Oxidative stress and macromolecular damage caused by pollutants and repair of oxidised DNA in the gill of *Mytilus edulis*

by

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Abstract

Mussels (*Mytilus edulis*) are able to bioaccumulate many chemicals including potential genotoxicants in their soft tissues. Certain genotoxicants damage DNA and other macromolecules via production of reactive oxygen species. Genotoxicants can also interfere with DNA repair processes in the cell. This project examined the potential of such agents to produce oxidative damage in gill of mussels both under laboratory and field exposures. Indigenous mussels from a polluted (New Brighton) and a reference (Llandudno) estuary were collected in 2005-2006 and were examined for DNA damage, lipid peroxidation, glutathione depletion and effects on repair of DNA damage (8-oxo-deoxyguanosine). From the parameters tested, 8-oxo-deoxyguanosine levels (assessed using the formamidopyrimidine glycosylase-modified comet assay) were higher in mussels from the polluted site only during summer whereas repair (cutting) of 8-oxo-deoxyguanosine in DNA was lower in the polluted site at most collection times. Lipid peroxidation varied according to season. Subsequent maintenance of mussels under clean laboratory conditions for one month abolished the differences between sites in most cases for all the parameters. *In vivo* exposure of mussels to the metals hexavalent chromium and cadmium also verified DNA damage via reactive oxygen species and the suppression of DNA repair mechanisms (towards either 8-oxo-deoxyguanosine or ethenoadenosine) was shown to be a potential contributor to genotoxicity. Overall, mussels were shown to be sensitive to the genotoxic potential of different aquatic pollutants. The study emphasises the need to measure specific oxidative DNA lesions rather than a simple measurement of frank DNA strand breaks and supports the strategy of "recovery" experiments to determine recoverable, field-induced damage to DNA and other macromolecules.

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Dedicated to Mum, Dad and George

Contents

Figures

Tables

List of abbreviations

$1N^2$εdG	$1,N^2$-ethenodeoxyguanosine
$^1O_2$	singlet oxygen
2-ABP	2-aminobiphenyl
2 OH ATP	2-hydroxy adenosine 5′ triphosphate
2 OH dATP	2-hydroxy deoxy adenosine 5′ triphosphate
3meA	3-methyl-adenine
3-PUA	3′-α,β unsaturated aldehyde
4-HNE	*trans*-4-hydroxy-2-nonenal
4-NQO	4-nitroquinoline-1-oxide
5′dRP	5-deoxyribose phosphate
7meA	7-methyladenine
7meG	7-methylguanine
8-oxo-dA	8-oxo-7,8 dihydro-2'-deoxyadenosine
8-oxo-dG	8-oxo-7,8-dihydro-2′-deoxyguanosine
^{32}P	^{32}phosphorus
α-MHCH	α-methyl hexachlorocyclohexane
β-HCH	β-hexachlorocyclohexane
γ-HCH	γ-hexachlorocyclohexane
εdA	$1,N^6$-ethenoadenosine
εdC	3, N^4-ethenodeoxycytidine
AAG	3-methyladenine *N*-glycosylase
AhR	aryl hydrocarbon receptor

AlkA	alkyl-adenine *N*-glycosylase
Asc	ascorbate
Asp	aspartic acid
APE1	human apurinic endonuclease 1
AP site	abasic site
BaP	benzo[a]pyrene
BER	base excision repair
BSA	bovine serum albumin
CAR	constitutively active receptor
CAT	catalase
$CdCl_2$	cadmium chloride
$CdSO_4$	cadmium sulphate
CpG	cytosine phosphate guanine
Cr(III)	trivalent chromium
Cr(V)	pentavalent chromium
Cr(VI)	hexavalent chromium
Cr(V)-EHBA	bis(2-ethyl-2-hydroxybutyrato)oxochromate(V)
$(CrO_4)^{2-}$	chromate anion
CYP450	cytochrome P-450
Cys	cysteine
DDT	dichloro-diphenyl-trichloroethane
DMT-1	divalent metal transporter protein 1
DMSO	dimethylsulfoxide
dRP	deoxyribosophosphate

dsDNA	double stranded DNA
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1
EROD	ethoxyresorufin-O-deethylase
Fapy	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FapyA	4,6-diamino-5-formamidopyrimidine
FapyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FEN1	flap structural specific endonuclease-1
Fpg	formamidopyrimidine glycosylase
GSH	glutathione (reduced)
Glu	glutamic acid
GR	glutathione reductase
GPx	glutathione peroxidase
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
HhH	helix-hairpin-helix
HIF-1	hypoxia inducible factor 1
HPLC	high performance liquid chromatography
$KBrO_3$	potassium bromate
k-ras	Kirsten rat sarcoma 2
LL	Llandudno
LMPA	low melting point agarose

Lys	lysine
LO·	lipid peroxyl radical
LPO	lipid peroxidation
M1dG	N^2, 3-ethenodeoxyguanosine
MDA	malondialdehyde
Mefp-1	*Mytilus edulis* foot protein 1
Met	methionine
MMR	mismatch repair pathway
MMS	methanosulfonic acid
MN	micronucleus test
MSA	methanosulfonic acid
MSH2	mutS homolog 2
MT	metallothionein
MTF1	metal responsive transcription factor 1
N^2-3εdG	N^2, 3-ethenodeoxyguanosine
$Na_2Cr_2O_7$	sodium dichromate
NAD(P)H	reduced nicotinamide adenine dinucleotide
NB	New Brighton
NER	nucleotide excision repair
NF-κB	nuclear Factor kappa B
NMPA	normal melting point agarose
NO·	nitric oxide
O_2	molecular oxygen
$O_2^{\cdot -}$	superoxide anion

OGG1	8-oxoguanine DNA glycosylase 1
OGG2	8-oxoguanine DNA glycosylase 2
OH·	hydroxyl radical
·OONO	peroxynitrite
OPT	*o*-phthalaldehyde
PAH	polyaromatic hydrocarbons
PAPR-1	poly (ADP Ribose) Polymerase-1
PCB	polychlorinated biphenyls
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PNK	polynucleotide kinase
PXR	pregnane-X receptor
ROO·	peroxyl radical
ROS	reactive oxygen species
SOD	superoxide dismutase
Sp1	specificity protein 1
SSB	single strand breaks
TBAR	thiobarbituric acid reactive substances
TBT	tributyl-tin
TFIIA	transcription factor II A
TG	thymine glycol
TGG1	thymine glycol glycosylase 1
TGG2	thymine glycol glycosylase 2
TOSC	total oxyradical scavenging capacity

Tyr	tyrosine
USF-1	upstream stimulator factor
UV	ultra-violet
Val	valine
XRCC1	X-ray cross-complementing group 1
XPA	xeroderma pigmentosum, complementation group A

1.0 General Introduction

1.1 Mussels and their use in pollution monitoring programs

Bivalves such as oysters, hard clams and mussels are valuable organisms for environmental monitoring and as such have been used for many years. Mollusks in particular are widely distributed in marine waters of the northern hemisphere (Wilson *et al.*, 1998; Rank and Jensen, 2003) and are easy to collect (Klobuçar *et al.*, 2003). They are filter-feeding and can bioaccumulate contaminants (Labienec *et al.*, 2003; Gielazyn *et al.*, 2003), especially metals (Torres *et al.*, 2002). Furthermore, they have a suitable size for biochemical analysis and they are tolerant both to reduced salinity conditions (Wilson *et al.*, 1998) and to a wide range of pollutants, the latter partly because of their remarkably active immune system (Wooton *et al.*, 2003).

For many years these organisms (*Mytilus edulis* L. and other mytilid species) have been used as bioindicators of chemical pollution of coastal waters in the Mussel Watch Programmes (Canova *et al.*, 1998). The Mussel Watch Program, originally designed by Goldberg in 1975 consists of the use of marine mollusk bivalves to test for qualitative and quantitative existence of numerous aquatic pollutants (LePennec and LePennec, 2003). Because mollusks are able to concentrate various chemicals from their surrounding environment, the subsequent analysis is more sensitive than that for water (Baumard *et al.*, 1999). Also they are sessile and fairly resistant to chemical pollution and can be found even in places where other less hardy species cannot operate (O'Connor, 2002). In Mussel Watch Programmes, specimens of indigenous or transplanted mussels are collected and the levels of pollutants in the pooled tissues are determined. The results are then compared to the levels expected to prevail in the region examined and/or the acceptable tissue levels set for mussel consumption (e.g from Food and Drug Administration, US), and deductions regarding

plan of action are made. These programmes have been, and still are, important tools in monitoring of environmental pollution. Evidence of their global scale employment and their abundant use is depicted in following table.

Species	Area	Pollutant	Detection Method	Reference
M.edulis	Shetland Islands (transplanted)	PAH	GC-MS	Webster et al, 1997
M.edulis *M.galloprovincialis*	Baltic Sea, Atlantic Coast, Mediterranean Sea	PAH	GC-MS	Baumard et al, 1999
M.edulis	Mersey Estuary	PCB	CG-ECD	Connor et al, 2001
30 molluscan species	West Taiwan Coasts	Cu, Zn, Cd, Pb, Ni, Cr, As, Sn	AAS DPASV	Hung et al, 2001
M.edulis *M.galloprovincialis*	Basque Coast, Spain	Cd, Cu, Zn, Pb, Cr, Ni, Hg, As, Ag	AAS	Franco et al, 2002
M.edulis	South Korea Coasts	BT	GC-FPD	Hong et al, 2002
M.trossulus *C.grayanus*	Okhotsk Sea	Cd, Cu, Fe, Pb, Mn, Ni, Zn	AAS	Kavun et al, 2002
P.perna	Rio de Janeiro Bay	Hg, MeHg	AAS GC-ECD	Kehring et al, 2002
M.edulis *M.coruscus*	South Korea Coasts	CP, PCB	GC-MS	Kim et al, 2002
M.edulis	Bay of Fundy, Canada	PAH, PCB	CG-MS	Chou et al, 2003
Perna viridis	Asian coasts	PCB, CP	GC-ECD	Monirith et al, 2003
M.edulis	Arctic Sea	Pb, Ni, Cd, Cu	AAS	Zauke et al, 2003
P.perna	Atlantic Coast	Cd, Cu, Pb, Hg, Zn	AAS	Banaoui et al, 2004
M.galloprovincialis	Sardinia Coast (transplanted)	Hg, MeHg	GC-MS	Ipolyi et al, 2004
Brachidontes sp.	Egyptian Red Sea	CP, PCB	GC-ECD	Khaled et al, 2004
M.galloprovincialis	Black Sea	CP, PCB	GC-ECD	Kurt and Ozcok, 2004
P.viridis	Malaysian Coasts	Cd, Cu, Pb, Zn	AAS	Yap and Tan, 2004
M.edulis *M.galloprovincialis*	Atlantic Coast English Channel Mediterranean Sea	Ag	AAS	Chifolleau et al, 2005
M.californianus	San Fransisco Estuary (transplanted)	PAH, PCB	GC-MS	Oros and Ross, 2005
M.edulis	English Channel Southern North Sea	PCB	GC-MS	Danis et al, 2006
M.edulis *M.galloprovincialis*	English Channel Atlantic Coast Mediterranean Sea	PBDE	GC-MS	Johansson et al, 2006
M.galloprovincialis	Adriatic Sea	Pb, As, Cr, Mn, Fe, Ni, Cu, Zn	EDXRF	Orescanin et al, 2006

Abbreviations: AAS (Atomic Absorption Spectroscopy), BT (Butyl Tin), CP (Organochlorine pesticides), DPASV (Differential Pulse Anode Strip Voltametry), EDXRF (Energy Dispersive X-ray Fluorescence), GC-ECD (Gas Chromatography-Electron Capture Detector), GC-FPD (Gas Chromatography-Flame Photometric Detector), GC-MS (Gas Chromatography-Mass Spectroscopy), MeHg (Methylmercury), PAH (Polyaromatic Hydrocarbons), PCB (Polychlorinated Biphenyls).

1.2 The blue mussel (*Mytilus edulis*)

Our organism of interest is one molluscan species commonly found in UK, the blue mussel. However we note that the word "mussel" does not have real taxonomic status and can describe several unrelated taxa. In this way, the freshwater mussels of the superfamily *Unionaceae* or the "zebra mussels" of the superfamily *Dreissenaceae* are also referred to as mussels and are commonly used in ecotoxicology studies. However the "real" mussels belong to the family *Mytilidae*, a diverse group of bivalves with broad distribution in marine waters which can further be divided into 4 separate subfamilies: *Mytilinae, Modiolinae, Lithophaginae* and *Crenellinae* (Distel, 2000). The common ancestor of these and other molluscs is thought to be *Fordilla troyensis* (Gosling, 1992). The calcification of the external skeleton of bivalves which gave rise to the shape and morphology of mussel as we know it today, happened between 545 and 535 million years ago as a result of higher atmospheric oxygen, increasing calcium levels in the ocean and active predation against bivalves (Marin *et al.*, 1996).

The blue mussel *Mytilus edulis* belongs to the *Mytilinae* subfamily and possesses some distinct anatomic characteristic like an inequilateral shell with beaks at the anterior end. The colour of the shell varies from purple to blue and sometimes brown on the outside to pearl-white on the inside and the length of the shell varies from less than 5.08 to 22.86 cm (Tebble, 1966). The gills are flat and filamentous and serve both for respiration and feeding (Gosling, 1992), whereas the digestive gland is found in the visceral mass of the mussel and is the major tissue of xenobiotic metabolism and biotransformation enzymes (Mitchelmore *et al.*, 1998b).

M. edulis existence is hindered by high temperature with an upper thermal tolerance limit of 29°C (Almada-Villela *et al.*, 1982) however this organism is found further than earlier suspected. The animal is present in Iceland and Hudson Bay and it may be present in Greenland, Novaya Zemlya and the Arctic coast of Canada. In the southern hemisphere it has been found in Falkland Islands and along the east and west coast of South America. (Seed, 1992)

Mussels have a clearly defined annual reproductive cycle and are sexually mature after their first year of growth (Brake *et al.*, 2004). However this cycle changes both from year to year and from habitat to habitat (Bayne, 1976). There are two separate sexes in *Mytilus* even though hermaphrodites exist in a small percentage (Gosling, 1992). Triploid individuals are also found in *Mytilus* species but they are sterile (Brake *et al.*, 2004). Reproductive cycle is influenced by complex interactions between hormonal cycle, genotype, salinity, ambient temperature and food availability (Gosling, 1992). *Mytilus* meat weight and condition indices are, in their turn, significantly affected by reproductive activity and by food availability (Okumuş and Stirling, 1998)

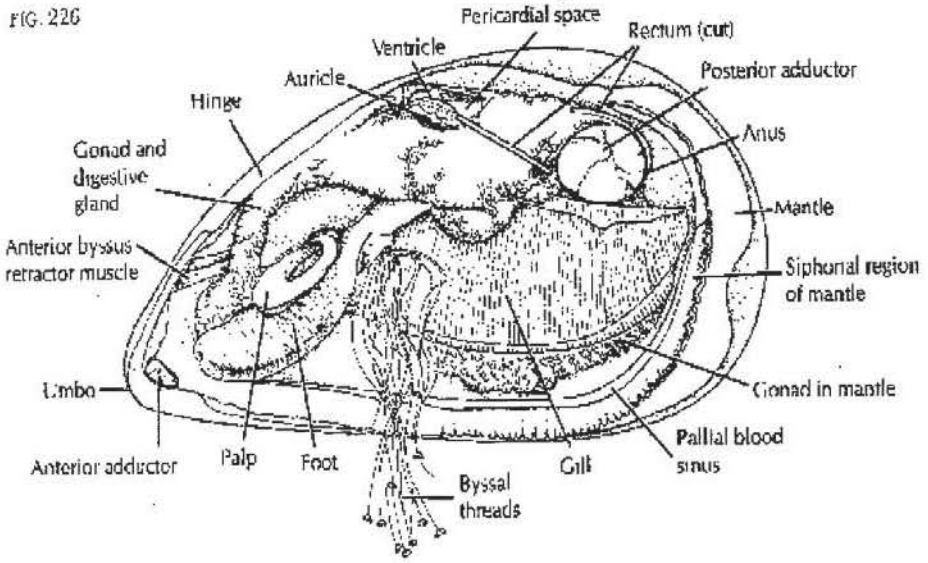


Figure 1: Schematic representation of *Mytilus edulis* anatomy (From Pierce and Maugel, 1987: www.usm.maine.edu/~gainey/mytc.gif).

1.3 Biomarkers of effect in mussels

The fact that bivalves are able to bioaccumulate pollutants in their tissues has consequences both on the health of the animal (Dyrynda *et al.*, 1998; St-Jean *et al.*, 2004) and on the structure and size of the population in the long term (Kurelec, 1993; Ma *et al.*, 2000). A reliable evaluation of these effects is possible through the integrated use of relevant biomarkers. Biomarkers can be defined as "measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response" (McCarthy and Shugart, 1990). Biomarkers of effect measure a toxic response or a disease progression as a consequence of exposure to pollutants. DNA damage is a well-known biomarker of effect and is of particular concern because it can give rise to heritable effects as well as potential diseases. Formation of a variety of DNA lesions including strand breaks (SSB), base modifications and bulky DNA adducts as well as

gross alterations (chromosomal aberrations or micronuclei formation) has been well documented in aquatic animals after exposure to genotoxic chemicals and they point towards a link between aquatic pollution and genetic related disorders (Marsh *et al.*, 1992). DNA adducts for example may be used to determine the critical levels of environmental genotoxins that have reached the target tissue, integrating also the influence of a number of pharmacokinetic factors (Harvey and Parry, 1998; Farmer, 2004). When mussels (*Mytilus* sp.) were the experimental model, substantial but not persistent rise in the level of bulky adducts (dG-C8-4NQO) was successfully induced after treatment with 4-nitro-quinoline 1-oxide (4-NQO) as measured by ^{32}P post-labeling (Harvey and Parry, 1998b) and similar and more persistent results were found when *Mytilus galloprovincialis* or *Mytilus edulis* was exposed to benzo[a]pyrene (BaP) (Akcha *et al.*, 2000c; Skarphéðinsdottir *et al.*, 2003 respectively). Also wild mussels (*Mytilus edulis*) from Reykjavik harbor showed increased DNA adduct levels in their gills compared to pristine sites (Ericson *et al.*, 2002). In contrast, some other field results were less promising: in a field survey after an oil spill, vertebrate fish species showed an elevated incidence of adducts whereas the invertebrates *Halichondria panacea* and *Mytilus edulis* did not, possibly with the aid of a multixenobiotic resistance mechanism (Harvey *et al.*, 1999). We have to mention though that the analysis was performed on whole body tissue and not on specific organs (gills, digestive gland) in direct contact with the spill. However, the fact that the metabolism of well-known carcinogens is relatively slow in bivalves in comparison to fish or mammals, enables them to accumulate xenobiotics with limited adduct formation (Dolcetti *et al.*, 2002) and perplexes even more the relationship between genotoxic exposure and effect. In general, however, DNA damage evaluation

in mussels is a reliable and indicative marker of exposure to pollutants that can interfere with DNA integrity.

1.4 Mussel disorders potentially related to DNA damage by pollutants

Pollutants may not only directly influence the genetic constitution of the nucleus of the individual cell but also lead to whole-organism adverse effects. Genotoxic effects in somatic cells may lead to potential dysfunction, cell death, organ failure and organism morbidity. In contrast, genotoxic effects in germ cells may be passed on to future generations resulting in a "forced" allele selection (in relation to normal evolutionary rates) (Depledge, 1998). Both scenarios pose an important ecological threat on aquatic animals. Some possible disorder outcomes of DNA damage are cited below

1.4.1 Haemocytic leukaemia

Haemocytic leukaemia has been reported in 15 marine or estuarine molluscan species and is characterised by abnormal proliferation of large nucleated circulating cells (Ciocan and Sunila, 2005). The neoplastic cells gradually replace all normal haemocytes and the disease is fatal for the organism. Genes similar to mammalian p53 have been found in *Mya arenaria*, *Spisula solidissima* and *Mytilus* sp. (Dondero *et al.*, 2006) and mutated p53 alleles were found in cancerous *Mya* cells (St-Jean *et al.*, 2005). Molluscan p53 probably has the same role as the mammalian p53 in monitoring the DNA integrity of the cells and initiating DNA repair or apoptosis (Boelsterli, 2003). The origin of cancer in mollusk remains controversial (Smolowitz *et al.*, 1989; Depledge, 1998; St-Jean *et al.*, 2004). Quinones created during the intracellular metabolism of the common contaminant benzo(a)pyrene (BaP) by *Mytilus edulis* tissues (Chapter 1.9.1.3 vi) possess mutagenic capacities and in the

clam (*Mercenaria mercenaria*) BaP is metabolised to a direct bacterial mutagen (Stegeman, 1985). Aromatic amines (such as 2-aminofluorene) are also activated through metabolism in the freshwater mussel *Dreissena polymorpha* (Britvic and Kurelec, 1999) and in *Mytilus edulis* (Marsh *et al.*, 1992) to mutagenic metabolites as detected by *Salmonella typhimurium* in the Ames test. However, a relationship between pollution and neoplasia in marine invertebrates has not yet been proven (Depledge, 1998). St-Jean *et al.*, (2004) found a strong correlation between leukaemia induction in transplanted mussels exposed to municipal and industrial wastes whereas Krishnakumar *et al.*, (1999) did not find any significant link between body burdens of PAH, PCB and metals and neoplasia prevalence. Furthermore, chronic *in vivo* exposure of *Mytilus edulis* to polyaromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB) failed to induce tumours (Krishnakumar *et al.*, 1999). What should be taken into consideration though is that most studies were performed on molluscs, which represent only a small portion of invertebrate species and until today little is known about neoplasia incidents in other members of the invertebrate phyla (Depledge, 1996).

1.4.2 Genotoxic Disease Syndrome

Cancer is generally uncommon in marine invertebrates (Stegeman, 1985). On the other hand the Genotoxic Disease Syndrome as first described by Kurelec is the clearest manifestation of the detrimental impact of pollution on invertebrates. The Genotoxic Disease Syndrome (a series of consequences of pollution) includes some or all of the following: "impairments in enzyme function, altered protein turnover, impairment of general metabolism, production of initiators of cytotoxic injuries, inhibition of growth, degenerative processes and atrophy in tissues and organs, decreased scope for growth, faster ageing, decreased fitness and well being,

impairments in immunoresponse and reproduction, increased frequency of diseases and neoplasia, impairment of adaptation, survival and succession and finally extinction of species" (Kurelec, 1993). Actual field experiments on oysters from a PAH-contaminated site have indeed shown that these animals have a lower phagocytotic ability compared to those dwelling in reference sites (Sami *et al.*, 1993). Grundy *et al.*, (1996) reached similar conclusions after observations on heavily PAH-burdened mussels that exhibited pronounced lysosomal damage and reduced resistance to infectious diseases. PAH comprise an important group of aquatic contaminants and light-activated PAH caused slower growth of mussel soft-tissues (Steinert *et al.*, 1998b). BaP, an aromatic hydrocarbon and common aquatic pollutant, apart from its apparent genotoxicity (Chapter 1.9.1.3), can act as an endocrine disruptor in invertebrates: Blue mussels experimentally exposed to PAH showed toxic effects in their gonads in the form of degenerated ovarian follicles in females, melanomacrophage-like centres in males, induction of vitellogenin-like proteins in both sexes and probable endocrine disruption in chronic terms (Aarab *et al.*, 2004). Furthermore, a high production of reactive oxygen metabolites was recorded in blue mussels after laboratory exposure to the aromatic hydrocarbon fluoranthrene (Coles *et al.*, 1994). Finally mussels (*Mytilus edulis*) from metal-contaminated sites exhibited increased reactive oxygen species (ROS) production from their haemocytes (Dyrynda *et al.*, 1998) and haemocyte cytoskeletal disruption in *Mytilus galloprovincialis* was evident after Cd or Cu treatment (Gómez-Mendikute and Carajaville, 2003). All these field or *in vivo* observations show a general trend: pollution affects negatively many physiological and biochemical mechanisms of the animal, particularly via impairment of its immune system and via increase of cytotoxic products.

1.4.3 Alterations in genetic structure

The ultimate problem of pollution is its impact on the level of the population (size, structure) rather than on the level of the individual. Polluted environments pose an intense pressure to their inhabitants. Resistance is therefore developed in these organisms and its basis could be either physiological/epigenetic acclimatisation or genetic adaptation. (Wirgin and Waldman, 2004). Adaptation occurs at the population level leading to selection of more resistant genotypes so in each subsequent generation the resistant genotypes increase exponentially (Shugart and Theodorakis, 1996). For example, in the parthenogenic cladoceran *Daphnia magna*, there was a clear genotype-orientated selection after exposure to genotoxic chemicals: different genotypes showed different levels of resistance towards cadmium and dichloroaniline (Depledge, 1994). The genotypes recorded as resistant to cadmium however may be ill-fitted towards dichloroaniline and vice versa. Similar research on mosquitofish (*Gambusia holbrooki*) dwelling in metal polluted site showed a highly significant reduction in their genetic variability (Guttman, 1994). This loss of diversity gives rise to genetically depauperate populations probably unable to cope with different kinds of stressors and more susceptible to extinction even if, according to some authors, the process of genetic variability reduction can be slowly reversed when remediation takes place (Guttman, 1994). A correlation between loss of diversity (thus increased danger of extinction) and inbreeding has indeed been found and is given by the following formula

$$H_t/H_o = (1-1/[2N_e])^t = 1-F$$

where Ht is heterozygosity (or gene diversity) at generation *t*, Ho is initial heterozygosity, N_e the genetically effective population size and F the inbreeding coefficient. As a result, higher inbreeding leads to an exponential decrease of

heterozygosity in small size populations (Frankham, 2005). Mussels and especially *Mytilus edulis* are widely distributed (Rank and Jensen, 2003) and there is no imminent risk of extinction, however massive inbreeding of genetically identical populations could theoretically lead to a progeny less fit to cope with changes in stress, in a given dwelling site. Field observations with wild *Mytilus galloprovincialis* populations have indeed proven that mussels from polluted sites exhibited reduced genetic diversity in comparison with specimens from clean sites, even though population sizes were not yet afflicted (Ma *et al.*, 2000).

However, pollution does not always lead to a decrease of genetic diversity: Differences in genetic composition were found for a population of *Gambusia affinis* dwelling in a radionuclide-contaminated pond compared to reference sites with the contaminated samples exhibiting more genetic diversity (Shugart and Theodorakis, 1994). We have to note though that, even in this case, among the variations recorded in the pond, only a specific genotype was fitter than the others, showing the minimal DNA breakage and the maximal fecundity.

