environmental pollution can have detrimental consequences on these organisms. DNA damage is of particular concern because it can give rise to heritable effects as well as potential diseases. Some of the most commonly measured DNA lesions are single strand breaks (SSB). SSB are sensitive biomarkers of genotoxicity and can reflect the presence of potentially pre-mutagenic lesions (Bolognesi et al., 1996; Mitchelmore and Chipman, 1998). Oxidative DNA damage, in the form of modified bases, is another important category of DNA modification and has been related to carcinogenesis as well as to the ageing process (Cooke et al., 2003). 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxo-dG) is the main lesion caused by oxidation of deoxyguanosine and is highly mutagenic since it can mispair with deoxyadenine at the same rate to its match with deoxycytosine (Kawanishi et al., 2001). SSB can be measured by the alkaline 'comet' assay, a method that is simple, quick, sensitive and cost effective (Mitchelmore and Chipman, 1998). 8-oxo-dG levels can also be quantified indirectly using the ''coupled'' comet assay with the aid of the lesion-specific glycosylase formamidopyrimidine glycosylase (Fpg) (Collins et al., 1997). The enzyme recognizes the oxidized bases and because of its glycosylase activity cleaves them, resulting in additional SSB. Lipid membranes are another possible target for oxidative damage and substantial lipid peroxidation has been noted in mussels after *in vivo* exposure to various heavy metals (Viarengo et al., 1996). Gill lipid peroxidation may therefore be a reliable marker of oxidative stress in mussels (e.g. Gravato et al., 2005).

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The aim of our project was to compare indigenous mussels collected from the urban/industrialized site of New Brighton, Merseyside, UK to the reference site of Llandudno, North Wales regarding baseline DNA SSB and 8-oxo-dG. Lipid peroxidation was also compared as a general indicator of oxidative stress. The tests were repeated at two time-points: within 24 h post-collection and after 1-month maintenance in clean laboratory conditions. We tested the hypothesis that mussels from the relatively polluted site have relatively high macromolecular oxidation and that this could be lowered by depuration. The populations were verified to contain pure *Mytilus edulis* specimens by PCR amplification of the non-repetitive domain of the *Mefp-1* gene (Inoue et al., 1995).

2. Materials and methods

2.1. Reagents

Sodium chloride (NaCl), ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), Tris buffer, sodium hydroxide (NaOH), ethidium bromide, phosphate-buffered saline (PBS), *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), potassium chloride (KCl), dimethylsulfoxide (DMSO), Triton X, sodium lauryl sarcosinate and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Poole, UK). Magnesium chloride ($MgCl_2$) was purchased from BDH Chemicals (Poole, UK). Normal melting point agarose (NMPA), low melting point agarose (LMPA), Taq polymerase, 10 mM dNTPs, 10× NH_4 reaction buffer, 50 mM $MgCl_2$ were from Bioline (London, UK). Formamidopyrimidine glycosylase (Fpg) was purchased from Trevigen (Gaithersburg, USA), primers from Alta Bioscience (Birmingham, UK) and total DNA isolation kit from Qiagen (Crawley, UK). Lipid peroxidation was measured by Calbiochem Lipid Peroxidation Assay Kit (Darmstadt, Germany).

2.2. Mussel sampling and maintenance

Mussels of similar size (4.0–4.5 cm) were handpicked from Llandudno (LL), North Wales (a reference site) and New Brighton (NB), Liverpool, UK (an industrialized site) on the same day and at the same intertidal region and transported to our laboratory in perspex boxes on ice in late July. After overnight acclimatization in running seawater a subset of the animals were sacrificed and assays were performed within 24 h of collection. The rest of the animals were maintained in 20 l plastic tanks of running seawater (16 °C, pH 8.4, salinity 34‰) at low densities (40 mussels per aquarium, maximum) with a light regime of 12 h light/dark for 1 month. The mussels were fed with a combination of powdered kelp pellets (2 g) and 1 ml per 6 l of *Nanochloropsis oculata* (cell density 250×10^6 cells/ml, cell diameter ~2.5 μ, Shirley Aquatics, Birmingham, UK) every 2 days. Nitrate levels were checked every week (Nitrate test kit, Aquatic Pharmaceuticals, Canada). Some differences in contamination levels between the two sites are depicted in Table 1.