1.5 Reactive Oxygen Species (ROS)

1.5.1 ROS and their sources in the cell

Reactive oxygen species is a term collectively used to describe (free) oxygen-containing radical species or species that can produce radicals (Halliwell and Aruoma, 1991). Free radicals are molecules that contain one or more unpaired electrons. These electrons give to the moiety a considerable degree of reactivity compared to non-radicals (Guetens *et al.*, 2002; Valko *et al.*, 2004). Molecular oxygen itself is a free radical since it contains two unpaired electrons (McCord, 2000). Species like

hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) (Wang *et al.*, 1998; Slupphaug *et al.*, 2003), singlet oxygen $^1O_2$ (Cadet *et al.*, 1999) and O_2 (Halliwell, 1999) can all be classified as ROS.

Free radicals are continually produced in the cell. Aged mitochondria significantly contribute to intracellular ROS burden due to compromised membrane integrity and consequently intense ROS release (Cohen and Nyska, 2002). Other enzymes like xanthine oxidase produce $O_2^{\cdot -}$ and H_2O_2 during the oxidation of hypoxanthine to uric acid. Further possible sources are neutrophils and activated macrophages that release ROS during their "respiratory burst" phase against pathogens (Halliwell, 1999; Valko *et al.*, 2004), including myeloperoxidases, which catalyse hypochlorous acid production, (McCord, 2000; Valko *et al.*, 2004). This intense ROS production, which is essential for microbial elimination, may actually be damaging because of concomitant injury to adjacent cells (Ames *et al.*, 1993). Transition metals like Fe^{+2} and Cu^{+} found intracellularly, may cause cleavage of the O-O bond of H_2O_2 producing $OH^{\cdot}$, one of the most potent free radicals known (McCord, 2000). Finally, reaction of the weak free radical nitric oxide ($NO^{\cdot}$) with superoxide yields the highly reactive peroxynitrite ($^{-}OONO$), which is able to attack macromolecules like DNA (Halliwell, 1999). $NO^{\cdot}$ in its turn is produced during the oxidation of L-arginine by nitric oxide synthetases (Tamir and Tannenbaum, 1996). Exogenous agents are also responsible for superfluous ROS production. Ionising and non-ionising radiation are able to produce a variety of ROS including $^1O_2$, H_2O_2, $OH^{\cdot}$ and $O_2^{\cdot -}$ (Wang *et al.*, 1998). Pollutants like exhaust and cigarette smoke, industrial contaminants, certain drugs like bleomycin and adriamycin and a variety of xenobiotics are other major exogenous sources of ROS (Kohen and Nyska, 2002; Chapter 1.8-1.9). Finally,

oxidation of food in the gastrointestinal tract may lead to ROS by-products since food contains a number of pro-oxidants including peroxides, aldehydes, fatty acids and redox metals (Ames, 1986). A summary of the major free radicals produced in cells is given in Figure 2.

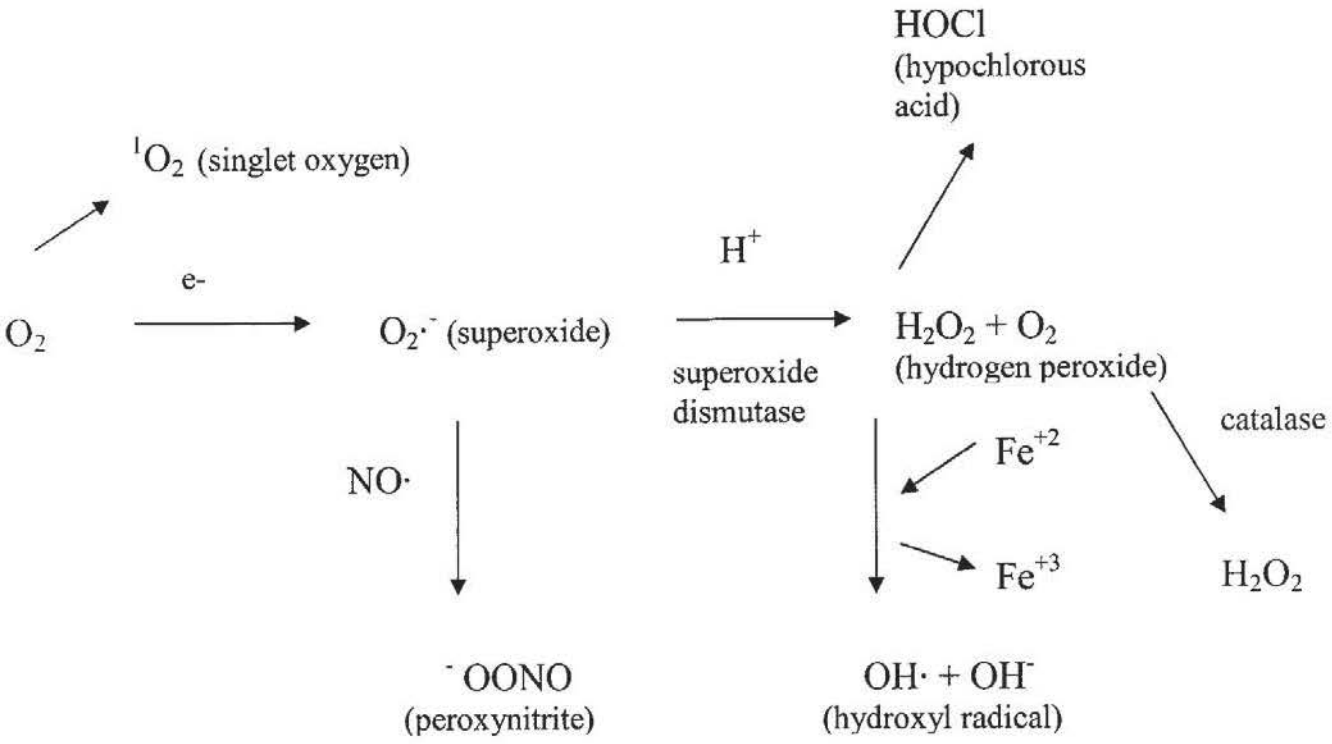


Figure 2: Schematic representation of most common ROS production

Some of the most important reactive oxygen species are cited below:

i) **Superoxide**: Oxygen itself has a low oxidizing potential and is incapable of reacting with DNA (Halliwell, 1999), but one of the primary produced ROS is $O_2\cdot^-$, which is created during the reduction of oxygen by other ROS or with the aid of xanthine oxidase. Then superoxide takes part in dismutation reactions: a superoxide radical reacts with another superoxide radical as depicted below by means of the enzyme superoxide dismutase (McCord, 2000; Kohen and Nyska, 2002)

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{2H^+} H_2O_2 + O_2 \quad k \sim 10^6\ M^{-1}s^{-1}$$

Furthermore, in a hydrophilic environment $O_2^{\cdot -}$ is able to reduce ferric (Fe^{+3}) to ferrous (Fe^{+2}) ions and under stress conditions is able to release Fe^{+2} from sequestering enzymes facilitating further the Fenton reaction (Valko *et al.*, 2006) as explained in the next sections.

ii) Hydrogen peroxide: H_2O_2 is produced through this dismutation reaction mentioned above. One molecule of H_2O_2 reacts with another H_2O_2 molecule yielding water and oxygen by means of the enzymes catalase and glutathione peroxidase, in a detoxification reaction (McCord, 2000, Valko *et al.*, 2004). Hydrogen peroxide may cause cellular damage directly by degradation and inactivation of haem proteins and oxidation of –SH groups and keto acids (Kohen and Nyska, 2002), however both $O_2^{\cdot -}$ and H_2O_2 are weak DNA oxidizers unless they are present in more than physiological quantities (Halliwell and Aruoma, 1991; Halliwell, 1999). DNA damage due to hydrogen peroxide therefore is indirect and it is limited in its capacity to undergo Fenton reactions yielding the highly active $OH^{\cdot}$ as depicted below

$$M^{n+} (=Cu^{+}, Fe^{+2}, Ti^{+3}, Co^{+2}) + H_2O_2 \rightarrow M^{(n+1)} (=Cu^{+2}, Fe^{+3}, Ti^{+4}, Co^{+3}) + OH^- + OH^{\cdot}$$

where M^{n+} is a transition metal, normally iron or copper (Valko *et al.*, 2004).

Under physiological pH most of the intracellular iron is oxidized and chelated in the form of Fe^{+3}. Reducing agents like ascorbic acid, metals in a higher oxidation state

and superoxide radicals [Chapter 1.5.1i)] enable the continual supply of Fe^{+2} for the Fenton reaction as depicted below

$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH\cdot + OH^-$ (1)

$O_2\cdot^- + Fe^{+3} \rightarrow O_2 + Fe^{+2}$ (2)

$O_2\cdot^- + H_2O_2 \rightarrow O_2 + OH\cdot + OH^-$

The sum of the reactions (1) and (2) is the Haber-Weiss reaction which can happen thermodynamically *in vivo* although at an extremely slow pace (Kohen and Nyska, 2002).

iii) Hydroxyl radical: The hydroxyl radical is produced during the course of the aforementioned reactions. Hydroxyl radicals may also be produced directly through decomposition of H_2O by ionizing radiation (Kawanishi *et al.*, 2001). The hydroxyl radical OH· is extremely reactive and *in vivo* it persists for less than 1 μsec (Guetens *et al.*, 2002). It can attack all cellular components and all subunits of DNA in various ways resulting in sugar radicals, altered sugars which give rise to alkali labile sites, abasic sites and modified bases and crosslinks of DNA with amino acids, to name a few (Halliwell and Aruoma, 1991; Cadet *et al.*, 1999; Chapter 1.5.3.1). Since OH· possesses high reactivity and low diffusibility it has been postulated that only metals bound closely to DNA or released from their chelators due to ROS attack are close enough to generate OH which will oxidise DNA (Halliwell and Aruoma, 1991).

1.5.2 ROS production in mussels

There are many xenobiotics found in the aquatic environment, which can produce ROS in living organisms. The limited removal of metabolites in invertebrates (Livingstone, 1998), may pose these animals in a precarious situation. ROS production by aquatic pollutants may be generated via one of the following mechanisms a) redox cycling of quinone-like xenobiotics by NAD(P)H-dependent reductases b) redox reactions of non-redox cycling compounds (e.g. Ni, Cr) with O_2 or pre-existing ROS c) uncoupling of enzyme oxidation (e.g. CYP450) d) enzyme induction (e.g. Phase I enzyme induction) e) uncoupling of oxidative phosphorylation f) depletion or deficiency of anti-oxidant mechanisms. Ionising radiation may also cause ROS or exacerbate a pro-oxidant situation (Livingstone, 2001; Manduzio *et al.*, 2005). Direct ROS production has been successfully evaluated *in vitro* in subcellular fractions of invertebrate and vertebrate organisms, however their production has seldom been measured in whole animals *in vivo* (Livingstone, 2001; Valavanidis *et al.*, 2006). On the other hand, manifestations of ROS effects [single strand breaks (SSB), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), lipid peroxidation, activity of antioxidant enzymes and depletion of antioxidant defences] are commonly used as biomarkers of exposure.

1.5.3 Macromolecular damage caused by ROS

1.5.3.1 Damage to DNA

i) **SSB:** Single strand breaks can be caused by a number of factors: direct action of various xenobiotics on DNA, action of xenobiotics after their biotransformation to genotoxic metabolites, attack by primary ROS, intrinsic excision of oxidative or alkylated lesions, intrinsic excision of other DNA modifications such as bulky adducts

or transformation of alkali labile sites in highly alkaline experimental conditions (Tice *et al.*, 2000; Winter *et al.*, 2004). SSB are therefore common DNA modifications (Mitchelmore *et al.*, 1998b). As a result, the detection of single strand breaks in individual cells has become a sensitive but not specific (Laffon *et al.*, 2005) biomarker of genotoxicity (Depledge, 1996; Coughlan *et al.*, 2002). Furthermore, SSB are an early snapshot of the consequences of a genotoxic agent (Nacci *et al.*, 1996; Nigro *et al.*, 2002) and are potentially pre-mutagenic lesions (Kammann *et al.*, 2000; Bolognesi *et al.*, 1996).

A frequent source of SSB is attack of DNA by ROS (Lee *et al.*, 1996; Horváthová *et al.*, 1998). The model pro-oxidant H_2O_2 was able to cause SSB in human lymphocytes *in vitro* (Singh *et al.*, 1988) and it has been used since as a SSB-inducing positive control. Ozone also caused SSB in alveolar carcinoma cells A549, possibly via H_2O_2 production (Cheng *et al.*, 2003). SSB were noted in the eel *Anguilla anguilla* after *in vivo* exposure to BaP or 2,3,7,8 tetrachlorodibenzo-p-dioxin and were negatively correlated with the capacity of the organism to neutralise OH· (Nigro *et al.*, 2002). Finally the model redox cycling agent nitrofurantoin (producing ROS during the course of redox reactions) caused SSB in *Salmo trutta* hepatocytes (Mitchelmore and Chipman, 1998).

From a mechanistic point of view, SSB are created by H abstraction from the sugar moiety by ROS, (Cadet *et al.*, 1999), which leads to fragmentation or loss of base [abasic (AP) site] that also fragments under alkaline conditions (Collins *et al.*, 1997; Kumaravel and Jha, 2006). A possible mechanism of SSB formation by attack at C4′ is depicted in Figure 3

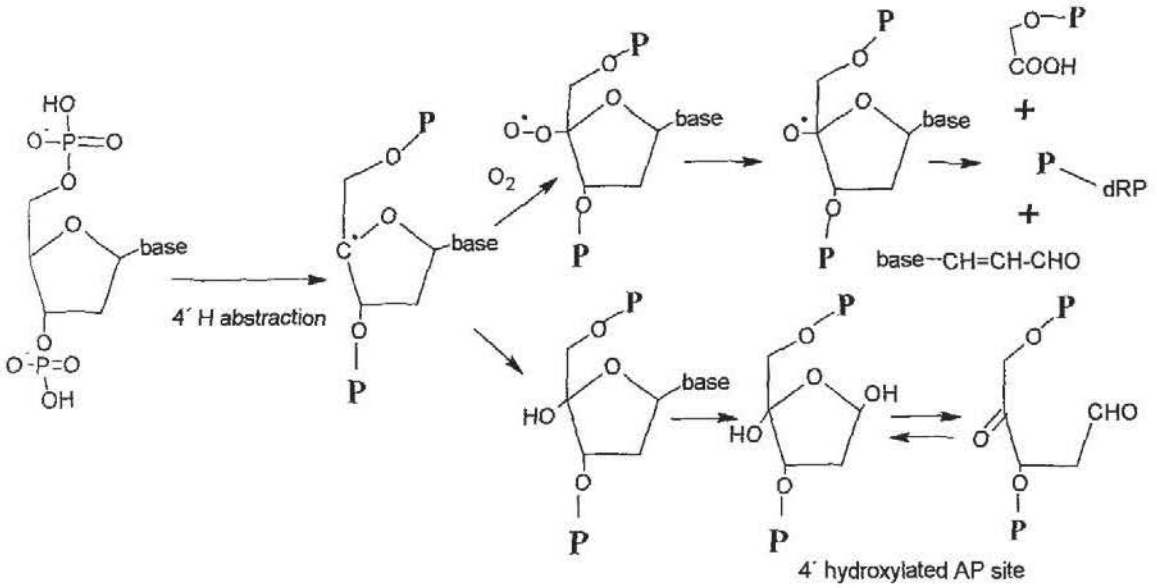


Figure 3: Schematic representation of H abstraction from C4′ by ROS and subsequent formation of SSB or AP sites (adapted from Casadevall *et al.*, 1999).

SSB may also be the indirect result of ROS-mediated base damage. Oxidative DNA damage in the form of modified purines or pyrimidines is attentively processed within the cell and the excision of the base by a bifunctional glycosylase leads to a "free" 5′ and a "blocked" 3′strand break (Izumi *et al.*, 2003; Chapter 1.6.1) as active intermediates. In the case of a monofunctional glycosylase SSB are also produced, this time further downstream, after the excision of the abasic site by an endonuclease (Memisoglu and Samson, 2000; Chapter 1.6.1).

ii) SSB in mussels: SSB are commonly measured in mussel tissues exposed *in vitro* to known or suspected genotoxic agents (Bihari *et al.*, 1990; Labieniec *et al.*, 2003) or in whole animals exposed *in vivo* to pollutants (Bolognesi *et al.*, 1998; Jha *et al.*, 2005). Field studies of indigenous or transplanted mussels from a polluted site (Large *et al.*, 2002; Akcha *et al.*, 2004) or from an impacted site after an ecological disaster

(e.g. oil spill) (Harvey *et al.*, 1999; Bolognesi *et al.*, 2006) are also frequent. SSB are mainly detected by the alkaline single cell gel electrophoresis (Comet assay) or the alkaline elution assay. Evidence of their global scale employment as a biomarker is depicted in the following table.

Species and tissue	Site	Change	Method	Reference
In vitro				
M. edulis digestive gland	BaP, H_2O_2, MX, 1-NP, NF	increase	SCGE	Mitchelmore et al. 1998
Unio tumidus gill	tannins	increase	SCGE	Labieniec et al. 2003
Scaphoria unequivalvis haemolymph	tin coumpounds	increase	SCGE	Gabbianelli et al. 2006
In vivo				
Mytilus galloprovincialis haemolymph	BaP, NQO	increase	Alkaline elution	Bihari et al. 1990
M edulis gill	Cu, MNNG	no increase, increase	Alkaline unwinding assay	Nacci et al. 1992
Mytilus galloprovincialis gills	DMBA	increase	Alkaline elution	Bolognesi et al. 1996
Patunopecten vessoensis, Tapes japonica gills	BaP, MNNG, EMS, MX	increase	SCGE	Sasaki et al. 1997
Mytilus galloprovincialis gill	Cu, Cd, Hg	increase	Alkaline elution	Bolognesi et al. 1999
Perna viridis digestive giand	BaP	increase	SCGE	Ching et al. 2001
Dreissena polymorpha haemolymph	PCP	increase	SCGE	Pavlica et al. 2001
Mytilus edulis digestive gland	crude oil fractions	increase	SCGE	Hamoutene et al. 2002
Mytilus edulis gill	$CdCl_2$, $CdCl_2$+H_2O_2	no increase, increase	SCGE	Pruski and Dixon, 2002
Unio tumidus digestive gland	PCP	increase	SCGE	Milowska et al. 2003
Mytilus edulis haemolymph	MMS	increase	SCGE	Rank and Jensen, 2003
Perna viridis haemolymph	BaP	increase	SCGE	Siu et al. 2003
Perna viridis digestive gland	Arocior 1254, BaP/Aroclor 1254	no increase/increase	SCGE	Siu et al. 2003
Mytilus edulis haemolymph	crude oil	increase	SCGE	Taban et al. 2004
Mytilus edulis haemolymph	TBTO	increase	SCGE	Hagger et al. 2005
Mytilus edulis haemolymph	tritiated water	increase	SCGE	Jha et al. 2005
Mice fed with *Mytilus* sp.	Erika oil spill	increase	SCGE	Lemiere et al. 2005
Corbicula fluminea gill, digestive gland, haemolympl.	MMS	increase	SCGE	Rigonato et al. 2005
Mytilus edulis gill	Cr(VI)	increase	SCGE	Emmanouil et a., 2006
Scaphoria uneauivalvis haemolympl.	Tin coumpounds	increase	SCGE	Gabbianelli et al. 2006
Limnoperna fortunei haemolympl.	PCP $CuSO_4$	increase increase	SCGE	Villela et al. 2006
Species and tissue	**Site**	**Change**	**Method**	**Reference**
Field				
Mytilus galloprovincialis (native) gills	New Bedford Harbour, Massachusetts	increase	Alkaline elution	Nacci et al. 1992
Anodonta grandis foot, mantle, adductor muscle	Strip mine pond, Oklahoma	no increase	Alkaline elution	Black et al. 1996
Mytilus galloprovincialis (native) gills	La Spezia Gulf, Italy	increase	Alkaline elution	Bolognesi et al. 1996
Patunopecten vessoensis, Tapes japonica (transplanted) gills	Tokyo, Osaka, Kobe	increase	SCGE	Sasaki et al. 1997
Mytilus edulis (transplanted) haemolymph	San Diego Bay	increase	SCGE	Steinert et al. 1998b
mussels (transplanted) haemolymph	PAH photoactivation/San Diego Bay	increase	SCGE	Steinert et al. 1998
Mytilus galloprovincialis (native) gills	Orbetello Lagoon, Italy	increase	SCGE	Frenzilli et al. 2001
Dreissena polymorpha (transplanted) haemolymph	River Sava, Croatia	increase	SCGE	Pavlica et al. 2001
Mytilus edulis (transplanted) gill	New Brighton, UK	increase	SCGE	Shaw et al. 2002
Dreissena polymorpha (transplanted) haemolymph	River Drava, Croatia	increase	SCGE	Klobucar et al. 2003
Mytilus edulis (native) digestive gland	Loch Leven, UK	no increase	SCGE	Large et al. 2003
Mytilus edulis (native) haemolymph	Koge Bay, Denmark	increase	SCGE	Rank and Jensen, 2003
Mytilus spp. (native) gill, haemolymph	French Atlantic Coast	no increase	SCGE	Akcha et al. 2003
Mytilus galloprovincialis (native, transplanted) gills	Ligurian Coast, Italy	increase, increase	Alkaline elution	Bolognesi et al. 2004
Mytilus edulis (transplanted) gill, haemolymph	Revkjavik Harbour	increase, increase/no increase	SCGE	Halldorson et al. 2004
Mytilus galloprovincialis (native) gill	Prestige oil spill	increase	SCGE	Laffon et al. 2005
Mytilus edulis (native) gill	Koge Bay, Denmark	increase	SCGE	Rank and Jensen, 2005
Mytilus galloprovincialis (native, transplanted) gill	Cecina River Estuary, Italy	increase, increase	SCGE	Nigro et al. 2006
Dreissena polymorpha, Mytilus edulis (native) haemolymph	Seine estuary	increase/no increase?	SCGE	Rocher et al. 2006

BaP: benzo[a]pyrene, Cd: cadmium, $CdCl_2$: cadmium chloride, Cu: copper, DMBA: dimethylbenzylidianthracene, EMS: ethylnitrosourea, H_2O_2: hydrogen peroxide, Hg: mercury, MMS: methanemethylsulphonate, MNNG: N-methyl-N'-nitrosoguanidine, MX: 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone, NF: nitrofurantoin, 1-NP: 1-nitropyrene, Pb: lead, PCP: pentachlorophenol, TBTO: tributyltin oxide

iii) Oxidative base modifications (e.g. 8-oxo-dG): DNA base modifications are the most common form of DNA damage after ROS exposure (Wang *et al.*, 1998) since hydroxyl radicals add to the double bonds of purines or pyrimidines at diffusion controlled rates (Evans *et al.*, 2004). Therefore both purines and pyrimidines can be modified by ROS leading to a wide variety of lethal and/or mutagenic lesions. The most common products of oxidative damage to DNA bases are depicted in Figure 4.

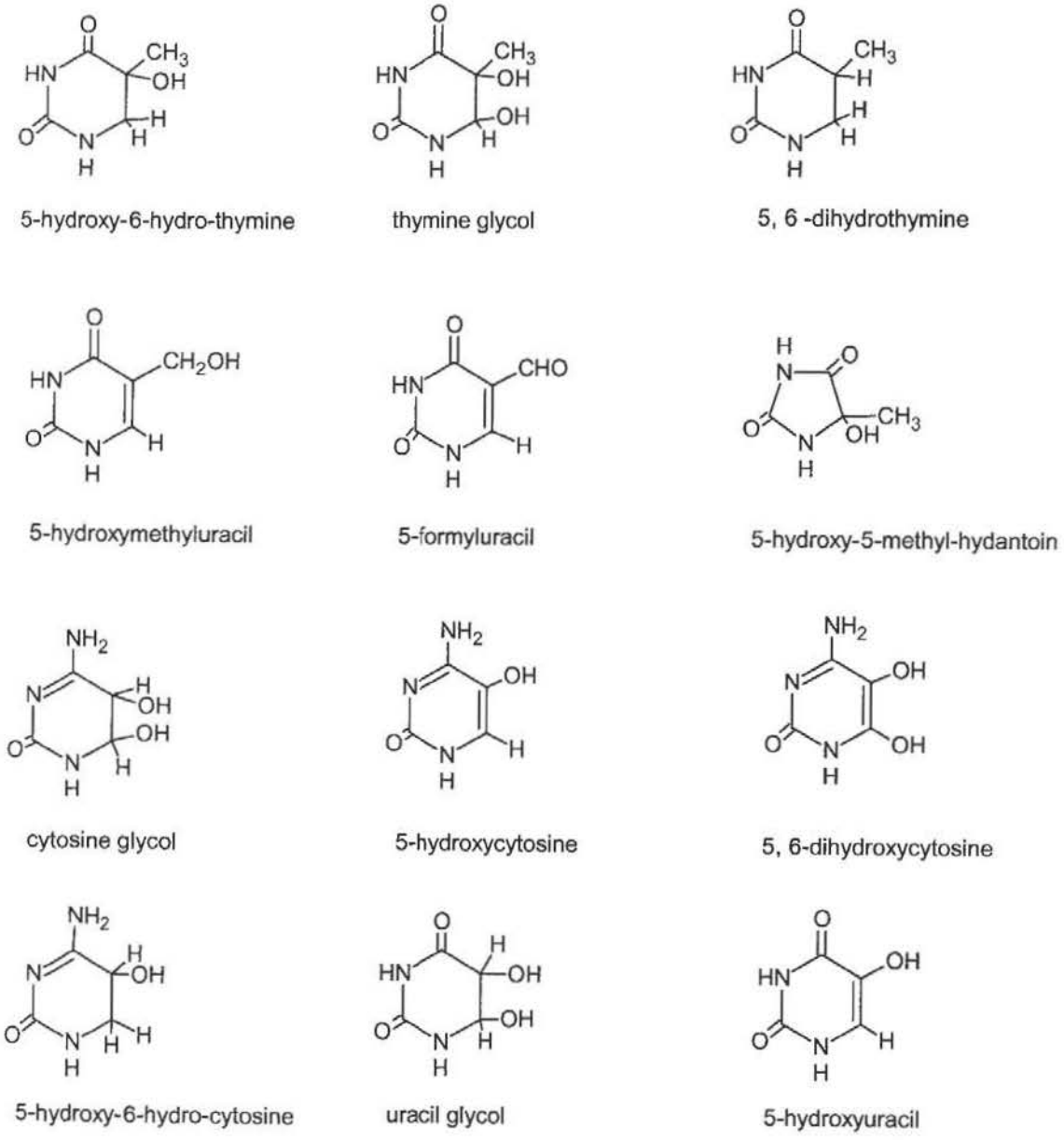


Figure 4a: Schematic representation of major pyrimidine modifications caused by ROS (adapted from Evans *et al.*, 2004).

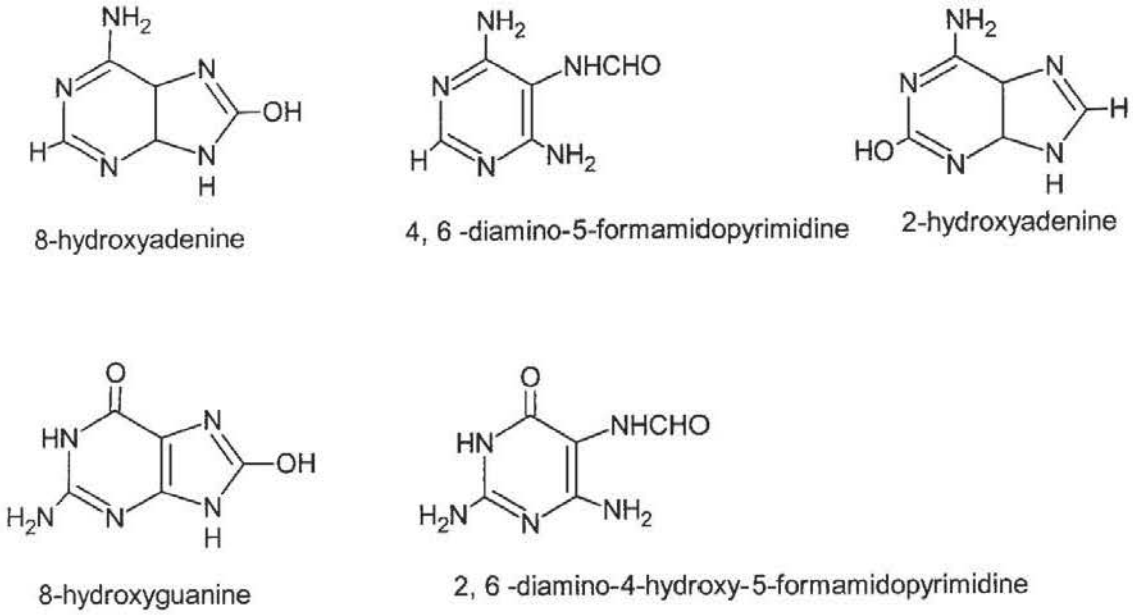


Figure 4b: Schematic representation of major purine modifications caused by ROS (adapted from Evans *et al.*, 2004).

Out of the four DNA bases, guanine has the lowest ionisation potential (Cadet *et al.*, 1999; Kawanishi *et al.*, 2001). Thus it can be easily oxidised by $^1O_2$, OH· and peroxynitrite (Cadet *et al.*, 1999). OH· can abstract H either from C8′ or from C4′ of guanosine so that in duplex DNA in aquatic solution there is a minor end product of an oxazolone and a major of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) respectively, with the latter in competitive equilibrium with the corresponding open-ring derivative formamidopyrimidine (Fapy) (Cadet *et al.*, 1999) and in keto-enol tautomerism with 8-hydroxyguanosine (Valko *et al.*, 2006).

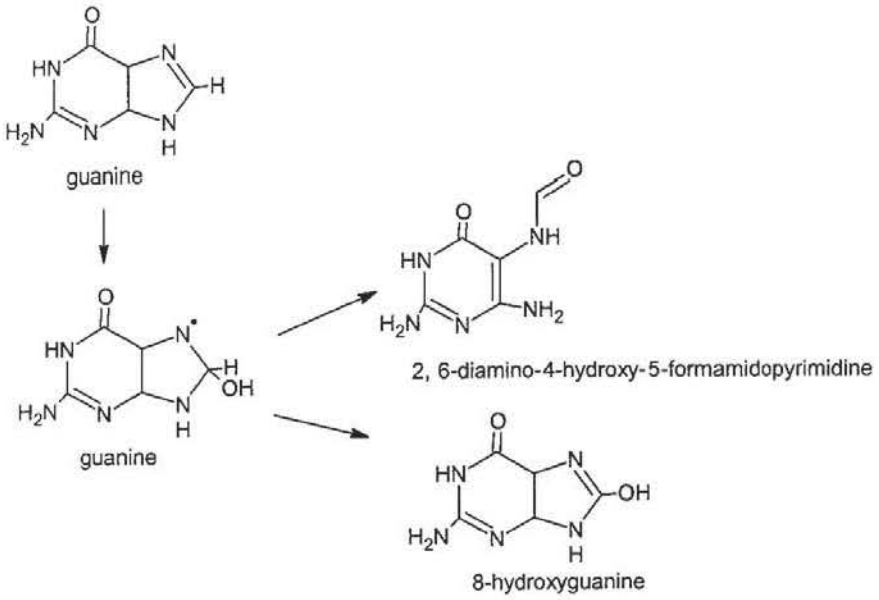


Figure 5: Formation and fate of the radical created by hydroxyl attack on C8′ of guanine. Oxidation generates 8-OH-dG in equilibrium with 8-oxo-dG and reduction the ring-opened product formamidopyrimidine (From Halliwell, 1999).

Oxidative DNA damage plays a crucial role in several human diseases, particularly cancer (Kawanishi *et al.*, 2001) and ageing (Cadet *et al.*, 1999). 8-oxo-dG seems to be an important intermediate because it mispairs with adenine and eventually leads to G-T transversions (Cheung *et al.*, 1992; Kasai, 1997; Kawanishi *et al.*, 2001). When 8-oxo-dG assumes a *syn* conformation it can form a stable Hoogsteen pair with A (Wang *et al.*, 1998). Point mutations like these facilitated by 8-oxo-dG mispairing have been found in K-ras and C-Raf1 oncogenes and in p53 and retinoblastoma tumor suppressor genes (Valko *et al.*, 2004). Furthermore, substitution of guanine with 8-oxo-dG in CpG dinucleotides blocked methylation of cytosine in nascent DNA strands. Abnormal methylation patterns may activate or inactivate oncogenes and tumor suppressor genes respectively and are commonly found in cancer cells (Cerda and Weitzman 1997). Cohort studies have also shown that there is a positive correlation between urine 8-oxo-dG levels and risk of lung cancer in non-smoking

individuals (Loft *et al.*, 2006). 8-oxo-dG levels measurement (base/nucleoside) therefore a good marker of oxidative DNA damage and potential for carcinogenesis, provided that it is done with accuracy (Kasai, 1997; Halliwell, 1999).

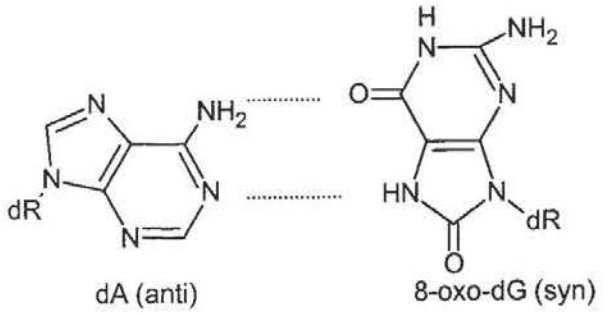


Figure 6: Base pairing properties of 8-oxo-dG with A (From Wang *et al.*, 1998).

iv) 8-oxo-dG in mussels: 8-oxo-dG as a biomarker of oxidative DNA damage has been observed in mussels either in *in vivo* exposures or in field pollution studies. For example, *M. galloprovincialis* exposed to BaP showed an increase in 8-oxo-dG levels in its digestive gland (Akcha *et al.*, 2000) but not in its gill (Akcha *et al.*, 2000c), via contaminated feed, or both in gill and digestive gland (Canova *et al.*, 1998; Livingstone, 2001). Other experimental studies also found an increase in 8-oxo-dG within the digestive gland of *M. galloprovincialis* after exposure to BaP, but this was not dose dependent (Machella *et al.*, 2005). Furthermore, transplanted mussels (*Unio tumidus*) to a contaminated site suffered from elevated gill 8-oxo-dG after 21 days of exposure (Charissou *et al.*, 2004). Indigenous mussels (*Perna perna*) from polluted sites also showed elevated digestive gland 8-oxo-dG when compared to a reference site (Torres *et al.*, 2002). Exposure to xenobiotics through water may be an important reason for the elevated oxidative DNA damage observed in mussels in comparison to mammalian DNA (de Almeida *et al.*, 2003). Seasonality and tidal oscillations

(subtidal versus intertidal populations) may also affect the actual 8-oxo-dG levels (Machella *et al.*, 2005; de Almeida *et al.*, 2007, Chapter 3).

1.5.3.2 Damage to lipids

i) Lipid peroxidation: Damage to lipids by ROS, known as lipid peroxidation (LPO) is typically divided into 3 stages. In the initiation stage a free radical abstracts a hydrogen atom from a methylene group within the lipid. Double bonds adjacent to the methylene group weaken the C-H bond and facilitate H abstraction. Rearrangement to a conjugated diene follows. When ambient O_2 is in sufficient quantities the fatty acid radical reacts with oxygen yielding a lipid peroxyl radical (ROO·). During the propagation stage ROO· abstracts H from vicinal unsaturated fatty acids so a single oxidation leads to a chain reaction of oxidations, resulting in peroxidation of all the unsaturated fatty acids of the membrane. The reaction terminates when ROO· reacts with another radical or when an antioxidant molecule intervenes and "breaks" the cascade. The short-lived lipid hydroperoxides decompose to a variety of end products namely malondialdehyde (MDA), hydrocarbons, cyclic endoperoxides, isoprostanes and unsaturated aldehydes (Kohen and Nyska, 2002; Valko *et al.*, 2004; Valko *et al.*, 2006). A representation of the lipid peroxidation cascade is shown in Figure 7.

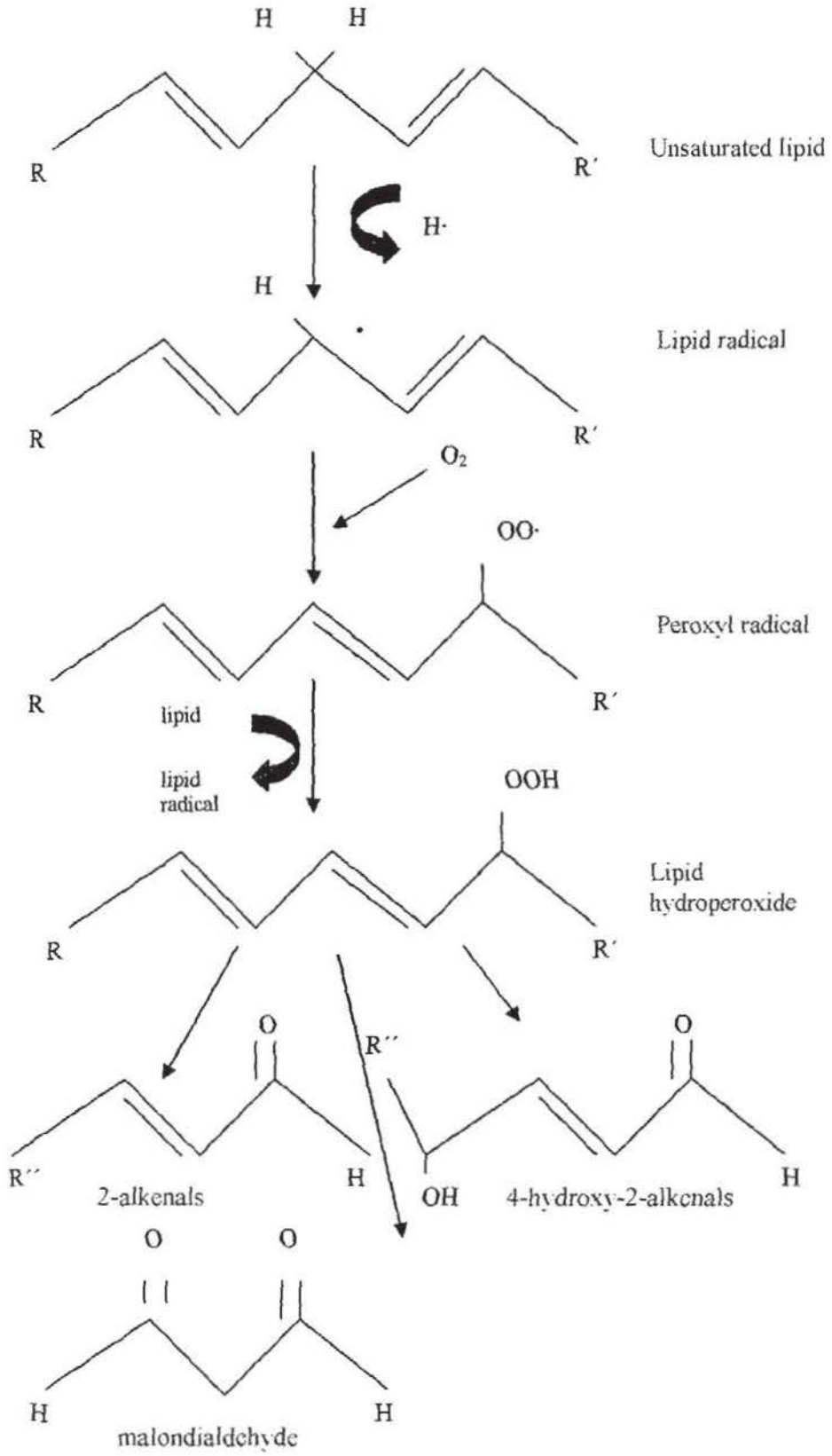


Figure 7: Schematic representation of fatty acids peroxidation.

4-hydroxyalkenals are biologically the most significant products of LPO because of their high reactivity (Karlhuber *et al.*, 1997). *Trans*-4-hydroxy-2-nonenal (4-HNE) is the major 4-hydroxylkenal product and it can form the miscoding exocyclic DNA adducts 1, N^6-ethenodeoxyadenosine (εdA) and 3, N^4-ethenodeoxycytidine (εdC) (Bartsch and Nair, 2005). These adducts were detected in rat kidneys after intraperitoneal administration of the pro-oxidant $KBrO_3$ (Chipman *et al.*, 1998). 4-HNE-induced adducts were also found in the liver of cancer-prone patients with hereditary metal storage diseases (Nair *et al.*, 1999). Malondialdehyde (MDA) is also mutagenic because it can form the exocyclic adduct N^2, 3-ethenodeoxyguanosine (M1dG) (Bartsch and Nair, 2005) or oxopropenyl adducts with A and C (Marnett, 2002). In humans, MDA-DNA adducts were elevated in larynx tissues of larynx cancer patients compared to control groups (Munnia *et al.*, 2004).

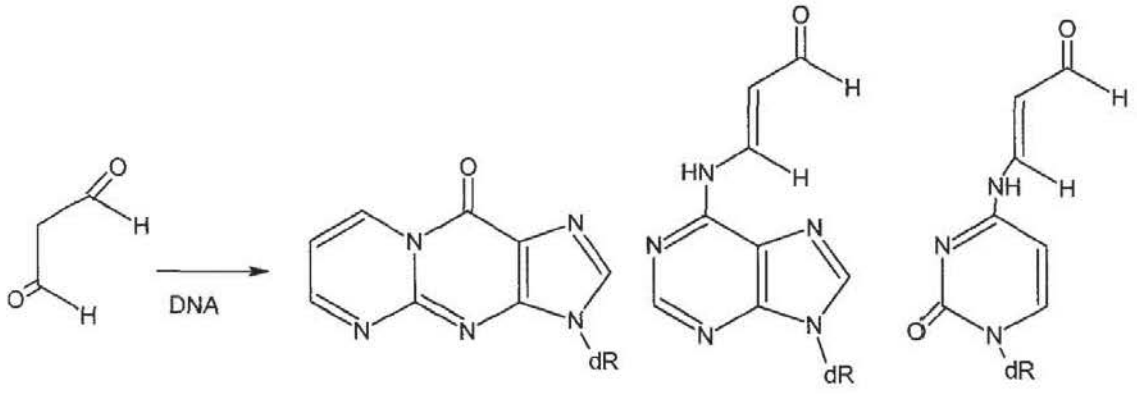
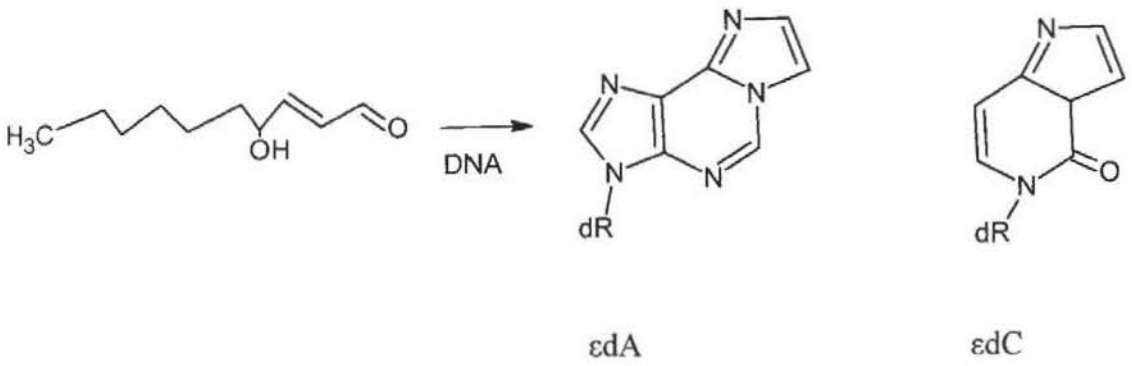


Figure 8: Generation of miscoding adducts from MDA reaction with DNA (adapted from Marnett, 2002).



εdA εdC

Figure 9: Generation of miscoding adducts from 4-HNE reaction with DNA (adapted from Bartsch and Nair, 2005).

ii) Lipid peroxidation in mussels: Lipid peroxidation (LPO) is a well-known mechanism of cellular injury and is indicative of oxidative damage in mussels (Pampanin *et al.*, 2005). A variety of xenobiotics and/or pro-oxidant conditions have caused LPO in molluscs in a series of experimental studies. For example, resin acids, implicated in redox cycling, were able to cause increase in MDA levels in *Mytilus galloprovincialis* gills after *in vivo* exposure (Gravato *et al.*, 2005). The pro-oxidant H_2O_2 caused a transient but significant increase in MDA in *Mytilus galloprovincialis* (Cavaletto *et al.*, 2002) and so did the combination of copper and thiram in *Unio tumidus* (Doyotte *et al.*, 1997). A negative correlation between the activity of mussels to neutralise peroxyl radical ROO· [as total oxyradical capacity (TOSC) for peroxyl] and MDA levels was found in *Mytilus galloprovincialis* (Pampanin *et al.*, 2005). Cu, which produces ROS, stimulated LPO in mussel tissues (Panfoli *et al.*, 2000) and Cd, which may also produce non-Fenton related ROS, did the same in *Bathymodiolus azoricus* gill (Company *et al.*, 2004) and in *Perna viridis* (Prakash and Rao, 1995). Furthermore, mussels impacted from oil spills, which contain highly oxidative PAH, showed a strong increase in LPO as an acute effect (Solé *et al.*, 2004). Finally metals like Cu and Zn, produced LPO in native *Mytilus galloprovincialis* species (Funes *et*

al., 2005) and ROS created during hypoxia- and/or re-oxygenation-simulating tidal oscillations oxidised *Perna perna* tissues (Almeida *et al.*, 2005).

1.6 Repair of oxidative DNA damage

1.6.1 Base Excision Repair overview

Base alterations like 8-oxo-dG are very common and have both exogenous and endogenous sources. Endogenous damage can be summarised into the following categories: a) misincorporation of uracil in the genome or spontaneous deamination of cytosine (Sung and Demple, 2006) b) hydrolysis of all four bases or oxidation by reactive oxygen species (ROS), oestrogens, chlorine agents, reactive nitrogen species, heme precursors and amino acids (Nilsen and Krokan, 2001; Wood *et al.*, 2001) c) alkylation of purines and pyrimidines by lipid end products (Sung and Demple, 2000) or other parameters (e.g. S-adenyl-methionine) (Chapter 1.6.3). Abasic sites are also common lesions and 10000 purines are detached from DNA per human genome per day (Wilson and Kunkel, 2000; Nilsen and Krokan, 2001). Exogenous agents as xenobiotics and radiation are able to also cause the aforementioned damage. All these small, non helix-distorting lesions are rectified by Base Excision Repair (BER) (Krokan *et al.*, 2000; Cabelof *et al.*, 2002). BER was discovered by Tomas Lindahl in 1974 (Krokan *et al.*, 2000), it is a tightly conserved pathway from bacteria to mammals (Izumi *et al.*, 2003; Didzaroglu, 2005) and it must be preserved in a highly coordinated way to be effective (Moustacchi, 2000; Allinson *et al.*, 2004).

BER is initiated by the cleaving of the damaged base by a specialized enzyme: a DNA *N*-glycosylase. The glycosylases implicated in BER fall into two main groups regarding their mechanisms of action: monofunctional and bifunctional glycosylases

(Fortini *et al.*, 1999; Krokan *et al.*, 2000; Cabelof *et al.*, 2002). In the case of the monofunctional glycosylases, an aspartic acid (Asp) residue activates a water molecule, which in its turn performs a nucleophilic attack on the *N*-glycosidic bond. In bifunctional glycosylases, the Asp residue activates an amino group of a lysine (Lys) residue. The amino group forms a Schiff base with C1′ followed by β-elimination at the 3′ side of the deoxyribose (Bailly *et al.*, 1989; Nilsen and Krokan, 2001). In the case of a monofunctional glycosylase, the net result is an apurinic or apyrimidinic site (AP site) and in the case of a bifunctional glycosylase the net result are two single strand breaks: one with a 3′-α,β unsaturated aldehyde end (3′PUA) and the other with a 5′-phosphate end (Izumi *et al.*, 2003). However, some of the bifunctional glycosylases (namely the bacterial Fpg and Nei and the mammalian NEIL1) are able to further process 3′PUA via δ-elimination bearing a 3′phosphate end (Nilsen and Krokan, 2001; Gros *et al.*, 2002; Wiederhold *et al.*, 2004).

The AP site created (as well as the SSB) must be quickly processed further since they are highly cytotoxic (Allinson *et al.*, 2004) and mutagenic (Nilsen and Krokan, 2001). This is done by an AP endonuclease (APE1 for mammalian organisms) resulting in the formation of a 3′-hydroxyl end (3′OH) and a 5′ abasic sugar phosphate end (5′dRP) (Memisoglu and Samson, 2000). AP endonuclease APE1 is also involved in the "trimming" of the blocked 3′ end created by bifunctional glycosylases (Mitra *et al.*, 2001; Cabelof *et al.*, 2002; Izumi *et al.*, 2003). However some researchers argue that the phosphatase activity of APE1 is low and that polynucleotide kinase (PNK) is the only enzyme that cleaves successfully the products of βδ-elimination (Mitra *et al.*, 2002; Wiederhold *et al.*, 2004).

The formation of SSB by APE1 is a critical point in the BER process since two sub-pathways may follow: the short-patch or the long-patch pathway (Christmann *et al.*, 2003; Sung and Demple, 2006). The short patch may be initiated by *N*-glycosylases whereas the long-patch may be the pathway of choice for spontaneous hydrolysis of bases (Didzaroglu, 2005). Cell cycle stage may also affect the choice of sub-pathway (Krokan *et al.*, 2000). Bifunctional glycosylases point to a short-patch mode of action whereas damage rectified by monofunctional glycosylases may follow either pathway (Fortini *et al.*, 1999). The long-patch process may have evolved as a more efficient or as a redundant mechanism for abasic moieties (Wilson and Thompson, 1997). In some cases these moieties are refractory to 5′phosphodiesterase activity. Indeed oxidized abasic sites do not give rise to deoxyribose phosphate (dRP) under physiological conditions. In this case the cleaving of the sugar-phosphate backbone must be done downstream towards the 3′end (Sung and Demple, 2006) and the same happens with reduced abasic sites (Zhang and Dianov, 2005).

No matter what the underlying reason for differentiation is, the two sub-pathways are substantially different. In the short-patch polymerase β attaches a single nucleotide to the trimmed 3′OH end, displacing the dRP at the 5′end (Wilson and Thompson, 1997; Schärer and Jiricny, 2001) and it also cleaves 5′dRP by its intrinsic lyase activity through a covalent Schiff intermediate (Sung and Demple, 2006). Ligase III/XRCC1 seals the gap and DNA integrity is restored (Wilson and Thompson, 1997; Cabelof *et al.*, 2002). Polymerase β does not have proofreading abilities and sometimes incorporates an incorrect nucleotide which is subsequently re-excised by APE1 (Noble, 2002). In the long-patch, polymerase β or polymerase δ/ε with the proliferating cell nuclear antigen (PCNA), add a few more nucleotides at the 3′OH

end (Christmann *et al.*, 2003). The number of extra nucleotides is according to researchers up to six (Schärer and Jiricny, 2001), up to ten (Christmann *et al.*, 2003) or up to 13 (Suttler *et al.*, 2003). This action creates a flap at the 5´dRP end. This flap is then excised by flap endonuclease (FEN1) and afterwards PCNA/ligase I seals the gap (Christmann *et al.*, 2003). A representation of BER pathways is depicted in Figure 10.