2.3. Mefp-1 based species determination

Genomic DNA was isolated from soft mussel tissues using the Total DNA Isolation Kit (Qiagen) according to the manufacturer's instructions. The presence of genomic DNA in the final eluant was verified by UV spectroscopy (UVIKON Spectrophotometer 922, Kontron Instruments, UK) by measuring absorbance at 260 nm. The standard PCR reaction mix contained 1 μl of genomic DNA, 0.02 units Taq polymerase, forward primer (10 pmol), reverse primer (10 pmol), 10 mM dNTPs (2 μl), 10× NH_4 reaction buffer (10 μl), 50 mM $MgCl_2$ (3 μl) and sterile water (to 50 μl). PCR reaction mixtures were initially denatured at 95 °C for 15 min followed by 30 cycles of: denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min (see Table 2 for individual annealing temperatures) and extension at 72 °C for 1.5 min. A final extension step was performed at 72 °C for 10 min, following completion of the cycling phase. All steps were performed in a PCR machine (Minicycler™, MJ Research, UK). The products of the PCR reaction were analysed by DNA gel electrophoresis [2% agarose in 1× TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM Na_2EDTA, pH 8.3)] containing ethidium bromide (0.5 μg/ml) at 80 V for 60 min and visualized by UV transillumination.

Table 1
Concentrations of various chemical contaminants in body tissues from specimens (*Mytilus edulis*) from New Brighton and Llandudno[a]

Chemical	New Brighton	Llandudno
Cu	8.9	5.0
Zn	147	50
Cd	0.37	Non-detected
Tributyltin	0.09	Non-detected
As	8.9	7.9
Hg	0.16	0.10
Dieldrin	0.016	Non-detected
Pb	3.6	2.0
DDT[b]	0.08	0.01
PCB[c]	0.184	0.004
PAH[d]	4.47 (max)/2.58	1.22
Se	3.3	10.4

All contaminants expressed as μg/g dry weight. Note: the oxidation-protective selenium was found to be higher at the reference site (adapted from Shaw et al., 2004; Widdows et al., 2002).

[a] Detection limit: Hg, 0.1; Cd, 0.01; tributyltin, 0.03; polychlorinated biphenyls, 0.002.

[b] DDT, 4,4′-(2,2,2-trichloroethane-1,1-diyl) bis (chlorobenzene); PAH, polyaromatic hydrocarbons; PCB, polychlorinated biphenyls.

[c] PCB, sum of 25 PCB congeners.

[d] PAH, sum of two- and three-ringed aromatic hydrocarbons.

2.4. ''Coupled'' COMET assay in mussel gill

Representative mussels were sacrificed and gill was collected. The animal valves were opened with a metal scalpel (17 cm) and gill tissues were extracted with small size metal tweezers. The comet assay protocol was essentially as described in Mitchelmore et al. (1998) with Fpg buffer as described in Gielazyn et al. (2003). Twin frosted microscope slides were coated with 0.5% normal melting point (NMP) agarose in phosphate buffered saline (PBS). Gill cells were suspended in HEPES buffered saline (HEPES 0.12 M, KCl 0.15 M and Na_2EDTA 6 mM, pH 7.2) and a fraction (15.0 μl) was added to preheated 0.5% low melting point (LMP) agarose in PBS (150 μl) and mixed. The solution was then evenly spread on a slide and a cover slip was added. The slides (4 slides per animal) remained on ice for 10 min at least, then the cover slips were removed and the slides were lowered into freshly made lysis solution for at least 1 h at 4 °C. Slides were rinsed in Fpg buffer (1 ml) three times (5 min each). Fpg enzyme (1 unit) in Fpg buffer (50 μl) was added to one in every pair of slides and Fpg buffer without enzyme (50 μl) was added to the remaining one. The slides were placed in a humid plastic box wrapped with foil and incubated for 1 h at 37 °C and then the slides were placed on a horizontal gel electrophoresis tank and covered with freshly made electrophoresis buffer allowing 30 min for DNA unwinding (pH 12.6; Miyamae et al., 1997). Electrophoresis was carried out at 30 V and 300 mA for 20 min. Slides were then removed and rinsed with neutralization buffer (1 ml each) three times (5 min each wash). Slides were then drained and

Table 2
Primers used in PCR-based characterisation of *Mytilus edulis*

Primer name	Primer DNA base sequence (5′→3′)	Predicted annealing temperature (°C)
Me 15	CCAGTATACAAACCTGTGAAGA	52.3
Me 16	TGTTGTCTTAATAGGTTTGTAAGA	51.9