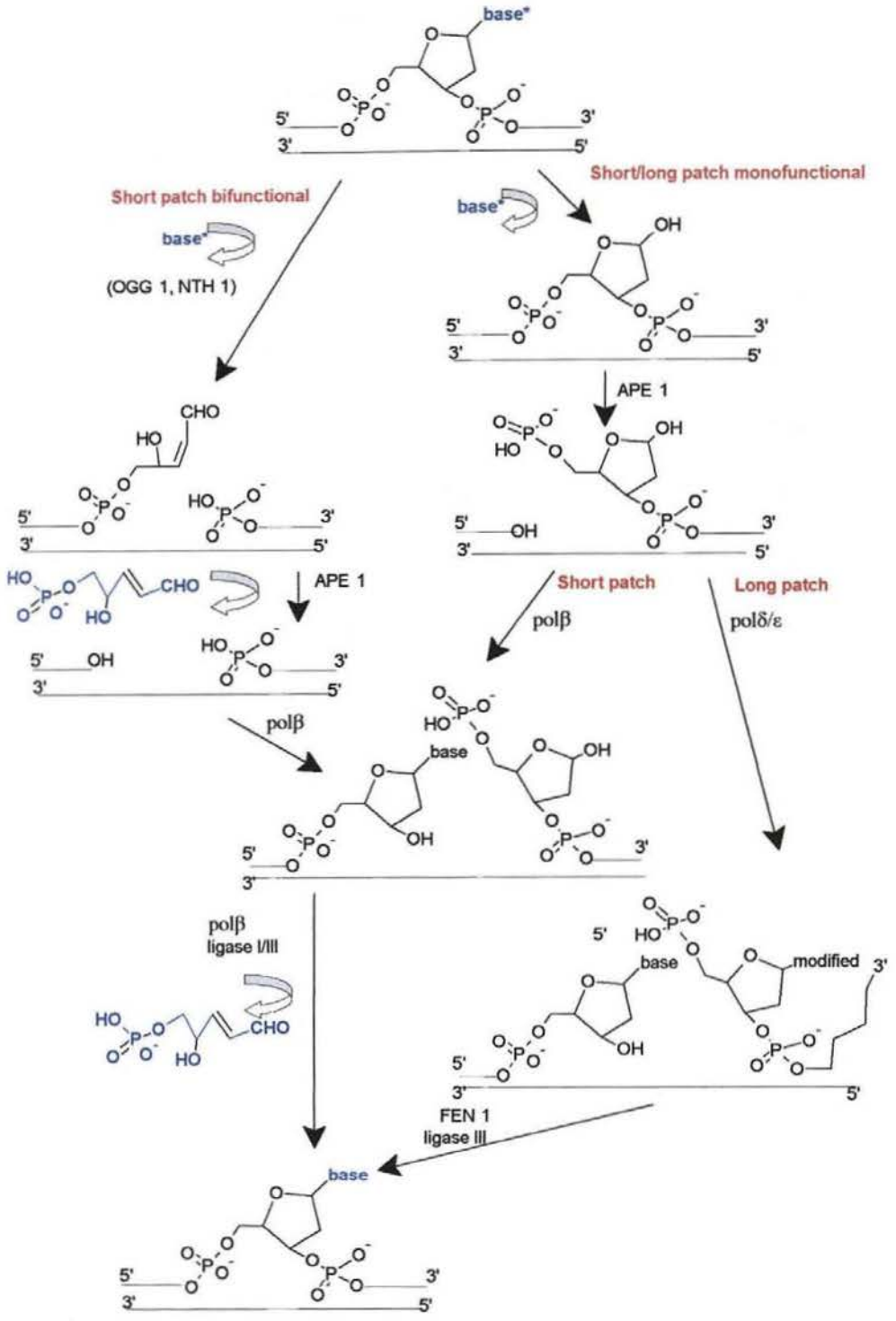


Figure 10: Schematic representation of mammalian Base Excision Repair pathway (adapted from Wiederhold *et al.*, 2004).

1.6.2 Specific BER *N*-glycosylases implicated in oxidised base removal

1.6.2.1 Prokaryotic organisms (*E.coli*)

i) Fpg (MutM): Formamidopyrimidine glycosylase (Fpg) is a glycosylase which excises 8-oxo-dG from 8-oxo-dG: C pairs (Beckman and Ames, 1997; Gros *et al.*, 2002; Russo *et al.*, 2006), but not from 8-oxo-dG: A mispairs because that would lead to a stable G-T transition (Wang *et al.*, 1998). One of the most extensively studied glycosylases, Fpg has been also found in *Deinococcus radiodurans* (Gros *et al.*, 2002), in yeast *Candida albicans* (Wallace, 2002) and in the plant *Arabidopsis thaliana* (Rosenquist *et al.*, 2003). It is a globular monomer of 269 aminoacids and 30.2 kDa, which cleaves 8-oxo-dG, its opened ring form Fapy and a variety of pyrimidines (5-hydroxycytosine, 5-hydroxyuracil and thymine glycol) (Gros *et al.*, 2002). Fpg possesses a C-terminal Zn finger motif, which stabilises the bound DNA and contributes to substrate interaction (Rosenquist *et al.*, 2003). Fpg has an AP lyase activity performing βδ elimination of the abasic site (Gros *et al.*, 2002; Russo *et al.*, 2006).

ii) Nei (endo VII): Nei exhibits an overlapping substrate-specificity with Fpg. It comprises 263 amino acids and a C-terminal Zn finger motif (Gros *et al.*, 2002; Rosenquist *et al.*, 2003).

iii) MutT: MutT is a GTPase which sanitises nucleotide pools from 8-oxo-GTP by hydrolysing it to monophosphate, thus preventing its misincorporation in DNA (Beckman and Ames, 1997; Fortini *et al.*, 2003). MutT deficient strains exhibit a strong mutator phenotype (Fortini *et al.*, 2003).

iv) **MutY**: MutY excises A which is wrongly incorporated opposite 8-oxo-dG (Beckman and Ames, 1997; Vidal *et al.*, 2001). MutY is a 39 kDa protein which can also excise A opposite to G, C and 8-oxo-dA (Le Page *et al.*, 1999; Gros *et al.*, 2002). Fpg/MutY double null strains exhibit a strong mutator phenotype (Wang *et al.*, 1998; Russo *et al.*, 2006).

v) **Nth**: Nth also excises Fpg substrates and it has a strong activity against thymine glycol and other oxidised pyrimidines (Gros *et al.*, 2002; Izumi *et al.*, 2003; Rosenquist *et al.*, 2003). Nth possesses a β-lyase activity besides its *N*-glycosylase activity (bifunctional glycosylase) (Izumi *et al.*, 2003). Nth mutants are not sensitive towards oxidative insult, however the double mutants *nth/nei* exhibit a mutator phenotype after exposure to ionising radiation or H_2O_2 (Gros *et al.*, 2002).

The triad MutT, MutY and MutM (Fpg) comprise the so called GO system which suppresses effectively Guanine Oxidation via the concerted actions of sanitising of the nucleotide pool, excising misincorporated A and excising 8-oxo-dG from duplex DNA respectively (Beckman and Ames, 1997).

1.5.2.2 Eykaryotic organisms (mammals)

i) **OGG1**: hOGG1 is the functional analogue of Fpg in humans. Besides 8-oxo-dG, OGG1 has activity against Fapy (Nohmi *et al.*, 2005) and very weak activity against FapyA (Wallace, 2002). The mammalian OGG1 contains a helix-hairpin-helix motif (HhH) with an Asp-activated Lys residue as an active site. The residue forms a Schiff base with the substrate and subsequently creates an AP site (Izumi *et al.*, 2003). OGG1 is a bifunctional glycosylase, however it acts as a monofunctional *in vivo* since

APE1 precludes the lyase activity of OGG1 (Vidal *et al.*, 2001; Fortini *et al.*, 2003). Given the crucial role of OGG1 in 8-oxo-dG control, it is rather surprising that *ogg1*- - mice are viable and do not show malignant phenotype (Klungland *et al.*, 1999; Gros *et al.*, 2002). Implications of other glycosylases or even other pathways (Nucleotide Excision Repair may also rectify 8-oxo-dG) are probably some of the reasons for this incident (Izumi *et al.*, 2003). However it has been shown in the same mice that the incidents of spontaneous lung carcinoma/adenoma and UV-induced skin tumours are elevated 1.5 years after birth (Sakumi *et al.*, 2003). In humans OGG1 polymorphisms have been incriminated for certain cancer types, especially the mutation Ser326Cys, since it is present in 20% of endometrial and ovarian cancer patients (de Boer, 2002; Gros *et al.*, 2002). It is postulated that Cys mutants have lower 8-oxo-dG excision capacity than wild type cells, however the glycosylase activity of human lymphocytes was not affected by polymorphisms at codon 326 (Nohmi *et al.*, 2005).

ii) NEIL: 3 human and 3 mouse homologues of the bacterial Nei have been cloned, namely NEIL1, NEIL2 and NEIL3. They contain a helix-two turn-helix motif and NEIL 2 and 3 may also contain Zn finger motifs (Rosenquist *et al.*, 2003). NEIL1 and NEIL2 perform a βδ elimination on their substrate with a 3′ and a 5′ phosphate strand as end products (Izumi *et al.*, 2003). NEIL excises thymine glycol (TG), FapyG and FapyA but shows only nominal activity against 8-oxo-dG (Rosenquist *et al.*, 2003). According to other researchers NEIL activity against 8-oxo-dG is significant and the tissue-specific high expression of NEIL3 may be a back up mechanism for removal of oxidised guanine (Slupphaug *et al.*, 2003).

iii) MTH1: the mammalian homologue of MutT is MTH1. MTH1 sanitises the nucleotide pool from 8-oxo-GTP and it also degrades 2OH-dATP and 2OH-ATP (Slupphaug *et al.*, 2003; Nohmi *et al.*, 2005). *Mth-/-* mice showed an increased rate of point mutations (Nakabeppu *et al.*, 2006). Some polymorphisms of MTH1 have been found in cancer patients but a correlation between cancer and MTH1 variations has not been established (Nohmi *et al.*, 2005). However the polymorphism Val83Met was dominant in female Japanese patients with Type I diabetes mellitus (Miyako *et al.*, 2004).

iv) MYH: the mammalian homologue of MutY is MYH. MYH removes A from 8-oxo-dG:A mispairs (Nagashima *et al.*, 1997; Fortini *et al.*, 2003). It also recognises A:G and A:C mispairs (Fortini *et al.*, 2003; Izumi *et al.*, 2003). It is mainly a monofunctional glycosylase with a weak AP lyase activity (Russo *et al.*, 2006). Important variants of MYH were found in siblings afflicted by multiple colon adenoma and carcinoma and further studies proved its role in colorectal adenoma and carcinoma predisposition (Gros *et al.*, 2002; Nohmi *et al.*, 2005). *Myh-/-* mice showed an age dependent accumulation of 8-oxo-dG in their liver. Also, double mutants for MYH and OGG1 had increased tumours in lung, small intestine and ovaries (Russo *et al.*, 2006). The unique action of MYH does not seem to have any back up from other glycosylases, which explain the importance of its mutations (Izumi *et al.*, 2003).

v) NTH1: The mammalian homologue of nth is NTH1. NTH1 has similar substrate specificity with Nth (Gros *et al.*, 2002). It possesses both an *N*-glycosylase and an endonuclease activity but a product inhibition dissociates the two activities (Izumi *et al.*, 2003; Marenstein *et al.*, 2003). Double mutant embryonic cells still showed TG

repair because of the accessory enzymes TGG1 and TGG2 (Gros *et al.*, 2002). Furthermore, *nth1*-/- mice stayed healthy but exhibited a slower excision activity for NTH1 substrates (Izumi *et al.*, 2003).

vi) OGG2: the novel glycosylase OGG2 has been found in human (Wang *et al.*, 1998; Bohr and Dianov, 1999) and yeast (Nash *et al.*, 1996) cells. OGG2 probably excises wrongly incorporated 8-oxo-dG opposite A, in a nascent strand (Izumi *et al.*, 2003).

The triad MTH1, MYH and OGG1 together with other accessory proteins like OGG2 and NEIL comprise the mammalian GO system which suppresses effectively Guanine Oxidation. A representation of the GO system is given in Figure 11.

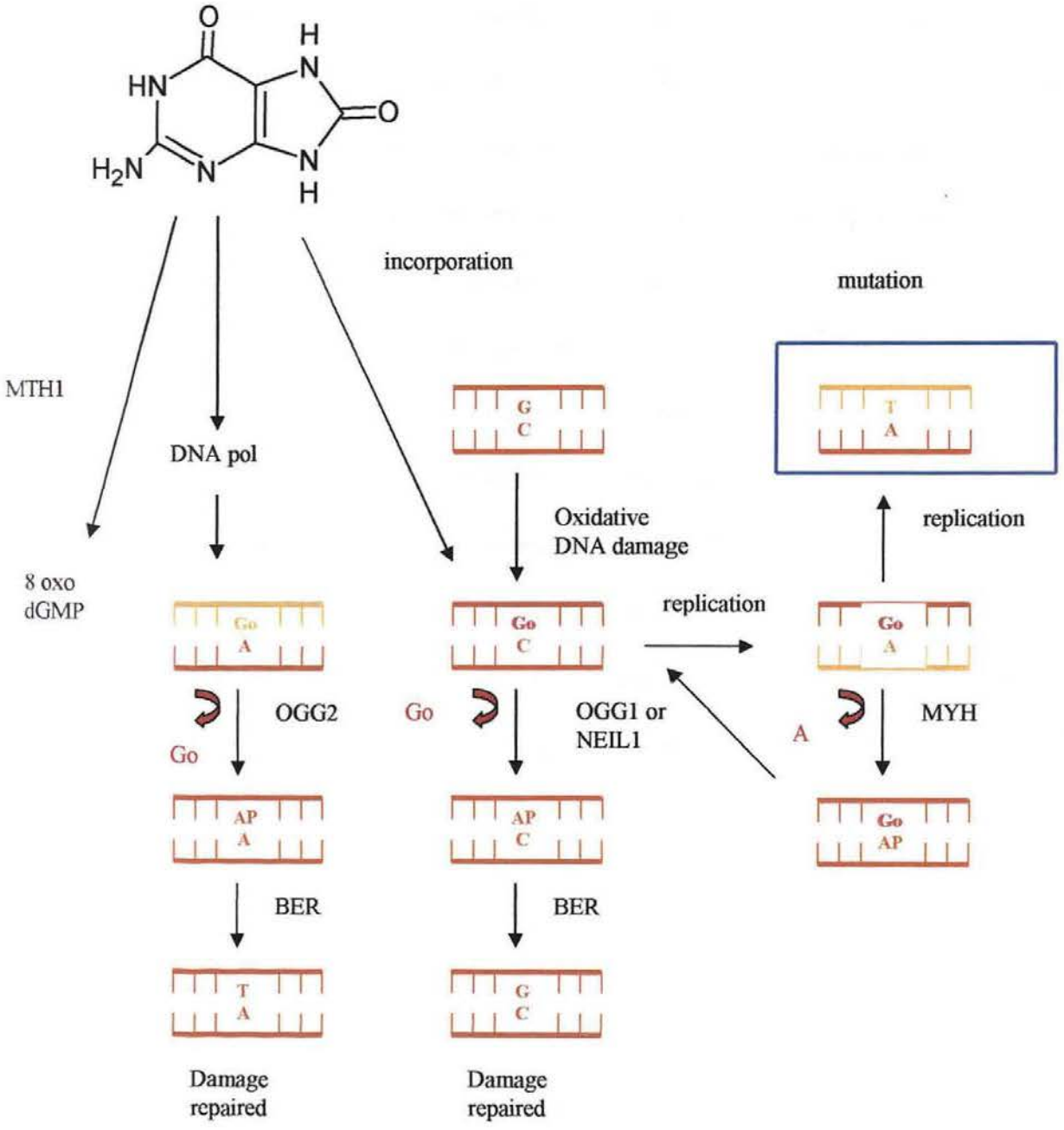


Figure 11: Schematic representation of the "GO-system" in mammalian cells (adapted from Slupphaug *et al.*, 2003).

1.6.3 Specific BER *N*-glycosylases implicated in alkylated base removal

It has been estimated that 20,000 DNA lesions are produced per cell per day but the contribution of alkylation damage is not well established (Drabløs *et al.*, 2004). However alkylation damage occurs frequently as part of the normal metabolism of the cell. It has been shown that the methyl donor *S*-adenyl-methionine can methylate spontaneously DNA to 3-methyl-adenine (3meA) (Seeberg *et al.*, 1995). Also alkylation occurs as a consequence of lipid peroxidation and of nitrosocompounds in the gastrointestinal tract. Furthermore, there are naturally occurring 7-methylguanine (7meG) residues in the genome (Xiao and Samson, 1993). Exogenous sources of alkylation include chloromethane gas by plants, fungi and industrial uses, *N*-nitrosocompounds in tobacco smoke and alkylating anticancer drugs like temozolomide, carmustin and lomustine (Drabløs *et al.*, 2004). Almost all the main mechanisms of DNA repair (direct damage reversal, BER, NER and recombination repair) are implicated in alkylation damage repair and almost all alkylating agents can form *O* and *N*-adducts in all bases and *O*-adducts in phosphodiesters (Drabløs *et al.*, 2004). Regarding BER, a series of *N*-glycosylases have evolved especially for excision of alkylated bases.

1.6.3.1 Prokaryotic organisms (*E.coli*)

i) Tag: The bacterial Tag excises 3meA and 3meG from dsDNA only (Bjeeland and Seeberg, 1996) and it is not inducible (Seeberg *et al.*, 1995). *E.coli* strains, which had enhanced 3meA glycosylase activity however, were more susceptible to mutations if they were simultaneously deficient in AP endonuclease activity (Taverna and Sedwick, 1996). The same happened with the functional homologue of *S. cerevisiae* when it was overexpressed in AP endonuclease deficient organisms (Xiao and

Samson, 1993). These data suggest that the expression of Tag should be carefully controlled for achievement of optimal activity.

ii) AlkA: The bacterial AlkA excises 3meA, 7meA, 7meG, O^2 methylcytosine, O^2 methylthymine and hypoxanthine (Seeberg *et al.*, 1995; Hollis *et al.*, 2000; Drabløs *et al.*, 2004). It is a monofunctional glycosylase (Labahn *et al.*, 1996) with a HhH motif and an Asp328 as an active site. Also the active pocket of AlkA is rich in aromatic residues, which interact with the alkylated bases (Cunningham *et al.*, 1997; Lau *et al.*, 1998). AlkA is using a base-flipping mechanism which projects the modified base into the active pocket of the enzyme. The charged, deficient bases may stack more tightly against the aromatic residues of AlkA than the uncharged bases (Hollis *et al.*, 2000). Double mutant strains of *E. coli* for Tag and AlkA are extremely sensitive to alkylating DNA damage (Seeberg *et al.*, 1995).

1.6.3.2 Eukaryotic organisms (mammals)

i) AAG (MPG, ANPG): The mammalian AAG shares the same broad specificity with the bacterial AlkA. It also removes intact guanines at very low frequencies but it cannot remove O^2-alkylated pyrimidines (Drabløs *et al.*, 2004). However. AlkA and AAG bear little or no sequence similarity between them: the yeast MAG and AlkA possess some common sequence characteristics but there is no sequence analogy between AlkA and the plant or mammalian AAG (Labahn *et al.*, 1996). The broad specificity of AAG is an interesting phenomenon. It is probable that AAG outstacks completely or partially nucleotides and scans along DNA searching for alkylation damage (Lau *et al.*, 1998). Base flipping of the modified base is accompanied by its

intercalation with Tyr162, its stabilisation and a nucleophilic attack by a water molecule deprotonated by Glu125 (Lau *et al.*, 1998; Hollis *et al.*, 2000).

As already mentioned. AAG is able to rectify exocyclic etheno adducts like εdA, 3,N^4-ethenodeoxycytidine (εdC), 1,N^2-ethenodeoxyguanosine (1,N^2εdG) and N^2, 3-ethenodeoxyguanosine (N2-3εdG) which are formed during lipid peroxidation or by vinyl chloride, vinyl fluoride, vinyl carbamate, urethanes and other carcinogens (Ham *et al.*, 2004). *In vitro* AAG was the only enzyme able to repair these kinds of adducts, however experiments with *Aag* -/- mice showed that there is still a weak activity against etheno-adducts via other unknown enzymes or via other pathways (Ham *et al.*, 2004). Overexpression of AAG may enhance the cytotoxicity of alkylating agents thus, protection from AAG or sensitization by AAG may be tissue-specific and its levels should be carefully controlled in order to achieve optimal activity (Drabløs *et al.*, 2004). It is probable that enhancement of its glycosylase activity creates a surplus of abasic sites which are not properly processed by endonuclases leading to a repair imbalance.

1.7 DNA repair in mussels

There is accumulating evidence of existence of DNA repair mechanisms in bivalves. The freshwater mussel *Unio tumidus* exhibited a time-dependent increase in 8-oxo-dG after transplantation to a contaminated site which was later accompanied by a partial recovery (Charissou *et al.*, 2004). This may be due to up-regulation of antioxidant defenses however 8-oxo-dG elimination via excision of the damaged base cannot be ruled out. Some evidence was also noted after *in vivo* exposure of *Mytilus galloprovincialis* to BaP contaminated feed, when 8-oxo-dG and bulky adducts were

initially elevated and then totally or partially (respectively) eliminated during remediation time (Akcha *et al.*, 2000). Of course cell turnover may partly explain this.

Despite the ambiguity of SSB origin, the Comet assay has been frequently used for time-course evaluation of repair. The rationale is that SSB measured after the genotoxic exposure are not due to DNA damage *per se* but should be mostly regarded as excision repair intermediates of the pathways BER and NER. Therefore, the initial increase of SSB is a measure of repair potency and their abnormally long persistence is a measure of inhibition of certain repair pathway steps (e.g. the ligation step) (Hook and Lee, 2003). Indeed mussels (*Perna viridis*) exposed to low concentrations of BaP showed an increase in SSB in the initial days of exposure but DNA integrity was rectified at day 12. Interestingly, higher concentrations of BaP did not cause increase in SSB at the time-points tested something that the authors attributed to "boosted" DNA repair mechanisms (Ching *et al.*, 2001). The existence of DNA repair mechanisms was also inferred by Black *et al.*, (1996) for *Anodonta grandis* and by Pruski and Dixon, (1999) for *Mytilus edulis*, based on the time- and concentration-dependent elimination of SSB. Finally, when the mussel *Limnoperna fortunei* was exposed to pentachlorophenol, it showed elevated SSB values, which however returned to control levels 2 hours post-exposure due to repair pathways (Villela *et al.*, 2006).

From a mechanistic point of view, there is insufficient information for repair pathways in bivalves, even though DNA repair mechanisms are tightly conserved among species (Didzaroglu, 2005). A search in genome databases (GenBank) verifies the lack of knowledge on key repair enzymes for these organisms. Until our study the only proven mussel repair enzyme activity is this of DNA polymerase β, via an [^{3}H]-

UTP incorporation assay in *Mytilus edulis* digestive gland and haemolymph, and it has been shown to decline in ageing mussels (Accomando *et al.*, 1999). Aphicolidin-sensitive polymerase activity (probably corresponding to polymerase α, δ and ε activity) has also been noted in the same mussels (Accomando *et al.*, 1999). With the exception of the apparent lack of inhibition of polymerase β by heavy metals (Cu, Hg) in isolated digestive gland nuclei (Accomando *et al.*, 1990), we are not aware of any other studies on the interactions of molluscan repair enzymes with common aquatic pollutants.

1.8 Metals implicated in oxidative DNA damage

1.8.1 Chromium

1.8.1.1 Sources of chromium

Chromium is widespread in the environment. It is used in various industry processes like textile dying and tannery [Cr(III)], metallurgy, metal electroplating, corrosion inhibition and wood preserving [Cr(VI)] and pigment production [Cr(III), Cr(IV)] (Dana Devi *et al.*, 2001; Gambelunghe *et al.*, 2003; Gheju and Iovi, 2006). As a result, a variety of steel alloy, cast irons, paints, metal finishes and wood treatments contain amounts of chromium (Bagchi *et al.*, 1995). Natural sources of chromium include certain kinds of rock and soil as well as volcanic dust and gases (Burger and Gochfeld, 1995). Other non-occupational exposures include automobile emission and cigarette smoke. Landfills, factories of chromium-related industries, autoroutes and hazardous waste disposal areas are other chromium "hot-spots" (O'Brien *et al.*, 2003).

Antifouling pigments	High temperature batteries
Antiknock coumpounds	Human joint replacement parts
Alloy manufacturing	Magnetic tape
Catalysts	Metal finishing
Ceramics	Metal primers
Corrosion inhibitors	Phosphate coatings
Dental constructions	Pyrotechnics
Drilling muds	Refractories
Electroplating	Tanning
Electronics	Textile preservatives
Emulsion hardeners	Textile printing and dying
Flexible printing	Wood preservatives

Table 3: Industrial uses of chromium (adapted from Guertin *et al.*, 2005).

1.8.1.2 Chromium in aquatic animals

The effluents of the Cr-related industries may be highly mutagenic (Mathur *et al.*, 2005) and since the aquatic environment is the ultimate recipient of these effluents (Pazos-Capeáns *et al.*, 2004), the latter pose a realistic threat to aquatic organisms (Çavaş and Ergene-Gözükara, 2005). Metals like chromium and others are of particular concern since they exist in many anthropogenic pollution sources, are persistent and, in excess, are toxic to aquatic animals (Cohen *et al.*, 2001). These are dangerous characteristics since chromium contamination may be present even in deep-sea environments (Galgani *et al.*, 2005).

A variety of adverse effects in aquatic vertebrates and invertebrates have been noted in sub-lethal *in vivo* exposures to Cr(VI). Namely, juvenile Chinook salmon (*Onchorynchus tshawytscha*) suffered from necrosis of kidney cells and other renal

lesions after chronic treatment with Cr(VI) (Farag *et al.*, 2006). In a different experiment, eels (*Anguilla anguilla*) exposed to 1 mM Cr(VI) (as potassium dichromate in water) for 24 hours showed a depletion of GSH (Ahmad *et al.*, 2006). Finally, *Oreochromis nilotucus* exposed to petroleum refinery and chromium plant processing effluents exhibited increased incidents of micronuclei (Çavaş and Ergene-Gözükara, 2005).

Regarding the organisms of interest here mussels are sufficiently able to accumulate chromium both from sediment and from the water column (Walsh and O'Halloran, 1997; Wang *et al.*, 1997). There are not many studies involving *in vivo* exposure of marine mussels to chromium, however Cr(VI) has been proved to be genotoxic for *Dreissena polymorpha*: Exposure of the bivalve to potassium dichromate (100 µg/l in water) for 12 days produced a statistically significant increase in micronuclei (MN) frequency (Mersch *et al.*, 1996). Furthermore, a generalised stress status was evident in *Mytilus galloprovincialis* following *in vivo* exposure to 10 ng/l Cr(VI) (as potassium dichromate in water) for 7 days (Fabbri and Capuzzo, 2006).

Cr(VI) contamination can move upwards in the food chain via consumption of Cr(VI)-rich bivalves as shown by incidents in herring gull (*Larus argentatus*) chicks (Burger and Gochfeld, 1995). The contaminated chicks exhibited behavioural deficits and reduced body weight and were therefore more susceptible to first year mortality. Mammals may also be affected by consumption as shown in mice models fed with *Mytilus galloprovincialis* specimens from heavily contaminated estuaries (Moustaid *et al.*, 2005). The animals exhibited decreased body weight concomitant with creatinuria after the consumption.

1.8.1.3 Chromium genotoxicity

i) General information: Much of the concern about chromium pollution arises from the fact that Cr(VI) causes DNA damage both *in vitro* and *in vivo* (Ning and Grant, 1999). Cr(VI) is mutagenic (Bagchi *et al.*, 1995; Blasiak and Kowalik, 2000) and a Group I human carcinogen as classified by the International Agency of Research on Cancer (O'Brien *et al.*, 2003). Epidemiological studies have found a correlation between Cr(VI) exposure and increased risk of lung cancer (Wise *et al.*, 2006) and numerous *in vitro* studies have corroborated the genotoxicity of Cr(VI) to various human tissues in the form of SSB (Blasiak and Kowalik, 2000; Trzeciak *et al.*, 2000; Lee *et al.*, 2004). *In vivo*, Cr(VI) is readily absorbed through occupational inhalation and skin contact and it produces primary DNA damage in the form of SSB (Gambelunghe *et al.*, 2003).

ii) Cellular absorption of Cr(VI): Even though there is no doubt about the genotoxic potential of Cr(VI), the exact mechanisms of DNA damage still need to be elucidated (Casadevall *et al.*, 1999; Chowdhury and Jamieson, 2006). The highly genotoxic profile of Cr(VI) and the apparent lack of carcinogenicity of Cr(III) (De Flora, 2000) is the first intriguing point. Cr(VI) is found as $(CrO_4)^{2-}$ in neutral aqueous solutions (Bagchi *et al.*, 1995) and is isostructural with phosphate and sulphate. It can therefore enter the cells via non-specific sulphate transport proteins (Martin *et al.*, 1998; Ning and Grant, 1999; Depault *et al.*, 2000). In contrast under the same conditions, Cr(III) forms cationic octahedral complexes which are unable to pass through cellular membranes. This difference in accessibility is mirrored well in red blood cells where

Cr(VI) is fully absorbed after 4 hours of exposure whereas only 5% of Cr(III) is absorbed during the same time (Ning and Grant, 1999)

iii) Enzymatic and non-enzymatic reduction of Cr(VI): Once inside the cells, Cr(VI) is sequentially reduced to the more stable Cr(III) via enzymatic and non-enzymatic reductants. This process is critical for the manifestation of Cr(VI) genotoxicity (Ning and Grant, 2000; O'Brien *et al.*, 2003). Cr(VI) alone towards isolated DNA is completely non-reactive (Kortenkamp *et al.*, 1998). A variety of cell components may act as Cr(VI) reductants with ascorbate and GSH being the most important agents in this procedure (Casadevall *et al.*, 1999; O'Brien *et al.*, 2003). The net result of the reduction of Cr(VI) and the oxidation of its reductants is a variety of DNA lesions namely DNA-protein crosslinks, DNA interstrands, SSB, alkali labile sites (ALS), DNA-amino acid crosslinks and modified bases.

iv) Specific Cr(VI) lesions: SSB: In an *in vitro* system of DNA, Cr(VI) and GSH without any traces of iron, SSB was the only kind of lesion noticed in an oxygen-rich atmosphere (Kortenkamp *et al.*, 1998). The researchers therefore concluded that Cr(VI) [in the form of Cr(V)], needs activation by molecular oxygen to exert its clastogenic potential. Levina, (2003) also noticed that a complex of Cr(V)-GSH was able to nick plasmid DNA. The "active" Cr intermediates responsible for DNA breakage are probably are hypervalent chromium species (Martin *et al.*, 1998). Indeed, a synthetic Cr(V) complex [bis(2-ethyl-2-hydroxybutyrato)oxochromate(V), Cr(V)-EHBA], which mimics the real Cr(VI)-Asc complex (Chowdhury and Jamieson, 2006) was able to react with oxidant-sensitive dyes directly, without the

previous production of a diffusible ROS (Martin *et al.*, 1998). This particular complex possessed two small bidentate ligands and it could easily bind to the phosphate backbone of DNA and abstract H4′. Subsequent $O_2^{\cdot -}$ implication and hydrolysis led to SSB formation and release of a base-propenal, which was measured by the MDA-thiobarbituric acid assay. A similar result was achieved after incubation of DNA with Cr(VI) and GSH (Casadevall *et al.*, 1999), verifying that H4′ (or, according to Bose *et al.*, 1998, H1′ abstraction) by Cr(V)-oxo- species is the underlying mechanism in Cr(VI)-mediated SSB generation.

v) Specific Cr(V) lesions: 8-oxo-dG:8-oxo-dG may be produced through a Fenton-type reaction of Cr(VI) with endogenous H_2O_2

Namely $M^{n} + H_2O_2 \rightarrow M^{n+1} + OH^{-} + OH^{\cdot}$

Ueno *et al.*, 2001 agrees that Cr(VI)/(V), Cr(V)/(IV) and Cr(III)/(II) redox couples can act as cyclical electron donors in Fenton-like reactions with H_2O_2. This theory is corroborated by the fact that co-incubation of DNA with H_2O_2 and Cr(V) or Cr(VI) greatly enhanced the production of 8-oxo-dG (Faux *et al.*, 1992). Also, 8-oxo-dG was not an evident product of a hypervalent Cr-oxo-species on DNA (Bose *et al.*, 1998) and no 8-oxo-dG or other modified bases were present in a Cr(VI)-GSH-DNA system devoid of iron (Kortenkamp *et al.*, 1998) which suggest that reduction by GSH does not produce "classic" ROS but only Cr-oxo-species. However, the same researchers deduced that no pseudo-Fenton between Cr(VI) and H_2O_2 exists, since the primary product of such a reaction (8-oxo-dG) was absent in iron-free conditions. Furthermore, some other hypervalent oxo-species (Cr-Salen) was able to oxidize guanine and to re-oxidize 8-oxo-dG to guanidinohydantoin and

spiroiminodihydantoin (Sugden *et al.*, 2001). Thus, there are well-founded arguments both for and against the implication of ROS in 8-oxo-dG formation, which may be affected by the experimental system chosen (O'Brien *et al.*, 2003). However, 8-oxo-dG is a prevalent or in some cases major (Lee *et al.*, 2004) product of Cr(VI) genotoxicity.

vi) Specific Cr(VI) lesions: Cr(III)-DNA adducts: The final reductive product of Cr(VI) is Cr(III) which possesses a high affinity for the phosphodiester backbone (O'Brien *et al.*, 2003). Cr(III) may produce binary Cr(III)-DNA adducts or ternary adducts of Cr(III)-DNA together with GSH or some amino acid (cysteine or histidine). In the latter case, the peptide is bridged to DNA via Cr(III) (Voitkun *et al.*, 1998). Cr(III) adducts with tridentate aminoacids like cysteine, histidine and glutamic acid have very low lability thus readily intercalate with DNA (Zhitkovich *et al.*, 1996). Ternary Cr(VI) adducts constitute up to 50% of Cr-DNA adducts and have been proven to be highly mutagenic, causing mainly G-T transitions and G-A transversions.

vii) Specific Cr(VI) lesions: DNA-protein crosslinks: Even though Cr(III)-ternary adducts can be generally regarded as DNA-protein crosslinks, actual crosslinking of DNA with larger proteins is also facilitated by Cr(VI) (O'Brien *et al.*, 2003). These crosslinks are markedly different from the formaldehyde crosslinks since they comprise mainly non-histones (O'Brien *et al.*, 2003) and rarely happen in absence of other Cr(VI) lesions (Merk *et al.*, 2000).

In conclusion, intracellular Cr(VI) attacks the DNA molecule in a variety of interacting ways creating a cascade of mutagenic and/or cytotoxic lesions as depicted in Figure 12.

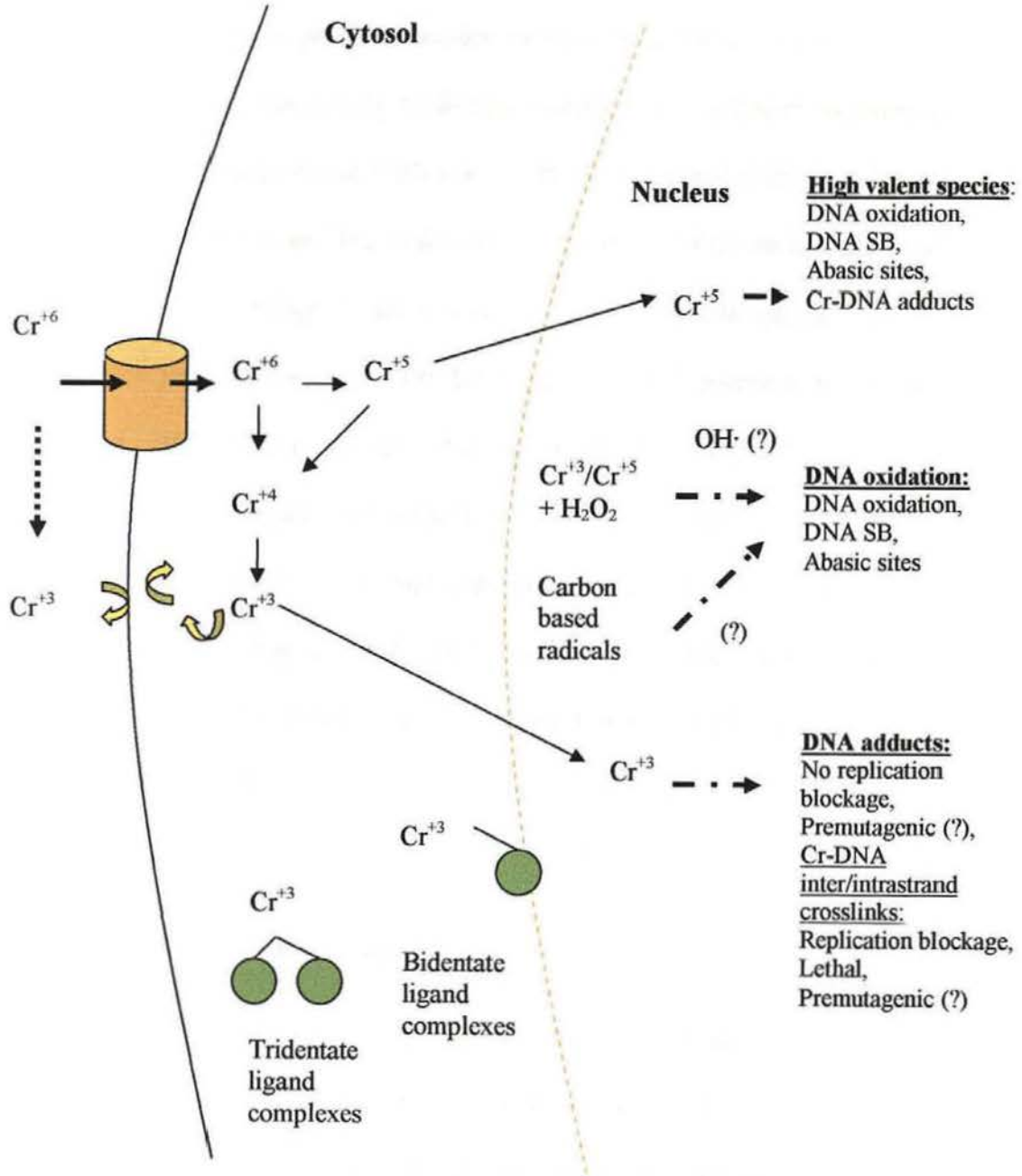


Figure 12: major pathways involved in the formation of DNA lesions by Cr(VI) (From O'Brien *et al.*, 2003).

1.8.2 Cadmium

1.8.2.1 Sources of cadmium

Cadmium has being widely used since the 1940s (Waisberg *et al.*, 2003). Roughly 15.000 tons of cadmium is produced worldwide every year for a variety of industrial uses (McMurray and Tainer, 2003). Cadmium coating by electroplating for corrosion protection is one of its major uses. Cadmium is also a component of several alloys and the anode in Ni-Cd batteries. The cadmium compounds chalcogenides are used as pigments in plastics, paintings, enamels and inks. Recently chalcogenides have been used for nanocrystals, which may exhibit wide industrial potential in the future (Martelli *et al.*, 2006). Natural sources of cadmium include volcanic dust, forest fires and windblown soil particles (Waisberg *et al.*, 2003). Other non-occupational exposures include cigarette smoke and contamination of food, soil, water and air (Filipič and Hei, 2004; Giaginis *et al.*, 2006). Outbreaks like the "itai-itai" disease in Toyoma, Japan, have been linked to accidental mixture of Cd effluents with potable water (Koutselinis, 1997).

1.8.2.2 Cadmium in aquatic animals

The wide use of cadmium and its ultimate accumulation in aquatic environments together with its lack of biological role in both terrestrial and aquatic organisms highlight the toxic potential of this metal (Gómez-Mendikute and Cajaraville, 2003; Mouchet *et al.*, 2006).

A variety of adverse effects have been noted in aquatic vertebrates and invertebrates after sub-lethal *in vivo* exposure to cadmium. Namely, exposure of *Xenopus laevis*

larvae to Cd caused induction of micronuclei in larvae, induction of antioxidant enzyme genes and induction or suppression of repair enzyme genes (Mouchet *et al.*, 2006). Antioxidant enzyme expression was also up-regulated in *Platichthys flesus* as a compensatory mechanism against possible oxidative stress (Williams *et al.*, 2006) after Cd exposure. Crabs (*Charybdis japonica*) exposed to Cd exhibited enhanced SSB in their tissues as measured by the alkaline elution assay (Pan and Zhang, 2006). Finally, the grass shrimp *Paleomonetes pugio* was unable to repair bulky BaP/UV DNA adducts when it was subsequently exposed to Cd, probably because of inactivation of NER components (Hook and Lee, 2004).

Regarding the organisms of interest here mussels bioaccumulate Cd avidly. The accumulation is done in a linear (Lares *et al.*, 2005) and temperature-dependent (Serra *et al.*, 1999) way in many molluscan species. The metal-inducible protein metallothionein finally sequesters this highly toxic metal (Serafim *et al.*, 2002; Soazig and Marc, 2003) intracellularly, however in pro-oxidant situations, massive re-release from metallothionein is possible (Potts *et al.*, 2001). Cd can be very toxic to mussels and it can cause lipid peroxidation (Geret *et al.*, 2002; de Almeida *et al.*, 2004), lysosomal enlargement (Marigómez *et al.*, 2005) and actin cytoskeleton disruption, the latter by direct denaturation of the protein (Gómez-Mendikute and Cajaraville, 2003).

1.8.2.3 Cadmium genotoxicity

i) General information

The metal and its salts are characterised as Group I human carcinogens (Filipič and Hei, 2004). Concern about Cd pollution arises from its extremely long biological half-life, which is 10-30 years in humans (Lynn *et al.*, 1997; McMurray and Tainer, 2003). Cadmium has been directly linked with lung cancer and it may participate in the onset or the propagation of prostate, renal, liver, hematopoetic, bladder and stomach cancer (Filipič *et al.*, 2006; Martelli *et al.*, 2006). Furthermore, cadmium caused tumours in various rodent organs, which were linked with the route of exposure (Waisberg *et al.*, 2003).

Even though there is no doubt about the carcinogenic potential of Cd its mechanisms of genotoxic action are not well understood (Fatur *et al.*, 2003). Cd has been considered as non-genotoxic because it is negative in bacterial tests and weakly positive in mammalian cell test systems (Fatur *et al.*, 2003; Filipič *et al.*, 2004). Furthermore, $CdCl_2$ did not produce SSB in human lung fibroplasts MRC-5 except in the highest dose, whereas $CdSO_4$ did in all concentrations (Mourón *et al.*, 2001). Conversely, Filipič *et al.*, (2006) used a specialised human-hamster hybrid cell line and noted large deletion mutations by Cd salts. Also cadmium facilitated the persistence of oxidative DNA damage in H_2O_2 treated alveolar epithelial cells (Potts *et al.*, 2001) and the persistence of SSB in MMS treated Chinese hamster ovary cells CHO-K1 (Lynn *et al.*, 1997) and in UV treated CHO-K1 cells (Fatur *et al.*, 2003). The latter results seem to support mainly the co-genotoxic potential of Cd (Lynn *et al.*, 1997; Zharkov and Rosenquist, 2002).

ii) Cellular absorption of Cd

In humans there is a low gastrointestinal absorption (5% of the dose) of Cd and a higher (>90%) from the lung. The metal is absorbed through the non-specific divalent metal transporter 1 protein (DMT-1) and rapidly cleared from the blood concentrating in several tissues (Filipič *et al.*, 2006). In a number of cultured cell lines the intracellular absorption of Cd is facilitated by Ca^{+2} voltage-gated channels (Beyersmann and Hechtenberg, 1997).

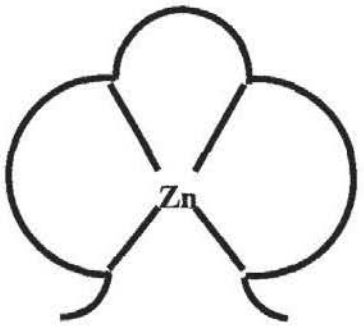
iii) Cd and ROS production

Cd is not a redox metal subsequently it cannot take part in Fenton-type reactions (Potts *et al.*, 2003; Filipič *et al.*, 2006). Furthermore, Cd induced SSB but not 8-oxo-dG, which is characteristic of ROS damage, in HeLa cells (Hartwig, 1998). However ROS have been implicated in Cd carcinogenicity in many different ways: Cd can decrease intracellular GSH and/or the activation of SOD, GPx and CAT (Hirano *et al.*, 1997; Waisberg *et al.*, 2003; Filipič *et al.*, 2004) thus lower the antioxidant defences of the organism. Furthermore, Cd may displace Fe and Cu from various intracellular proteins like ferritin and apoferritin. These metals will subsequently participate in Fenton reactions (Waisberg *et al.*, 2003; Filipič *et al.*, 2004). Cd may also have an inhibitory effect on the mitochondrial transport chain leading to direct transfer of electrons to oxygen (uncoupling) and it has been shown that Cd inhalation may cause an inflammatory oxidative burst from leykocytes and neutrophils (Potts *et al.*, 2003). On the other hand, it has been proposed that ROS production may be a negligible mechanism in Cd carcinogenicity (Witkiewicz-Kucharczyk and Bal, 2006).

iv) Effects of Cd on DNA repair

It is estimated that 3% of the human genome encodes for proteins containing Zn finger motifs (Hartwig *et al.*, 2003; Witkiewicz-Kucharczyk and Bal, 2006) and many proteins implicated in DNA repair possess Zn finger motifs. Zn finger motifs are protein domains which comprise a central Zn(II) complexed with four invariant cysteine and/or histidine residues. The classic Zn finger motif comprises (from its N- to its C-terminus) two Zn(II)-binding cysteines separated by usually two non-binding amino acids, followed by a longer chain of non-binding amino acids and ending with two Zn(II)-binding histidines separated by several amino acids. One example is poly(ADP-ribose) polymerase-1 (PAPR-1) which binds to SSB during the BER procedure, which contains two Cys3His1 Zn finger motifs. Another example is the well-known bacterial Fpg with one single Cys4 finger and the human Xeroderma pigmentosum group A protein (XPA) which is necessary for damage recognition and excision in the Nucleotide Excision Repair (NER) pathway, with one single Cys4 finger (Hartwig *et al.*, 2002; Hartwig, 2003; Witkiewicz-Kucharczyk and Bal, 2006). Other proteins are also UvrA, implicated in bacterial NER and ligase III, implicated in BER (Asmuß *et al.*, 2000). It is evident that damage or modification of these important enzymes will ultimately lead to compromised DNA repair. Cd is able to modify these proteins and it caused inhibition of XPA (Hartwig, 1998; Asmuß *et al.*, 2000; Hartwig *et al.*, 2002; Filipič *et al.*, 2006; Giaginis *et al.*, 2006), PAPR-1 (Hartwig *et al.*, 2002; Filipič *et al.*, 2006), Fpg (Hartwig, 1998; Asmuß *et al.*, 2000; Hartwig *et al.*, 2002; Giaginis *et al.*, 2006) and MTH1 (Asmuß *et al.*, 2000). It is possible that the inhibition takes place at the protein level and not on transcription level, by direct displacing of Zn(II) by Cd(II). This theory is corroborated, at least for the initial steps of NER which utilise XPA, by the fact that addition of Zn(II) to cell extracts reversed the inhibition of Cd(II) (Filipic *et al.*, 2006). The same principle may

apply also in the case of Fpg since simultaneous addition of Zn(II) reversed again the inhibitory effect of Cd(II) (Asmuß *et al.*, 2000). Furthermore it was shown *in vitro* that Cd(II) substituted easily for Zn(II) in XPA due to a very high binding constant and that this substitution changed the conformation of the enzyme (Witkiewicz-Kucharczyk and Bal, 2006). Also in the component of NER TFIIH, which contains a Zn finger in its subunit p44, there was again a direct substitution of Zn(II) by Cd(II) (Giaginis *et al.*, 2006). Finally, the inhibition of the Mismatch Repair Pathway (MMR) noted with Cd may be due to still unidentified Zn finger motifs in important enzymes of this pathway (McMurray and Tainer, 2003). It is also possible that oxidative damage caused by Cd(II) as noted in 1.8.2.3 iii), affect negatively Zn finger motifs: Oxidative stress has been shown to inactivate these proteins without direct metal action (Witkiewicz-Kucharczyk and Bal, 2006). Thus the physicochemical similarities between Zn(II) and Cd(II) may explain some (but not all) of the toxic actions of Cd (Martelli *et al.*, 2006).



XPAzf

Figure 13: Schematic representation of XPA Zn finger motif (adapted from Witkiewicz-Kucharczyk and Bal, 2006).

v) Cd and interference with transcription factors

DNA repair mechanisms (and thus genotoxicity) may be influenced not only by direct inactivation of enzymes but also by indirect manipulation of their transcription levels. Cd is able to interfere with a variety of gene transcription factors (Beyersmann and Hechtenberg, 1997). Namely Cd up-regulates the transcription of the proto-oncogenes c-fos and c-jun, the metal regulatory transcription factor 1 (MTF1), the upstream stimulator factor (USF) and the nuclear factor κB (NF-κB) and down-regulates the hypoxia-inducible factor 1 (HIF-1) and the specificity protein 1 (Sp1) (Beyersmann and Hechtenberg, 1997; Waisberg *et al.*, 2003). It is not known if some or all of these factors take part in regulation of important repair genes, however Sp1 is indeed a transcription factor for OGG1 (Youn *et al.*, 2005). It has also been shown that the transcription factor TFIIIA is a possible target for Cd, which can disturb the binding of the factor to its cognate DNA sequence (Giaginis *et al.*, 2006). TFIIIA regulates 5S RNA synthesis (Giaginis *et al.*, 2006), which in its turn directs the last assembly step for 50S RNA subunit (Dohme and Nierhaus, 1976). In many cases (e.g. TFIIIA) Cd inhibition is due to interference with Zn finger motifs in the enzyme (Beyersmann and Hechtenberg, 1997). In other cases, like Sp1, the inhibition is not yet clear (Chapter 6.4) but it may be facilitated by kinase(s) or phosphatase(s) (Martelli *et al.*, 2006). Finally it has been shown that Cd inhibits murine enzymes related to DNA repair (PCNA, Dnase 1, ERCC1, MSH2) at the transcription level (Zhou *et al.*, 2004).

vi) Cd and interference with antioxidant status

Cd interferes with antioxidant enzymes activity and reduced glutathione (GSH) levels, which may ultimately affect oxidative DNA damage. Generally Cd decreases GSH levels and activities of superoxide dismutase (SOD), glutathione peroxidase (GPx)

and catalase (CAT) both *in vitro* and *in vivo* short-term exposures (Waisberg *et al.*, 2003; Filipič *et al.*, 2006). GSH was indeed significantly decreased in testes of rats treated with $CdCl_2$, 3 days post-injection (Hirano *et al.*, 1997). However, extended periods of exposure may enhance GSH production and antioxidant enzymes activity (Waisberg *et al.*, 2003; Filipič *et al.*, 2006). Cd-adapted rat lung epithelial cells exhibited higher SOD, GPx and CAT activities compared to non-adapted (Potts *et al.*, 2001).

The most prominent change in antioxidant status after Cd exposure is the induction of the protein metallothionein (MT). Cd activates the transcription of MT in almost all tissues both *in vitro* and *in vivo* systems (Filipič *et al.*, 2006). MT facilitates efficient Cd sequestering; however Cd mobilisation from MT under periods of oxidant stress is possible. Extended oxidation of the thiol residues of this protein facilitated by oxidative stress severely compromise Cd sequestering, hence the "double-edge sword" role of MT (Potts *et al.*, 2001).

1.9 Organics implicated in oxidative DNA damage

1.9.1 Benzo[a]pyrene (BaP)

1.9.1.1 Sources of BaP

BaP belongs to the chemical group of polyaromatic hydrocarbons (PAH), which are produced by a variety of industrial and domestic activities. PAH (and BaP) are commonly found in automobile exhaust fumes, coal tars, pitch, coke oven emissions,

petroleum residues and tobacco smoke (McCoull *et al.*, 1999; Xue and Warshawsky, 2005). BaP is also found in a variety of food products with grilled/barbecued steaks and hamburgers and grilled/barbecued chickens with skin containing as high as 4 ng BaP/g of cooked meat (Kazerouni *et al.*, 2001). Because of their hydrophobic nature PAH are easily absorbed by suspended particles in air or water and thus are more refractory to biodegradation (De Luca *et al.*, 2005)

1.9.1.2 BaP in aquatic animals

Urbanisation and petrogenic and pyrolytic industries are highly responsible for PAH aquatic contamination. Civil and industrial wastewaters, street dust-off discharges, fossil-fuel combustion, particles and carbonized coal products spills have lead to a dangerous increase of PAH in aquatic environments (De Luca *et al.*, 2005). Ecological disasters like oil spills (Sole *et al.*, 1996; Webster *et al.*, 1997) also contribute to PAH accumulation.

BaP is genotoxic for aquatic animals as shown by *in vitro* production of SSB after incubation of *Salmo trutta* cells with BaP (Mitchelmore and Chipman, 1998). Also eels exposed *in vivo* to BaP by single intraperitoneal injection showed elevated levels of SSB and of apoptotic cells (Nigro *et al.*, 2002). Regarding our organisms of interest, the bioaccumulation of BaP in mussels is rapid and extensive (Akcha *et al.*, 2000b) enabling their use as sentinel species for PAH pollution in Mussel Watch Programmes (Webster *et al.*, 1997; Baumard *et al.*, 1999; Oros and Ross, 2005). PAH are highly toxic for invertebrates causing, among others, endocrine disruption (Aarab *et al.*, 2004) and reduced scope for growth (Widdows *et al.*, 2002). BaP in particular is also genotoxic as shown by the formation of SSB (Mitchelmore *et al.*, 1998; Ching

et al., 2001; Siu *et al.*, 2003; Bihari and Fafandel, 2004), of stable degenerative lesions like micronuclei and chromosomal aberrations (Venier *et al.*, 1997) or of bulky DNA adducts (Akcha *et al.*, 2000; Akcha *et al.*, 2000b) and by its bioactivation to a bacterial mutagen by mussel subcellular fractions (Michel *et al.*, 1992). BaP in itself is not genotoxic; its metabolic activation reveals its mutagenic potential (Xue and Warshawsky, 2005). In mussels this process gives rise to mainly three classes of products: dihydrodiols, quinones and phenols. There is a discrepancy between *in vivo* (acquired by extraction from digestive gland) and *in vitro* (formed by mussel microsomes) metabolites: The profile of *in vitro* metabolites consists of 80% quinones and 20% diols and phenols. In contrast, *in vivo* metabolites comprise 17% 9-10, 4-5 and 7-8 diols, 36% 9, 1 and 3 phenols and only 47% quinones. All the metabolites are capable to conjugate with glucuronic acid or sulfate and/or to bind covalently to cellular macromolecules (Michel and Narbonne, 1996). However this *in vivo* ratio is not always stable- it is believed that in bivalves the pathway leading to diol formation is catalysed by an inducible CYP1A-like enzyme whereas the pathway leading to quinone formation is non-inducible and related to a one-electron oxidation pathway (Michel *et al.*, 1992; Livingstone *et al.*, 1997) The different mechanisms by which BaP exerts its genotoxicity both in bivalves and mammals are analysed in the following section (1.9.1.3).

1.9.1.3 BaP genotoxicity

i) General information

As already mentioned, BaP is genotoxic and mutagenic and it is classified as a probable carcinogen for humans (Group 2A, IARC). When administered orally, by

skin contact or by inhalation, BaP caused a variety of tumours in mice, rats, guinea pigs, rabbits and subhuman primates. It also increased the incidence of lung adenomas and skin cancers in mice offspring (11th Report on carcinogens, U.S. Department of Health and Human Services, 2004). The principal metabolic pathways analysed here contribute to its genotoxicity, mutagenicity and probable carcinogenicity.

ii) Bay region dihydrodiol epoxides pathway

Metabolic activation of BaP involves its epoxidation by CYP450 (principally CYP1A1) and formation of an unstable arene oxide. Its hydrolysis by epoxide hydrolase leads to a dihydrodiol (*trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene). A second CYP450 catalysed epoxidation yields a vicinal diol epoxide (*trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene). This is a "bay region diol epoxide" with the epoxide ring within the bay region of BaP and sterically hindered by the molecule. Reaction of the benzylic carbon of the epoxide and the exocyclic amino-group of deoxyguanosine or deoxyadenosine forms stable miscoding DNA adducts. This preference of the epoxide for purinic moieties is not yet elucidated (McCoull *et al.*, 1999; Xue and Warshawsky, 2005).

iii) Radical cation pathway

The probability of a PAH to lose an e^- and form a cation is depended on many factors. A relatively low ionisation potential is a prerequisite and it has been shown that a cut-off value of 7.35 eV or less is essential. BaP therefore (I.P.<7.35 eV) may be oxidised by CYP450 or peroxidases and the BaP cation in its turn, may form depurinating

adducts. Subsequent base-loss leads to abasic (alkali-labile) sites (McCoull *et al.*, 1999; Xue and Warshawsky, 2005).

iv) PAH-*o*-quinone pathway

Trans-dihydrodiols formed by the epoxidation of BaP (Chapter 1.9.1.3 ii) may also be a substrate for dihydrodiol dehydrogenases, which under physiological conditions compete with CYP450 for oxidation of non k-region diols (Xue and Warshawsky, 2005). The enzyme forms initially a ketol, which rearranges to a catechol (hydroquinone). Hydroquinones are not stable and in an oxygen-rich atmosphere autoxidise to the corresponding quinones via semiquinones with concomitant production of ROS: oxygen is reduced to produce superoxide, which rapidly dismutates to H_2O_2. Redox metals and/or Haber-Weiss reactions ultimately produce OH· the proximate clastogenic species. Mild biological reducing agents regenerate the hydroquinones and enable the continuous cycling of these redox couples (Lorentzen and Ts'o, 1977). OH· produces a variety of DNA lesions (Chapter 1.5.3) but also quinones themselves may form stable or depurinating adducts with DNA (McCoull *et al.*, 1999). A schematic representation of the various BaP metabolic sub-pathways is given in Figure 14.

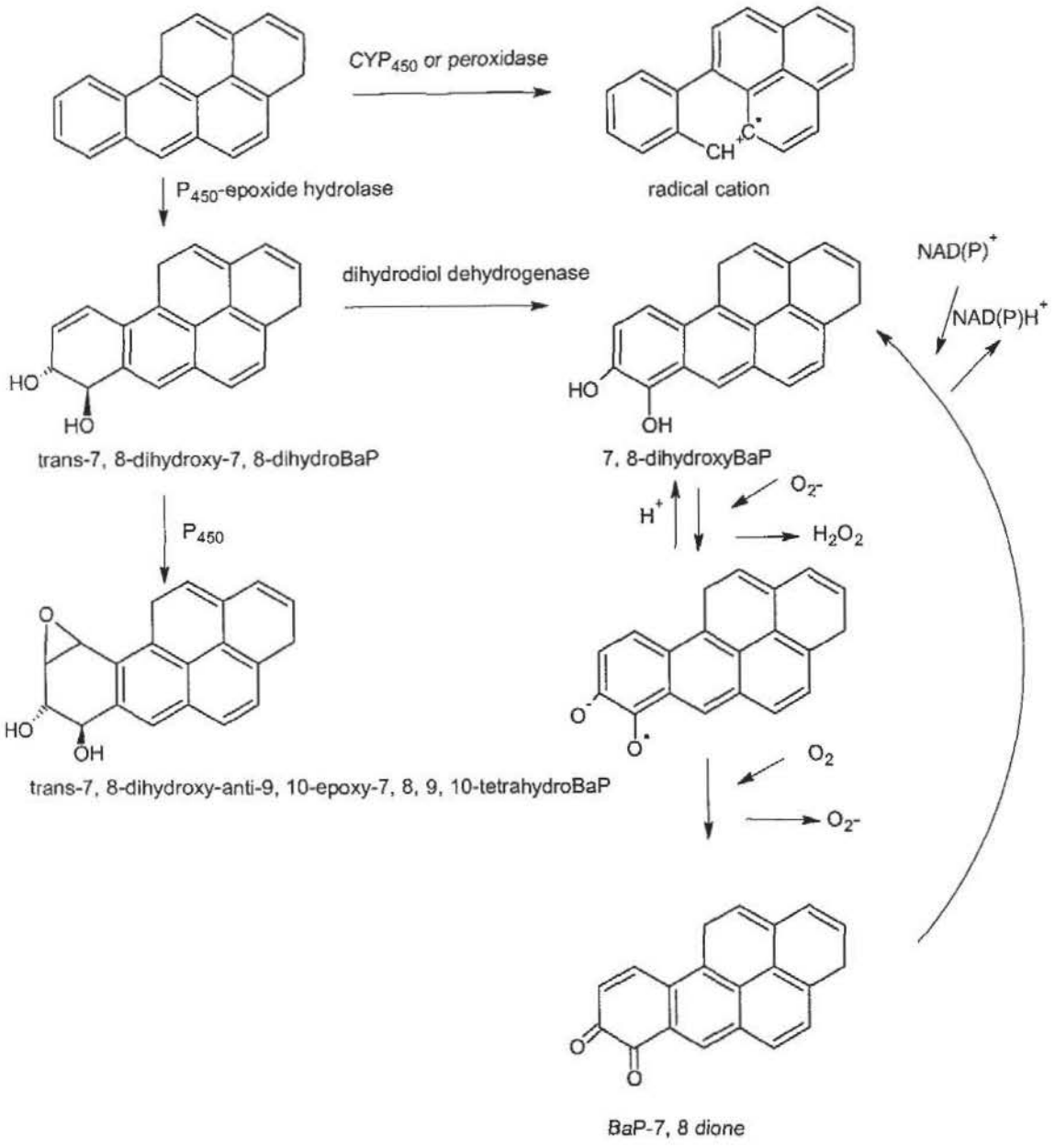


Figure 14: Proposed metabolic pathways for BaP (From McCoull *et al.*, 1999)

1.9.2 Polychlorinated biphenyls (PCB)

1.9.2.1 Sources of PCB

PCB are industrial chemicals which were introduced in the 1930s and were banned in most developed countries in the 1970s (Borell and Aguilar, 2006). They have been extensively used as dielectrics in capacitors, as flame-retardants, in hydraulic fluids and in carbonless paper to name a few of their applications (McLean *et al.*, 1996; Srinivasan *et al.*, 2001). Commercially they were available mainly as mixtures such as Clophen, Aroclor or Kanechlor (Ptak *et al.*, 2006). PCB are lipophilic and very persistent in the environment so they tend to bioaccumulate (Srinivasan *et al.*, 2001). Humans may be exposed to PCB via ingestion, skin contact or inhalation (Ptak *et al.*, 2006).

Studies on the PCB are difficult since there are 209 structural candidates. When linking structure with pharmacodynamic action, PCB can be divided in two sub-categories: coplanar and non coplanar with the former comprising congeners with no *o*-chloro substituents and the latter comprising *ortho*-substituted members (McLean *et al.*, 1996; Canesi *et al.*, 2003). Only the coplanar PCB are aryl hydrocarbon receptor (AhR) agonists (Schlezinger *et al.*, 2006). Another subdivision can be made between lower chlorinated PCB (with 3 or fewer chlorine atoms) like CB3 and higher PCB like CB169 and CB126. Lower PCB can be oxidised by CYP450 whereas higher members are relatively resistant to metabolism (McLean *et al.*, 2000). The general structure of PCB is given in Figure 15.

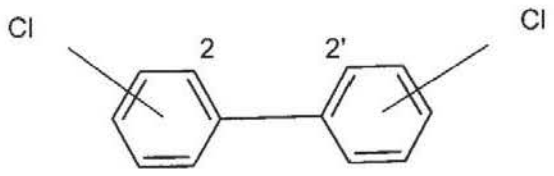


Figure 15: Schematic representation of the general structure of PCB

1.9.2.2 PCB in aquatic animals

Being lipophilic and resistant to degradation, PCB accumulate in the fatty tissues of animals and biomagnify. Furthermore, their trapping in soil and sediments results in leaching towards water reservoirs and ultimately towards aquatic environments. Nowadays, fatty tissue PCB concentrations of top predators like dolphins show that aquatic contamination (e.g. Mediterranean Sea) is still significant (Borrel and Aquilar, 2007). PCB in sub lethal concentrations caused oxidative stress in the fish *Girardinichthys viviparous* evident by increased LPO (Vega-Lopez *et al.*, 2006). In the same fish exposure to a commercial mixture of PCB (Inerteen) caused induction of vitellogenin in both males and females, verifying the endocrine disruptor profile of these chemicals (Vega-Lopez *et al.*, 2006b). Finally low doses of co-planar PCB caused production of ROS and counterbalancing up-regulation of antioxidant enzyme activities in *Stenotomus chrysops* (Schlezinger and Stegeman, 2001).

PCB are commonly measured in mussel soft tissues as part of Mussel Watch Programmes (Connor *et al.*, 2001; Kim *et al.*, 2002; Danis *et al.*, 2006). PCB can also interfere with a variety of biological and biochemical biomarkers in bivalves. For

example, exposure to CB126 resulted in reduced mussel tolerance to aerial exposure (Eertman *et al.*, 1996). Also, non coplanar PCB caused decreased lysosomal stability in mussel haemocytes and in some cases concomitant decrease in bactericidal activity (Canesi *et al.*, 2003).

1.9.2.3 PCB genotoxicity

i) General information

PCB were considered benign when they first appeared, however today there is enough information about their hepatotoxicity, neurotoxicity, immunotoxicity, immunosuppression and their implication in hormonal dysfunction and atherosclerosis (McLean *et al.*, 2000; Henning *et al.*, 2002; Canesi *et al.*, 2003). Furthermore, commercial PCB mixtures induced cancer in rodent models. Epidemiological studies have also implied a connection between PCB exposure and deaths from liver, gall bladder, biliary tract, digestive tract, lung, brain and breast cancer, malignant melanoma and non-Hodgkin's lymphoma (Srinivasan *et al.*, 2001). Some of these effects are facilitated by interactions with CYP450, and some are due to other distinct mechanisms as analysed in the following sections.

ii) Induction of CYP450

Both coplanar and non coplanar PCB were able to increase the expression of several CYP450 in animal models. PCB that are agonists for AhR, constitutively active receptor (CAR) and/or pregnane-X receptor (PXR) can induce CYP1A, 2B and 3A respectively. Generally *meta* and *para*-substituted PCB are ideal agonists for AhR,

whereas *ortho* and *para*-substituted PCB induce CYP2B and multi-*ortho* members induce CYP3A (Ptak *et al.*, 2003). Indeed, coplanar PCB, which are AhR agonists interacted significantly with CYP1A, whereas non coplanar PCB had little effect on this category of cytochromes (Schlezinger and Stegeman, 2001). More analytically, 3,3′,4,4′,5-pentachlorobiphenyl, 3,3′,4,4′-tetrachlorobiphenyl (CB-77) and 2,3,7,8-tetrachlorodibenzofuran elicited a significant induction in CYP1A mRNA in scup *in vivo* (Schlezinger and Stegeman, 2001). Also CB-77 and CB-153 caused CYP1A induction (increased EROD activity) in Sprague-Dawley rats (Fadhel *et al.*, 2002). CYP450 catalyse a variety of important Phase I "activation" reactions namely oxidations or reductions, aliphatic or aromatic hydroxylation, dealkylations, desulfurations and oxidative dehalogenations, thus their induction may yield a variety of reactive intermediates (Boelsterli, 2003). Furthermore, a variety of cellular products and hormones are synthesised and/or metabolised by CYP450, which also explains the endocrine disruptor profile of PCB. As a result, CB3-mediated CYP1A induction in porcine ovarian cells caused an up-regulation of estradiol (Ptak *et al.*, 2006).

iii) Inactivation of CYP1A

In most cases this induction is followed by CYP inactivation at higher or more prolonged doses of PCB, as has been proven especially for CYP1A. That was the case in scup liver where incubation with CB-77 caused an irreversible loss of EROD activity and loss of CYP450 (Schlezinger and Stegeman, 2001). This loss is probably due to *in situ* production of ROS, which inactivate and finally denature the enzyme (Livingstone, 2001; Schlezinger and Stegeman, 2001). This increased ROS production is due to the uncoupling (dissociation of electron transport from substrate

oxidation) abilities of PCB (De Matteis *et al.*, 2002; Schlezinger *et al.*, 2006). Only tight-binding, slow-metabolising xenobiotics are able to cause uncoupling, a theory verified by the presence of uncoupling consequences (persistent EROD inhibition, CYP450 loss and ROS production) in the case of coplanars (CB-77, CB-126 and CB-169) and the lack of uncoupling consequences in the case of the non-AhR agonist CB-52 and the "good" CYP1A substrate BaP (Schlezinger *et al.*, 2006).

iv) Redox cycling of PCB metabolites

The metabolism of higher PCB is slow and possibly the actual metabolites do not significantly contribute to PCB toxicity (Srinivasan *et al.*, 2001; Schlezinger *et al.*, 2006). However this is not the case for lower PCB, which are readily oxidised by CYP450 isoenzymes. Indeed rat microsomes were able to transform *in vitro* the PCB 4-monochlorobiphenyl into catechols and hydroquinones as shown in Figure 14 (McLean *et al.*, 1996). The same hydroquinone product could undergo oxidation and ultimately lead to quinone redox cycling *in vitro* in rat microsomes (McLean *et al.*, 2000) in a similar way with BaP quinones [Chapter 1.9.2.3 iii)]. Other low PCB quinones (dihydroxy- and quinoid-PCB with 1-3 chlorines) also verified the oxidative potential of these metabolites (superoxide production and DNA strand breaks) *in vitro* (Srinivasan *et al.*, 2001)

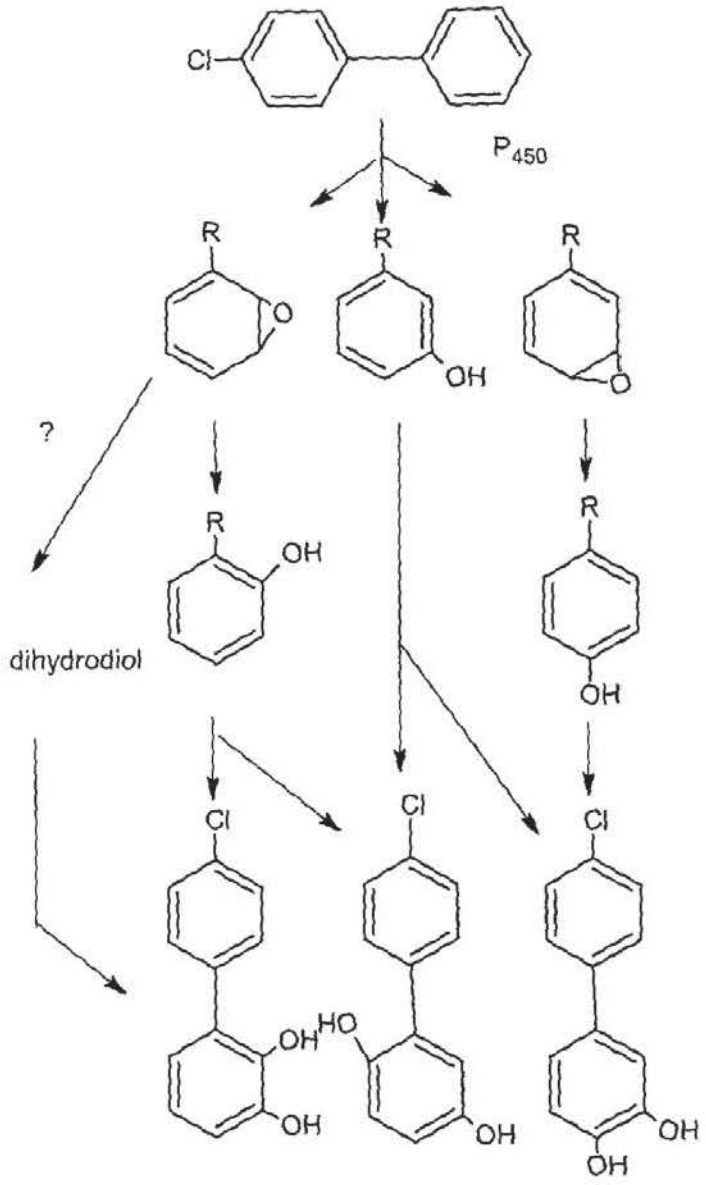


Figure 16: Proposed metabolism of 4-chlorobiphenyl to hydroxy-metabolites (From McLean *et al.*, 1996).

1.10 Project aims

Our overall hypothesis is that mussels chronically exposed to a range of pollutants may exhibit increased oxidative macromolecule damage and that the related DNA repair may also be modulated.

Our specific project aims were the evaluation of

- The discriminating ability of the conventional and the coupled comet assay in mussel populations chronically or acutely exposed to contaminants
- The persistence of the biomarkers measured by the aforementioned methods in a subsequent non-polluted environment
- The existence and performance of oxidative DNA repair in mussels
- The effect of selected pollutants on bivalve DNA damage and repair

For these aims, indigenous mussels from a polluted and a reference site were collected throughout the year 2005-2006 and examined for DNA strand breaks and oxidised bases in DNA at two time points: 24 hours post collection and after one-month laboratory maintenance, with or without acute pro-oxidant treatment. Lipid peroxidation and non-enzymatic antioxidant defences (reduced glutathione) were measured as indications of generalised oxidative stress levels. Excision capacity towards oxidised or etheno-adducted DNA bases was also evaluated in the same samples. Finally, the effect of *in vivo* exposures to Cr(VI) and Cd(II) on the measured parameters was evaluated.

2.0 Choice of environmental sites, mussel speciation and husbandry

2.1 Introduction

Mussel transplantation is a strategy that has been applied in monitoring of estuarine environments (Shaw *et al.*, 2002). Briefly, mussels from uncontaminated sites are deployed in mesh bags or cages around the contaminated sites for different time periods: e.g. from 7 days (Charissou *et al.*, 2004) for up to one year (Gagné *et al.*, 2004). After the end of the exposure period, appropriate biomarkers are measured and are compared to those found in the mussels still dwelling at the reference site or upstream from the source of pollution. Indigenous populations have also been used for monitoring studies (Large *et al.*, 2002; Rank and Jensen, 2005). Indigenous populations from natural habitats can reflect prevailing contamination of the habitat as well as the adaptive mechanisms of the animals. Also, alterations in physiological or biochemical mechanisms resulting from cumulative effects of pollutants are best measured in native mussel populations (Nigro *et al.*, 2006). On the other hand, a closely controlled environment such as a laboratory aquarium gives insight on the persistence of these adapting mechanisms. However remediation studies on mussel populations transported from natural estuaries to laboratory aquaria are limited (e.g. Laffon *et al.*, 2006). Since we have followed this protocol we have provided a standardised, non-contaminated environment in the laboratory aquaria, a normalized regime of light, food and water input and low mussel densities.

Our choice of sites for mussel collection was based on the following grounds

- The sites should contain pure *Mytilus edulis* species populations
- The "reference" site should be less contaminated than the "polluted" site with respect to both organic and inorganic anthropogenic chemicals.

- Tidal oscillations should follow similar time and duration patterns since hypoxia/reoxygenation affect oxidation parameters (Almeida *et al.*, 2005).
- The sites should exhibit comparable salinities (Westerbom *et al.*, 2004) and pH
- Mussel collection should be performed on the same intertidal region and if possible on the same day
- Sites should be accessible by land

Our choice of sites was limited by the fact that *M. edulis* and *M. galloprovincialis* form a mosaic of populations containing pure species mixed together with hybrids of different ratios in a broad coastal zone in southwest England (Hilbish *et al.*, 2002). There has been considerable controversy since the 1980s about the relationship between the blue mussel *Mytilus edulis* and the Mediterranean mussel *Mytilus galloprovincialis*. The latter has probably diverged from the former when the Mediterranean Sea was cut off from the Atlantic during a Pleistocene ice age (Gosling, 1992). It is possible that the warm climate developed in the Mediterranean together with the limited water exchange between Mediterranean Sea and Atlantic Ocean have hastened the differentiation procedure (Bayne, 1976). Since then there is a continuous northern expanding of *Mytilus galloprovincialis* onto the Atlantic coast of Western Europe (Gosling, 1992). Even though *M. edulis* and *M. galloprovincialis* are distinct species with different morphological, physiological and genetic content (Wu, 2001) they readily interbreed forming hybrids since there is no gamete incompatibility between them (Hilbish *et al.*, 2002). *Mytilus galloprovincialis* thrives in relatively warmer waters than *Mytilus edulis* (Beaumont *et al.*, 2004) so estuaries like our

sampling regions possibly do not favor migration and growth of this species. However, verification of the species was essential before any direct comparisons were made.

In this chapter information is given about the pollution profile of the sites in question and about laboratory animal husbandry. The detection method of the species of the samples is also recorded.

2.2 Materials and Methods

2.2.1 Reagents

All chemicals unless otherwise stated were of the highest quality available from Sigma-Aldrich, Dorset, UK

2.2.2 Mussel collection

Mussels of similar size (4 to 4.5 cm) were collected from the urban/industrial site of New Brighton, Merseyside and the reference site of Llandudno, North Wales at the same intertidal region (800 m from the beach) and if possible on the same day, approximately one hour before the lowest tide. The mussels were placed in perspex boxes (18x 18x 18 cm) containing ice packs (New England Biolabs, US), which were not in direct contact with the specimens and transported to Birmingham within 4 h of collection.

2.2.3 Animal husbandry

After arrival, the mussels were scraped clean of barnacles (*Balanus improvisus*) since barnacles are more sensitive than mussels and environment changes may be lethal for them (J.P. Shaw, personal communication). Rock crabs and sea anemones were also discarded. Results from specimens infested with pea crabs (*Pinnotheridae)* were not

included since pea crabs destroy gills forming fibrous masses (Grove *et al.*, 2000). No parasitic nematodes (Gosling, 1992) were found in any specimens. Dead mussels were discarded as soon as possible. All assays described in subsequent chapters were performed within 24 h after overnight acclimatization lasting 12 h. The rest of the mussels were maintained in plastic aquaria of 12 l each with artificial (UV-sterilized) seawater (salinity 34‰) at ambient temperature 17° C at a density of 20 mussels per aquarium maximum. The artificial seawater was continually filtered through an activated charcoal column by an external pump. The pH of the water was 8.4 (Seamaster kit, Aquatic Pharmaceuticals, Canada) and the light regime was 12 h light/12 h dark. After the end of the "24 h" assays the rest of the specimens were fed with a combination of commercially available *Nannochlopsis oculata* (Phyto-Aqua, Shirley Aquatics UK) (1ml/6 l) and 2 g of powdered kelp every second day for 2 h. During feeding time the water was not renewed (Krishnakumar *et al.*, 1999). Within the course of one week since the acclimation of the remaining batch, the whole volume of the circulating water had been replaced with fresh artificial seawater. Nitrate levels were checked every week (Nitrate test kit, Aquatic Pharmaceuticals, Canada). Before the arrival of every new batch the aquaria were cleaned thoroughly and dried.

For the *in vivo* exposures, mussels (*Mytilus edulis* L.) from a Conwy farm (Conwy Mussel Company, the Quay, Conwy) were kept on ice for transportation and then acclimatized for 24 h in the aforementioned aquaria. Five mussels (in 4 replicates per treatment group) were then placed in glass beakers (Simax, Czech Republic) filled with 2 l artificial seawater each, which was continually aerated (ball airstones, Animal House, UK) at temperature 17° C with a 12 h light/ 12 h dark regime. The mussels

were fed with *Nannochloropsis oculata* (1 ml/6 l) every two days. The animals used were attached to the aquarium walls and exhibited normal feeding habits evident by the rapid algae clearance and the production of (pseudo)faeces. Water was changed every other day for Cd exposure and every day for Cr exposure, approximately one hour after feeding.

2.2.4 Isolation of genomic DNA from mussel specimens

Mussels of average shell length 4.0-4.5 cm were collected from the estuaries of interest and transported to laboratory aquaria as described in 2.2.2. The animal valves were opened with a metal scalpel (17 cm) and gill tissues were extracted with small size metal tweezers. Genomic DNA was isolated using the Total DNA Isolation Kit (Qiagen, US) according to the manufacturer's instructions. Briefly, 0.25 g of wet gill tissue/per animal were cut into small pieces, added to sterile 1.5 ml tubes containing Tissue Lysis Buffer (180 μl) and mixed thoroughly. Proteinase K (20 μl) was added and thoroughly mixed with the lysate and the mixture was incubated at 55° C for 3 h. Lysis buffer (200 μl) was added to the sample and the mixture was incubated at 70°C for 10 min. Ethanol 98% was added to the sample and mixed thoroughly. Then the whole of the solution was placed onto a provided spin column (Qiagen, US) attached to a 2 ml collection tube and subjected to centrifugation for 6000x g for 1 min. The eluate was discarded, a new collection tube was attached to the column and Washing Buffer 1 (500 μl) was added onto the spin column. Following centrifugation at 6000x g for 1 min, the eluate was discarded, a new collection tube was attached to the column and Washing Buffer 2 (500 μl) was added onto the spin column. The columns were then centrifuged at 20,000x g for 3 min. The spin column was carefully detached from the collection tube and a plain 1.5 ml tube was attached to the column. Elution

Buffer (200 µl) was added directly onto the membrane and the mixture was incubated at room temperature for 1 min. The columns were then subjected to centrifugation at 6000x g for 1 min and the eluate was collected and used immediately for subsequent analysis. Total DNA was quantified by UV spectrometry (UVIKON Spectrophotometer 922, Kontron Instruments, UK) by measuring absorption at 260 nm.

2.2.5 Polymerase chain reaction

The polymerase chain reaction (Inoue *et al.*, 1995) was used to amplify the non-repetitive domain of the *Mytilus edulis* foot protein-1 gene (*mepf-1*) using sequence specific primers (Table 4). The standard PCR reaction mix contained genomic DNA (1 µl), Taq polymerase (0.02 units, Bioline, UK), forward primer (10 pmol), reverse primer (10 pmol), 10 mM dNTPs (2 µl), 10 x NH_4 reaction buffer (10 µl), 50 mM $MgCl_2$ (3 µl) and nuclease-free water (to 50 µl). PCR steps were performed in a PCR machine (Minicycler™, MJ Research, UK). The PCR program consisted of 2 min initial denaturation at 94° C, and 30 cycles of denaturation at 94° C for 1 min, annealing at 50° C for 1 min (Table 4 for individual annealing temperatures) and extension at 72° C for 1.5 min. A final step was performed at 72° C for 10 min, following completion of the cycling phase. The products of the PCR reaction were analysed by agarose (2%) gel electrophoresis

Primer Name	Primer DNA Base Sequence (5′ → 3′)	Predicted Annealing Temperature
Me 15	CCAGTATACAAACCTGTGAAGA	52.3° C
Me 16	TGTTGTCTTAATAGGTTTGTAAGA	51.9° C

Table 4: Primers used in PCR-based speciation of mussel specimens

2.2.6 DNA gel electrophoresis

DNA gel electrophoresis allows isolation and visualization of individual DNA fragments according to their size. General purpose agarose (2 g) was added to 1x TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM Na_2EDTA, adjusted to pH 8.3) containing ethidium bromide (0.5 μg/ml). DNA samples (10μl) were subsequently mixed with Orange G loading buffer (2μl) (0.5% orange G, 50% glycerol) and loaded in the gel wells. Additionally, DNA molecular weight markers (New England Biolabs, USA) (100bp) were also electrophoresed on the same gel. Electrophoresis was performed at 80 V for 60 min and the gel was visualized by UV transillumination.

2.3 Results and discussion

2.3.1 Choice of sites

New Brighton is an urban site at the outer part of the Mersey Estuary, UK. It is also the final station for the Seacombe Ferry in Merseyside. Merseyside is a major estuary in the northwest England, which receives drainage for the megalopolis of Liverpool and Manchester. It has a long-standing association with shipping activities and a pollution legacy dating from the industrial revolution (Harino *et al.*, 2005). Shipping has declined substantially in Merseyside but Liverpool is still considered a major port. Consumption of fish from Merseyside should be done with caution; some fish had ΣPCB values higher than the acceptable limits of the Environmental Protection Agency for the USA in 1990-1992 (Leah *et al.*, 1997). The legacy of high pollution status of Merseyside does not favour the maintenance of mussel farms in the area (Harino *et al.*, 2005b). Various environmental pollutants have been measured in soft

tissues of mussels from outer Merseyside generally or New Brighton more specifically as shown in Table 5.

Site	Chemical	Concentration	Reference
New Brighton	Σ21PCB	168 ng/g dry w.	Thompson *et al.*, 1996
Isle of Man (ref)	Σ21PCB	28 ng/g dry w.	" "
Rock Ferry	α-MHCH	11± 3 ng/g wet w.	McNeish *et al.*, 1999
" "	α-HCH	0.7 ± 0.1 ng/g dry w.	" "
" "	γ-HCH	1.2 ± 0.3 ng/g dry w.	" "
New Brighton	Σ7PCB	58.9 ng/g wet w.	Shaw *et al.*, 2002
Port Quin (ref)	Σ7PCB	2.8 ng/g wet w.	" "
New Brighton	ΣPAH	160-2580 ng/g wet w.	Shaw *et al.*, 2004
Port Quin (ref)	ΣPAH	50-260 ng/g wet w.	" "
New Brighton	TBT	30 ng/g dry w.	Harino *et al.*, 2005
Egremont	TBT	30 ng/g dry w.	" "

Table 5: Concentrations of chemicals in mussel (*Mytilus edulis*) tissue from Merseyside and reference sites.

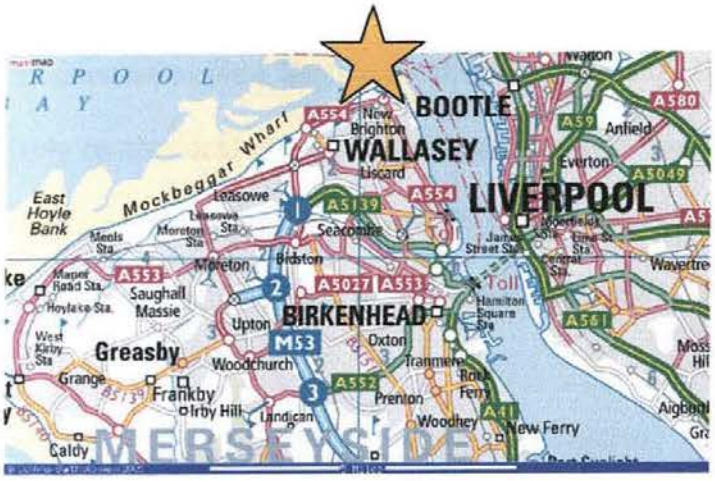


Figure 17: New Brighton is situated at the mouth of Merseyside towards the Irish Sea. Egremont, Seacombe and Rock Ferry are situated further inwards the Mersey estuary.

Merseyside is still burdened with some pollutants despite long remediation processes. Male flounder from Mersey estuary had significantly induced vitellogenin compared to samples from a number of other estuaries across UK (Kirby *et al.*, 2006). This is due to estrogenic-mimicking pollutants, which are commonly found in municipal wastewaters. Furthermore, rare organochlorines like hexachlorocyclohexane (HCH) with unknown biological consequences have been found in Rock Ferry, Merseyside (McNeish *et al.*, 1998). PCB pollution is still significant in Merseyside and sediment from Seacombe comprised the highest UK concentrations of Σ7PCB (Defra Status of the Seas Report, 2005). Quite elevated values (0.041-0.143 μg/l) for Hg were detected in Merseyside waters and also in sediments and dab flatfish possibly due to the chloralkaline industries of Liverpool. Also some mussel specimens exhibited concentrations higher than 50 μg of Hg/kg wet weight. No other metal or organic contaminant was above legislation standards for water in Merseyside (Defra Status of the Seas Report, 2005).

Llandudno, in contrast, is a resort in North Wales directly facing the Irish Sea. In further contrast to New Brighton and Merseyside very little recorded information is available on chemical exposures for Llandudno and coastal North Wales. The biggest towns in close proximity are Colwyn Bay and Conwy, which are also holiday resorts. Conwy is historically home to many mussel farms for human consumption.

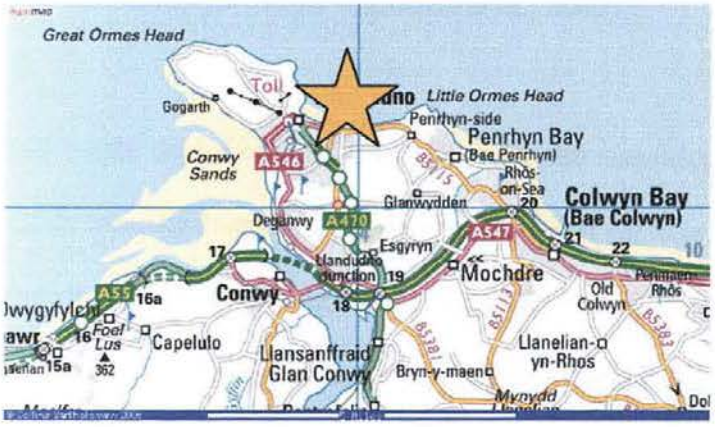


Figure 18: Llandudno is situated on coastal North Wales towards the Irish Sea.

The only direct comparison between specimens from New Brighton and Llandudno (and other Irish Sea stations) can be found in Widdows *et al.*, (2002). Some of the results are summarised in Table 6.

Sites	Metals										Organotin Organics		
	Cr	Ni	Cu	Zn	As	Se	Ag	Cd	Hg	Pb	TBT	Σ25CB	PAH
NB	0.6	0.8	8.9	147	8.9	3.3		0.37	0.16	3.6	0.09	0.184	4.47
LL	0.6	0.6	5.0	50	7.9	10.4		nd	0.10	2.0	nd	0.004	1.22
Detection limit								0.01	0.1		0.03	0.002	

Data in μg/g dry weight

Table 6: chemical contaminants in body tissues of Irish Sea mussels (mean values of duplicate samples) (from Widdows *et al.*, 2002).

Additional data from e.g. Port Quin (a site considered pristine) and from e.g. the highly industrialised Blackpool site show that there is no ideal "reference" site in the Irish Sea, at least among the sites tested: Port Quin samples for example presented relatively high levels of As (20.2 μg/g dry weight) and Blackpool had comparatively lower levels of ΣPCB compared to New Brighton (0.062 μg/g dry weight). According to Widdows *et al.*, (2002) New Brighton is considerably polluted in organic chemicals (PAH, PCB) whereas Llandudno is not. This is further corroborated by recent analysis of mussel and fish specimens for PCB (Figure 19). There was however a reduced scope for growth in Llandudno mussels, which in contrast to New Brighton mussels, could not be explained by the presence of PAH (Widdows *et al.*, 2002). The presence of sewage output near the area may explain this phenomenon (Figure 20). Still, the input of the wider Merseyside area, at the mouth of the estuary is stronger than the input of the sewage near Llandudno.

Exposure to heavy metals was evident in mussel specimens from both sites. From the metals analysed, levels were generally relatively high at NB (Table 6) and Cd

particularly was significantly elevated in Merseyside in comparison to coastal North Wales (Figure 21).

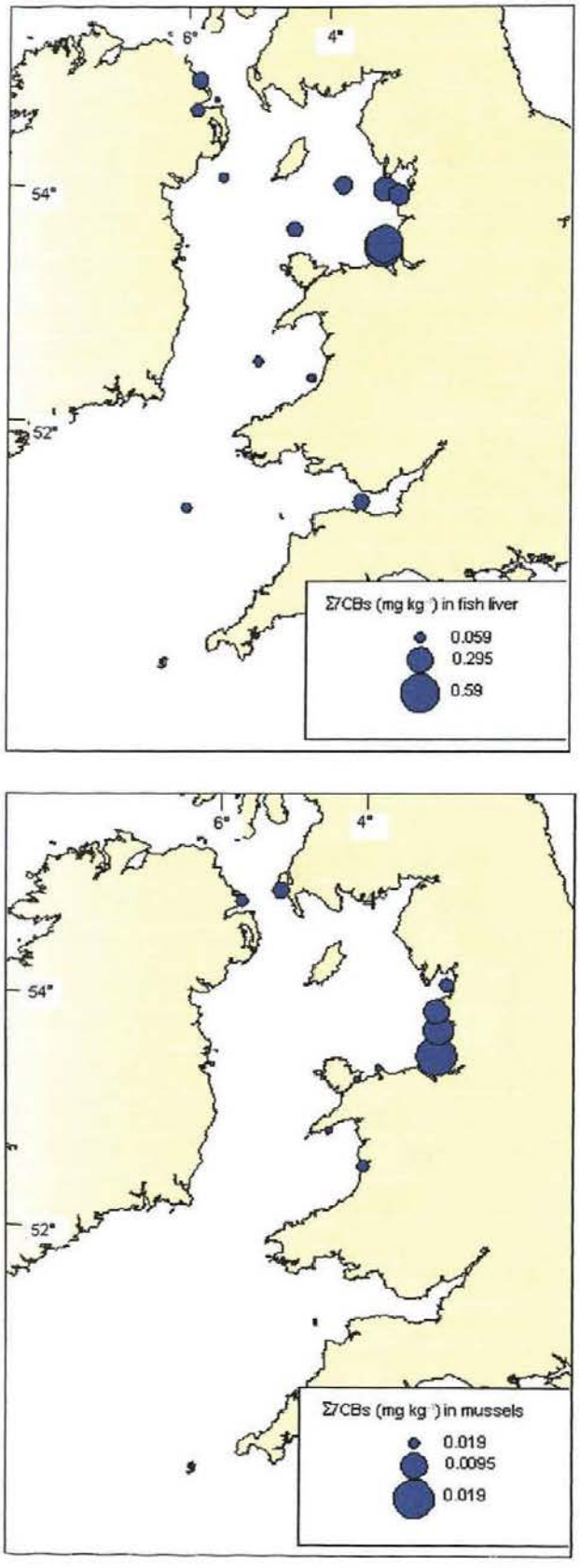


Figure 19: ΣPCB in fish liver (19A) and mussel tissues (19B) from Irish Sea (from Defra Quality Status Report of the Marine and Coastal Areas of the Irish Sea and Bristol Channel, 2000)

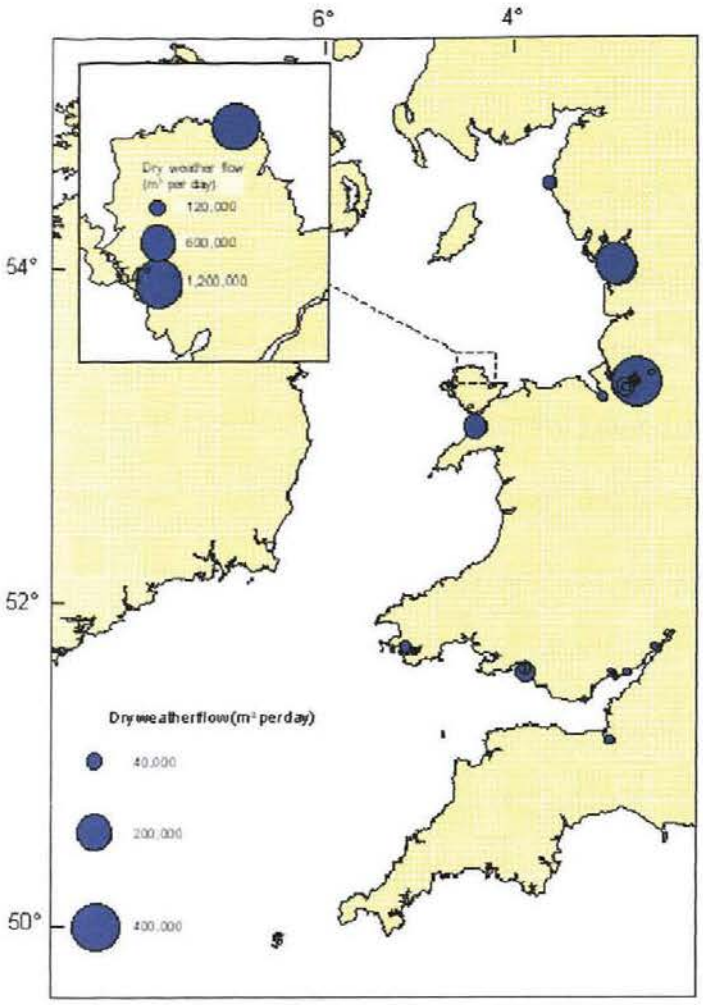


Figure 20: sewage discharged to tidal waters to Irish Sea (from Defra Quality Status Report of the Marine and Coastal Areas of the Irish Sea and Bristol Channel, 2000)

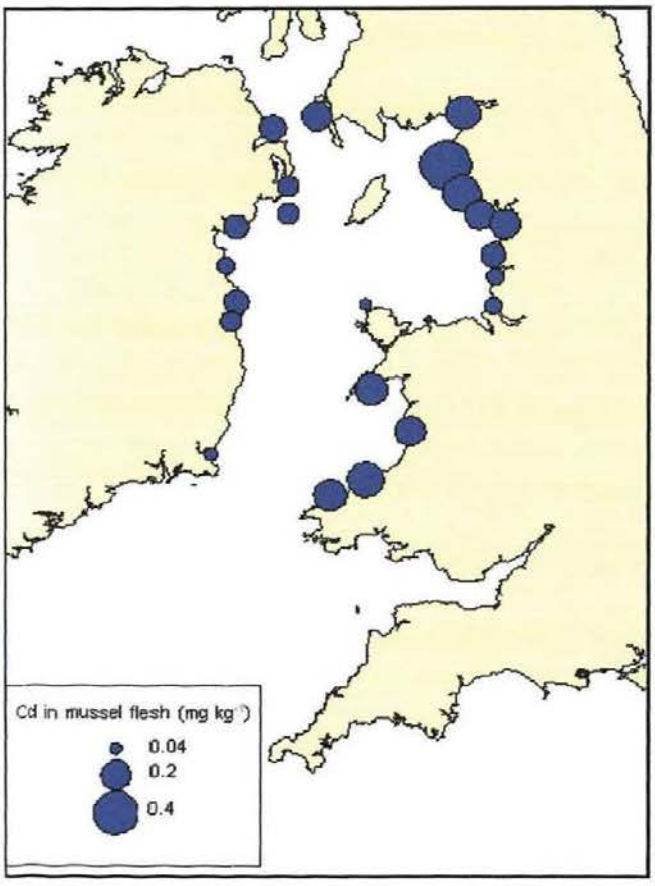


Figure 21: Cd in mussel tissues (from Irish Sea (from Defra Quality Status Report of the Marine and Coastal Areas of the Irish Sea and Bristol Channel, 2000)

2.3.2 Animal husbandry

Mussels are able to withstand long periods without feeding however starvation has a detrimental effect on animal well-being. Our initial diet of choice of powdered kelp was subsequently enriched with commercially available algae (*Nanochloropsis oculata*) that is within the mussel filtering range (cell density 250,000,000 cells/ml, cell diameter ~ 2.5 microns). This diet was finally used as a main diet with powdered kelp as a secondary. The endpoints used to test the success of the diet were the following: thickness and appearance of mantle, gill detachment, mantle detachment, aerial survival, filtering ability, response to stimuli (shell closure), (pseudo)feaces production. As a general observation the animals adapted well to the diet and in any case the diet followed was identical for all of our groups (different sites, different month collection). Furthermore, the mussels were able to filter rapidly the algae in our *in vivo* experiments within 1 h from feeding time (green aerated seawater becoming transparent again). The opportunistic mode of continuous and late spawning evident by the recruitment of juvenile mussels (spat) is another indication of sufficient food availability (Gosling, 1992).

2.3.3 Mussel speciation

Figure 22 shows representative results of PCR amplification of samples from animals from New Brighton and Llandudno (n=3). All animals gave a 180 bp fragment, which is characteristic for *M.edulis*. The experiment was repeated 2 more times with 3 further replicates for each site and gave equivalent results (data not shown).

M.edulis and *M.galloprovincialis* form hybrids in southwest English estuaries (Wilhelm and Hilbish, 1998; Hilbish *et al.*, 2002; Gilg and Hilbish, 2003) and

M.galloprovincialis has been detected even in northwest England (Skibinski *et al.*, 1983). Differences in growth rates (Lobel *et al.*, 1990) and fitness (Hilbish *et al.*, 2003; Ciocan and Sunila, 2005) are common between the two species and can affect various biomarkers; therefore pure species give more credible results. Speciation between *M. edulis* and *M. galloprovincialis* or between any other species of *Mytilus* is not easy, with simple morphometric criteria allowing for a high percentage of missclassification (Seed, 1992). There is no single morphological characteristic to discriminate effectively between allopatric *M.edulis*, *M.galloprovincialis* and *M.trossulus*, however a combination of 6 or 7 characteristics at least was able to distinguish *M.edulis* from *M.galloprovincialis* (Gosling, 1992; Toro, 1998). Diagnostic allozyme frequencies can also be used (Seed, 1992; Comesãna *et al.*, 1998). The introduction of PCR-based method greatly simplified the speciation process. Foot protein 1 or Mefp-1 is a highly basic and hydrophilic protein that enables adhesion of the mussel to its substrate. It is also responsible for forming a protective varnish along the attachment plaque (Frank and Belfort, 2002). The amino acid sequence of Mefp-1 in *Mytilus edulis* is different from this in *Mytilus galloprovincialis* (Inoue *et al.*, 1995). In the non-repetitive domain of the protein *M. edulis* has 18 amino acids that are completely deleted in *Mytilus galloprovinciallis*. As a result, amplification of the sequence corresponding to the non-repetitive domain of foot protein will give fragments of different size according to species: a 190 bp for *M.edulis* and a 126 bp for *M. galloprovincialis*. This observed difference in the non-repetitive domain between the two species was constant (Inoue *et al.*, 1995; 1997). Using this method the specimens from New Brighton and Llandudno were proved to be *M.edulis* as expected. Furthermore, no *Mytilus galloprovincialis* alleles were found northern of St. Ives in Cornwall when the authors examined mussel populations along the north Cornish Coast (Hilbish *et al.*,

2002). Samples from Conwy, North Wales have also been shown to be *M.edulis* by others (Beaumont *et al.*, 2004). As a conclusion, we have proved using this PCR-based method that our estuaries of interest contain pure *M.edulis* species.

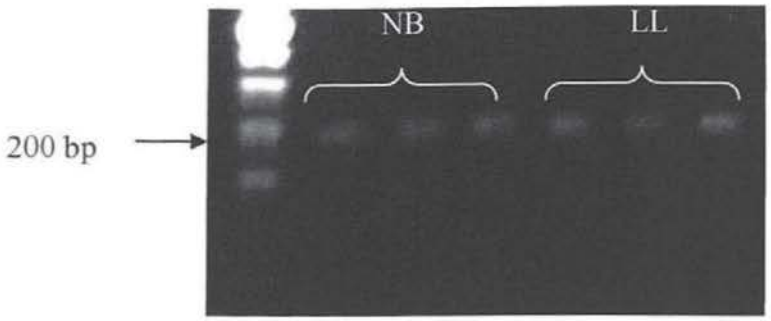


Figure 22: Agarose gel containing 0.5 µg/ml ethidium bromide. A DNA molecular weight marker (New England Biolabs, US) was also subjected to electrophoresis. Samples 1, 2 and 3 correspond to DNA extract from three mussels collected from New Brighton. Samples 4, 5 and 6 correspond to DNA extract from three mussels collected from Llandudno. No fragment corresponding to 168 bp (*M.galloprovincialis*) or 126 bp (*M.trossulus*) was found. All samples gave a 180 bp fragment indicating the species of the mussels sampled as *M.edulis*.

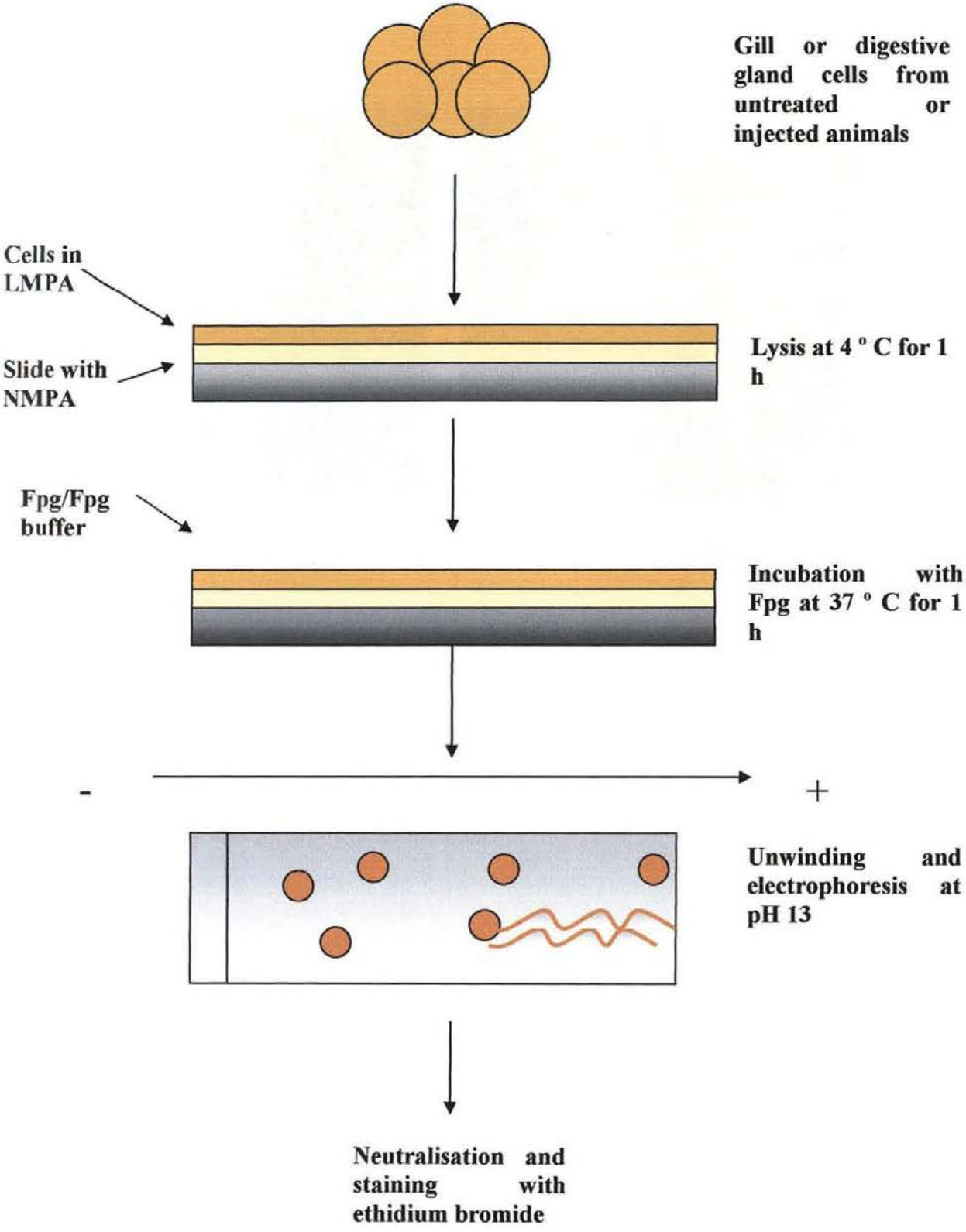
3.0 DNA strand breaks in gill of mussels from New Brighton and Llandudno populations

3.1 Introduction

DNA single strand breaks (SSB) have been widely measured in humans, other mammals, vertebrates and invertebrates for monitoring exposure to genotoxic agents (Danadevi *et al.*, 2004; Winter *et al.*, 2003; Table 2, p.22) and for detecting or verifying genotoxic properties of chemical agents (Blasiak *et al.*, 1999; Table 2, p.22). In recent years these measurements are mostly done via the alkaline Comet assay, a method commonly applied for detecting DNA damage (Møller *et al.*, 2006). Rydberg and Johanson introduced the method in 1978, which was later modified by Singh and colleagues in 1988 and Olive in 1989 (Møller *et al.*, 2006). Briefly, cells are embedded in agarose slides and lysed in high salt solution. Then they are subjected to electrophoresis under alkaline conditions, stained with a DNA intercalating dye and visualised under fluorescent microscopy (Singh *et al.*, 1988). DNA occurs as supercoiled loops in intact cells. The presence of SSB relaxes the supercoiling and under electric current they migrate towards the anode creating the characteristic comet tail (McKelvey-Martin *et al.*, 1993; Cotelle and Férard, 1999). Essentially, the comet tail is the halo of relaxed loops pulled to one side by the current. Stripping the nucleus of histones and membranes by high salt solution is a prerequisite for free DNA movement (Collins, 2004). The alkaline version of the comet assay detects a broad spectrum of DNA damage including single strand breaks, alkali labile sites and single strand breaks associated with incomplete excision repair (Hartmann *et al.*, 2003).

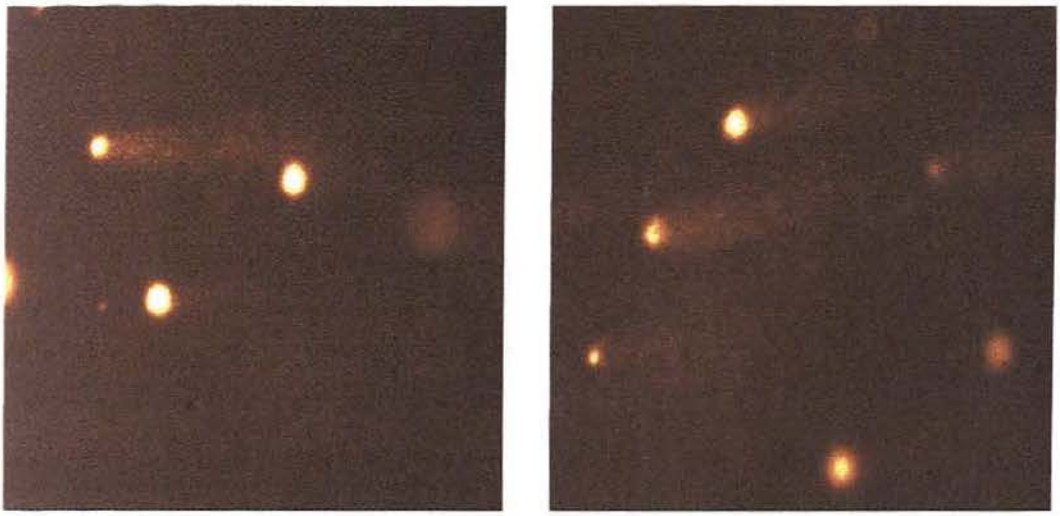
Although SSB are quickly repaired and are not considered highly mutagenic (Collins *et al.*, 1997), they can nonetheless be very predictive of the mutagenic potential of a chemical (Lee *et al.*, 1996). Coupling the conventional comet assay with a glycosylase-endonuclease extended further the applications of the assay. Incubation of

the cells with a lesion-specific endonuclease after the lysis step introduces additional strand breaks at the site of the lesion. Until today the comet assay has been coupled with Fpg for detection of 8-oxo-dG (Cheng *et al.*, 2003; Blasiak *et al.*, 2004), endo III (nth) for detection of oxidised pyrimidines (Collins *et al.*, 1997; Blasiak *et al.*, 2004), uvrABC for bulky DNA adducts (Collins *et al.*, 1997) and AlkA for alkylated bases (Woźniak and Blasiak, 2002; Blasiak *et al.*, 2004). The coupled comet assay can detect a wide range of lesions as long as a specific glycosylase is available. This modification enhanced the appeal of the technique, which is also relatively fast, simple, sensitive and inexpensive (Rojas *et al.*, 1999; Lee and Steinert, 2003). A schematic representation of the coupled comet assay is given in Figure 23.



Fpg: formamidopyrimidine glycosylase, **LMPA**: low melting point agarose, **NMPA**: normal melting point agarose

Figure 23: Schematic representation of the comet assay including the coupling with Fpg for detection of oxidised DNA.



Nuclei of untreated gill cells

Nuclei of gill cells from Cr(VI)-injected mussels

Figure 24: Images of gill nuclei stained with ethidium bromide visualised by fluorescent microscopy (200x magnification).

Mussels were collected from the sites of interest (New Brighton; NB and Llandudno; LL) at various time points of the year 2005-2006 and gills from representative animals were analysed by the alkaline comet assay with and without coupling with Fpg comet for detection of frank SSB and oxidative DNA damage (reflecting principally 8-oxo-dG) respectively. Another set of representative animals per site was injected with the pro-oxidant sodium dichromate ($Na_2Cr_2O_7$, 0.1μmol in 100 μl distilled water). Their gills were also analysed for SSB and 8-oxo-dG. Comparisons were made between sites (NB and LL) and between time points ["24 h" samples and "1 month" (post-maintenance samples)]. Temporal variations in SSB and 8-oxo-dG were also checked. Finally, correlations of oxidative DNA damage to 8-oxo-dG excision capacity by DNA repair enzymes were made (Chapter 4). Preliminary results on gill after treatment with selected genotoxic chemicals are presented. The effects of buffers (Fpg buffer) or procedures (injection, carrier) on recorded % tail DNA is also recorded as well as preliminary results on mussel digestive gland.

3.2 Materials and methods

3.2.1 Reagents: see 2.2.1

3.2.2 Buffers

HEPES buffered saline: HEPES (0.12 M), KCl (0.15 M) and Na_2EDTA (6 mM), pH 7.2.

HEPES buffered sucrose: HEPES (0.02 M), KCl (0.16 M), Na_2EDTA (1 mM), $MgCl_2$ (0.03 M) and sucrose 8% (w/v), pH 7.8. Before use DMSO (1% v/v) was added.

Lysis solution: NaCl (2.5M), Na_2EDTA (0.1M), sodium-*N*-lauryl-sarcosinate (SLS) solution (33.3 ml) and Tris base (10 mM), pH 10.0 made up to 1 litre with deionised water and stored at 4 ° C. Before use a fraction (89 ml) was removed and Triton-X (1ml) and DMSO (10 ml) were added.

Electrophoresis buffer: NaOH (0.1 M) and Na_2EDTA (1 mM), pH 13.0 made up to 2 litres before use and stored at 4 ° C for at least 1 h.

Neutralisation buffer: Tris base (0.4M), pH 7.5.

Fpg enzyme buffer: (according to Gielazyn *et al.*, 2003) NaCl (0.1M), HEPES (40 mm) and Na_2EDTA (0.5mM), pH 8.0 made up to 400 ml with deionised water and autoclaved. Bovine serum albumin (0.2 mg/ml) was added to the cooled solution. The solution was aliquoted (10 ml each) and stored at -20° C.

Fpg solution: 1 unit Fpg (Trevigen, US) / 50 µl Fpg enzyme buffer.

Staining solution: Ethidium bromide (20 µg/ml) filtered (0.2 µm filter, Pall Corporation US) and stored in the dark at 4° C.

Normal melting point agarose (NMPA) and low melting point agarose (LMPA): Normal or low melting point agarose solution (0.5 w/v in PBS) autoclaved and stored

at room temperature. Before use, LMPA was dissolved by heating in microwave (800W, 5 min).

Iodixanol gradient: 50% v/v Optiprep®/HEPES buffered sucrose (1 ml), 20% v/v Optiprep®/HEPES buffered sucrose (5 ml), 15% v/v Optiprep®/HEPES buffered sucrose (5 ml).

PBS: phosphate buffered saline (0.01M), KCl (0.0027M) and NaCl (0.137M), pH 7.4.

$Na_2Cr_2O_7$ solution: freshly made 1 M stock solution of $Na_2Cr_2O_7$ in deionised water diluted to 1mM.

3.2.3 Gill sample preparation for untreated animals

Mussels (n=3-4) from each of the two sites were randomly selected after overnight acclimatization. The animal valves were opened with a metal scalpel (17 cm) and gill tissues were extracted with small size metal tweezers. The tissue was gently disaggregated in a 25 ml glass beaker (Duran, Mainz Germany) containing HEPES buffered saline (5ml) and filtered through 2 layers of 150 μm gauze. The filtered solution was collected in sterile 1.5 ml microcentrifuge tubes (2 tubes per animal) and subjected to centrifugation (M.S.E., Sanyo, Japan) at 2000 x g for 5 min at room temperature. The cells were resuspended in buffered saline (1 ml) and a fraction (empirically determined) was discarded in order to keep a low density of cells on the final slide (Tice *et al.*, 2000). The remaining solution was diluted to final volume 1 ml and subsequently centrifuged for 10 min at 2000 x g at room temperature. The final pellet was resuspended in buffered saline (150 μl). An aliquot of the pellet (15.0 μl) was added to molten LMPA (150 μl). The same procedure was done with tissue from

animals (n=3-4), which were kept under laboratory clean conditions for one month post-collection.

3.2.4 Digestive gland sample preparation for untreated animals

Mussels (n=6) from NB were randomly selected after overnight acclimatization. The animal valves were opened with a metal scalpel (17 cm) and digestive gland tissues were extracted with small size metal tweezers. The tissue (pooled per 2 animals) was homogenised in a 20 ml glass homogeniser with Teflon pestle containing HEPES buffered sucrose (5ml) and filtered through 2 layers of 150 µm gauze. The filtered solution was collected in sterile 1.5 ml microcentrifuge tubes (2 tubes per pooled tissue) and subjected to centrifugation (M.S.E., Sanyo, Japan) at 2000 x g for 5 min at room temperature. The cells were resuspended in buffered sucrose (1 ml) and subsequently centrifuged for 10 min at 2000 x g at room temperature. The final pellet was resuspended in buffered sucrose (1.5 ml) and loaded onto a freshly prepared Iodixanol gradient (Optiprep®) in an ultra-centrifuge tube (Beckman centrifuge tubes, 14 x 95 mm). The tubes were balanced accurately and subjected to centrifugation (Beckman L7 65 Ultracentrifuge) at 25,000x g for 1 h at 4°C. After centrifugation the bottom layers of the gradient were discarded and the top layer, containing the yellow-coloured nuclei, was collected and subsequently centrifuged for 10 min at 2000 x g at room temperature. The final pellet was resuspended in buffered sucrose (150 µl). An aliquot of the pellet (15.0 µl) was added to molten LMPA (150 µl). The same procedure was done with animals (n=6), which were kept under laboratory clean conditions for one month.

3.2.5 Gill sample preparation for Cr(VI)-injected animals

Mussels (n=3) from each of the two sites were randomly selected after overnight acclimatization. In a fume hood (Faster™ cytosafe cabinet, Wolf Laboratories Ltd, York) the mussels were carefully opened on a metal tray with a metal scalpel (17 cm) so as not to damage any tissues and the excess water was taken out of the mussel body. Then each mussel was injected with 0.1 μmol $Na_2Cr_2O_7$/ in 100 μl deionised water (1 mM) with a 1 ml syringe and a 23G ultra-thin wall needle (Terumo Corp, Japan) through the posterior adductor muscle. A successful injection did not leave an orange coloured run-off on the metal tray. Then the animals were returned to clearly marked small plastic tanks (11 each) at ambient temperature of 16°C where they stayed for 4 h. After 4 h the mussels were sacrificed and their gills were analysed as described in 3.2.3. The same procedure was done with animals (n=3), which were kept under laboratory clean conditions for one month post-collection. Preliminary experiments were also completed with different concentrations of Cr(VI), benzo[a]pyrene (BaP) and the pyrethroid deltamethrin.

3.2.6 Slide preparation

Slides were initially prepared by dipping twin-frosted microscope slides (BDH, UK) in NMPA (0.5w/v) solution and leaving them to dry at room temperature for at least 48 h. Cell suspensions (15.0 μl) in LMPA agarose (150 μl, 2 suspensions per aliquot; 4 suspensions per animal) were added on the slide. Glass coverslips (BDH, UK, 22 x 64 mm) were added and the slides were placed on a thin metallic tray, which was in contact with ice for at least 15 min. Following solidification of the agarose, the coverslips were removed and the slides were lowered in a Coplin jar containing cold lysis solution and incubated for 1 h in the dark at 4°C. Following lysis the slides were washed in cold Fpg enzyme buffer (3x 5 min, 1 ml each slide). Fpg solution (1 unit of

Fpg, 50μl) was added on the slide made from the one suspension of the aliquot and Fpg buffer (without Fpg, 50 μl) was added on the slide made from the remaining suspension of the aliquot. Coverslips were added and the slides were placed in a moist plastic box for incubation at 37°C for 1 h in the dark.

3.2.7 Unwinding, electrophoresis and staining

Slides were removed from the moist box and placed into a horizontal electrophoresis tank (Pharmacia, GNA 200, US) containing cold electrophoresis buffer for 30 min in the dark. Following electrophoresis at 30 V/200 mA (Pharmacia GPS 200/300) for 20 min at room temperature, slides were removed and washed in neutralisation buffer (3 x 5 min, 1 ml). Finally, slides were stained with ethidium bromide (20 μg/ml, 50 μl), covered with a glass coverslip and stored in a plastic humid box at 4° C in the dark until scoring (within 48 h).

3.2.8 Analysis of cells using fluorescence microscopy

Each slide was analysed using a fluorescent microscope (Axiovert inverted fluorescence microscope, Zeiss, Germany) at 200 x magnification, with an excitation filter of 515-560 nm from a mercury lamp and a barrier filter of 590 nm and scored using an image analysis package (Comet 3.0 Europe Kinetic Imaging Ltd, Liverpool, UK). Fifty randomly selected nucleoids were analysed per slide in duplicates so that a total of 100 cells (per Fpg incubation) were scored.

3.2.9 Statistical analysis

SSB were assessed using the parameter %DNA in tail. This parameter is linearly related to strand breaks over a wide range of levels of DNA damage (Kumaravel and

Jha, 2006). Also, it is less prone to inter electrophoresis and interexperimenter variation than other comet parameters (De Boeck *et al.*, 2000). Differences in median % DNA in tail (Duez *et al.*, 2003) between time-points or between sites for frank SSB or Fpg-sensitive sites (8-oxo-dG sites) were assessed by a two-tailed Student's *t*-test for median values. Temporal differences within sites for SSB or 8-oxo-dG and response to chemical treatments were assessed by a one-way ANOVA using median values accompanied by a *post-hoc* Student's *t*-test as recommended by Duez *et al.*, 2003.

3.3 Results

- Contribution of Fpg buffer to recorded SSB

Fpg buffer for mammalian cells (Trevigen, US) created additional damage to the gill cells of mussels for reasons unknown. It was therefore substituted with the buffer used for oyster haemolymph by Gielazyn *et al.*, (2003). **Figure 25** shows the results from gill cells on slides incubated with deionised water (50 µl) [mean (n=3) of median values for % tail DNA: 13.65 ± 3.6] compared to gill cells incubated with Fpg buffer based on Gielazyn *et al.*, (2003), (50 µl) [mean (n=3) of median values for % tail DNA: 12.98 ± 4.41] for 1 h at 37 ° C. Results were compared by Student's paired *t*-test for medians. There was no statistically significant difference between treatments, meaning that the buffer used did not create additional SSB.

-Preliminary experiments on gill from mussels exposed to genotoxic chemicals

The conventional comet assay (measuring frank SSB only) was able to detect genotoxic effects at different levels of exposure and by different compounds as shown by preliminary experiments involving injection on specimens from NB. Mussels

(n=3) were injected with $Na_2Cr_2O_7$ in distilled water (0.1 and 1 μmol/ in 100μl), BaP in DMSO (0.4 and 1.59 μmol/ in 100 μl) (Bihari *et al.*, 1990) or deltamethrin in DMSO (2 and 20 μmol/ in 100 μl) as shown in **Figure 26A**, **26B** and **26C** respectively. There was a dose-related increase in SSB in mussel gill for all the compounds tested, 4 h post-injection.

-Contribution of injection and/or carrier to recorded SSB

Injection *per se* did not contribute to recorded % DNA in tail as shown by preliminary experiments. Mussels from NB (n=6) were either not injected or injected with deionised water (100 μl) or DMSO (100 μl) (**Figure 27**). There was no statistically significant difference between treatments, meaning that the injection and the carrier (distilled water or DMSO) did not create additional SSB.

-Effect of one-month laboratory maintenance on frank SSB in digestive gland of mussels from NB and LL

Frank SSB (as measured by the conventional comet assay) were recorded for digestive gland cells of NB samples in two separate experiments on September 2004 (**Figure 28**). The levels of SSB were slightly higher after one-month laboratory maintenance ($P< 0.05$, two-tailed Student's *t*-test), in accordance with the increase in SSB noted in gill after one-month laboratory maintenance (**Figure 31A, B**).

-Temporal variation in frank SSB in gill of mussels from NB and LL

Temporal variation in frank SSB was recorded for NB and LL samples at "24 h" (overnight acclimatisation group) throughout the year (**Figure 29A,B** respectively). Statistically significant differences between months were noted for both sites. Overall

there was no trend in the variability throughout the year although it is noted that the sampling in May gave the lowest response in mussels from both sites.

-Temporal variation in 8-oxo-dG in gill of mussels from NB and LL

Temporal variation in 8-oxo-dG was recorded for NB and LL samples at "24 h" throughout the year (**Figure 30A,B** respectively). There were no statistically significant differences between months for both sites. Overall there was no trend in the variability throughout the year although it is noted that higher 8-oxo-dG levels were recorded at summer (July 2005 and July 2006) for NB samples.

-Effect of one-month laboratory maintenance on frank SSB in gill of mussels from NB and LL

Frank SSB (as measured from the non-Fpg incubated samples) were recorded for gill cells of both NB samples (**Figure 31A**) and LL (**Figure 31B**) at "24 h" and at "1 month" (post-collection) for July and September 2005 and February, May and July 2006. Although not consistent in all the samples, there was a general trend for relatively high levels of SSB after one-month laboratory maintenance for mussels of both sites. The aforementioned levels (SSB at " 1 month") were the same, irrespective of site or month (two-way ANOVA for median values).

Frank SSB in gill cells of NB mussels were not statistically different from frank SSB in gill cells from LL mussels ("24 h" samples) with the exception of February 2006 when more SSB were recorded for NB samples ($P < 0.05$, two-tailed *t*-test for median values).

-Effect of one-month laboratory maintenance on 8-oxo-dG in gill of mussels from NB and LL

8-oxo-dG (as Fpg-sensitive sites) was also recorded for gill cells of both NB samples (**Figure 32A**) and LL samples (**Figure 32B**) at "24 h" and at "1 month" (post-collection) for July and September 2005 and February, May and July 2006. There was a general trend of lower levels of 8-oxo-dG after one-month maintenance of mussels for NB. For individual samples this was statistically significant only for July 2005 sampling ($P<0.05$). However, when a paired *t*-test was performed between the mean values (n=3-4) of median % tail DNA for "24 h" samples against "1 month" samples, there was an overall decrease ($P<0.05$) in 8-oxo-dG after laboratory maintenance (**Figure 33A**). In contrast, there was no trend of lower levels of 8-oxo-dG after one-month maintenance of mussels for LL. When a paired *t*-test was performed between the mean values (n=3-4) of median % tail DNA for "24 h" samples against "1 month" samples, again there was no statistically significant decrease (**Figure 33B**).

8-oxo-dG (as Fpg-sensitive sites) in gill cells of NB mussels were not statistically different from 8-oxo-dG in gill cells from LL mussels ("24 h" samples) with the exception of July 2005 when more 8-oxo-dG was recorded for NB samples ($P< 0.05$, two-tailed *t*-test for median values). By one-month, the relative values for 8-oxo-dG at each site were equivalent (two-way ANOVA for median values).

-Effect of one-month laboratory maintenance on frank SSB in gill of mussels from NB and LL after injection with $Na_2Cr_2O_7$.

Frank SSB (as measured from the non-Fpg incubated samples) were recorded for gill of both NB samples (**Figure 34A**) and LL samples (**Figure 34B**) after $Na_2Cr_2O_7$

treatment (injection with 0.1 μmol $Na_2Cr_2O_7$ in 100 μl distilled water). The experiment was performed at two time points: within 24 h post-collection ("24 h" samples) and after one-month maintenance in laboratory conditions ("1 month" samples) for February, May and July 2006. There was an increase in SSB after laboratory maintenance only in May for both sites ($P<0.01$). However, we have to note that the baseline SSB on other untreated specimens of the same batch (May 2006) was also elevated after one-month maintenance. Thus the increase in SSB after Cr(VI) injection at the "1 month" samples is possibly due to the higher baseline SSB recorded for this month in particular.

-Effect of one-month laboratory maintenance on 8-oxo-dG in gill of mussels from NB and LL after injection with $Na_2Cr_2O_7$

8-oxo-dG (as Fpg-sensitive sites) were recorded for gill of both NB samples (**Figure 35A**) and LL samples (**Figure 35B**) after $Na_2Cr_2O_7$ treatment (injection with 0.1 μmol $Na_2Cr_2O_7$ in 100 μl distilled water). The experiment was performed at two time points: within 24 h post-collection ("24 h" samples) and after one-month maintenance in laboratory conditions ("1 month" samples) for February, May and July 2006. There was a decrease in 8-oxo-dG after laboratory maintenance only in July 2006 for NB ($P<0.05$). The interference of baseline 8-oxo-dG (8-oxo-dG before the injection) for this particular month is minimal as shown in Figure **35A,B**.

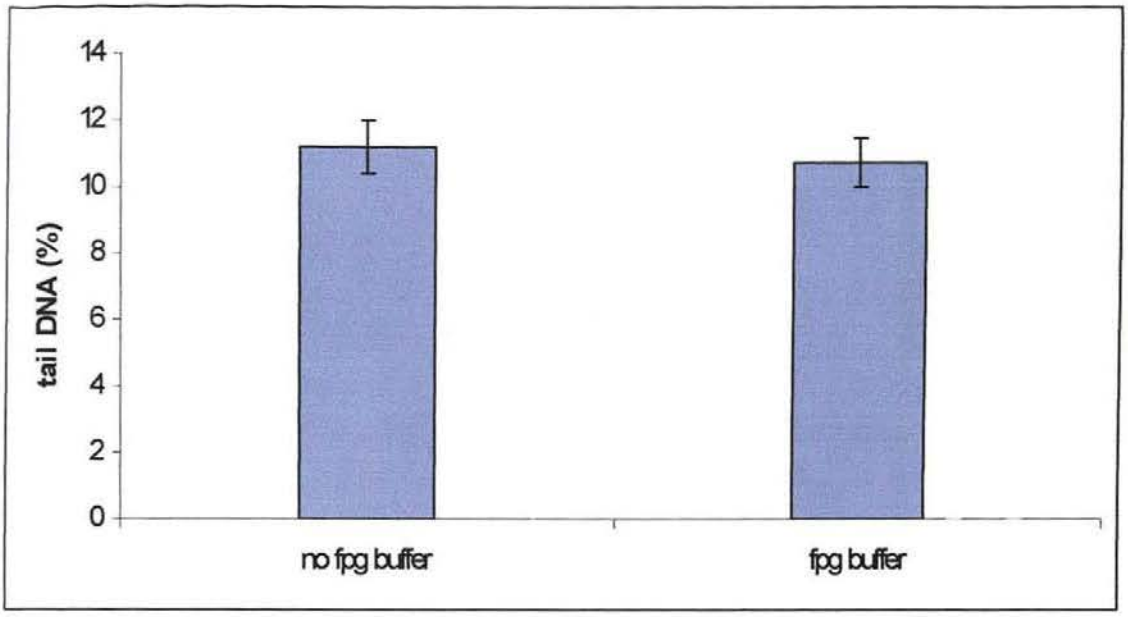


Figure 25: Effect of Fpg buffer on frank SSB for mussel gill cell (n=3 slides, ± SEM). No significant difference (P>0.05) between treatments as assessed using Student's paired *t*-test for medians.

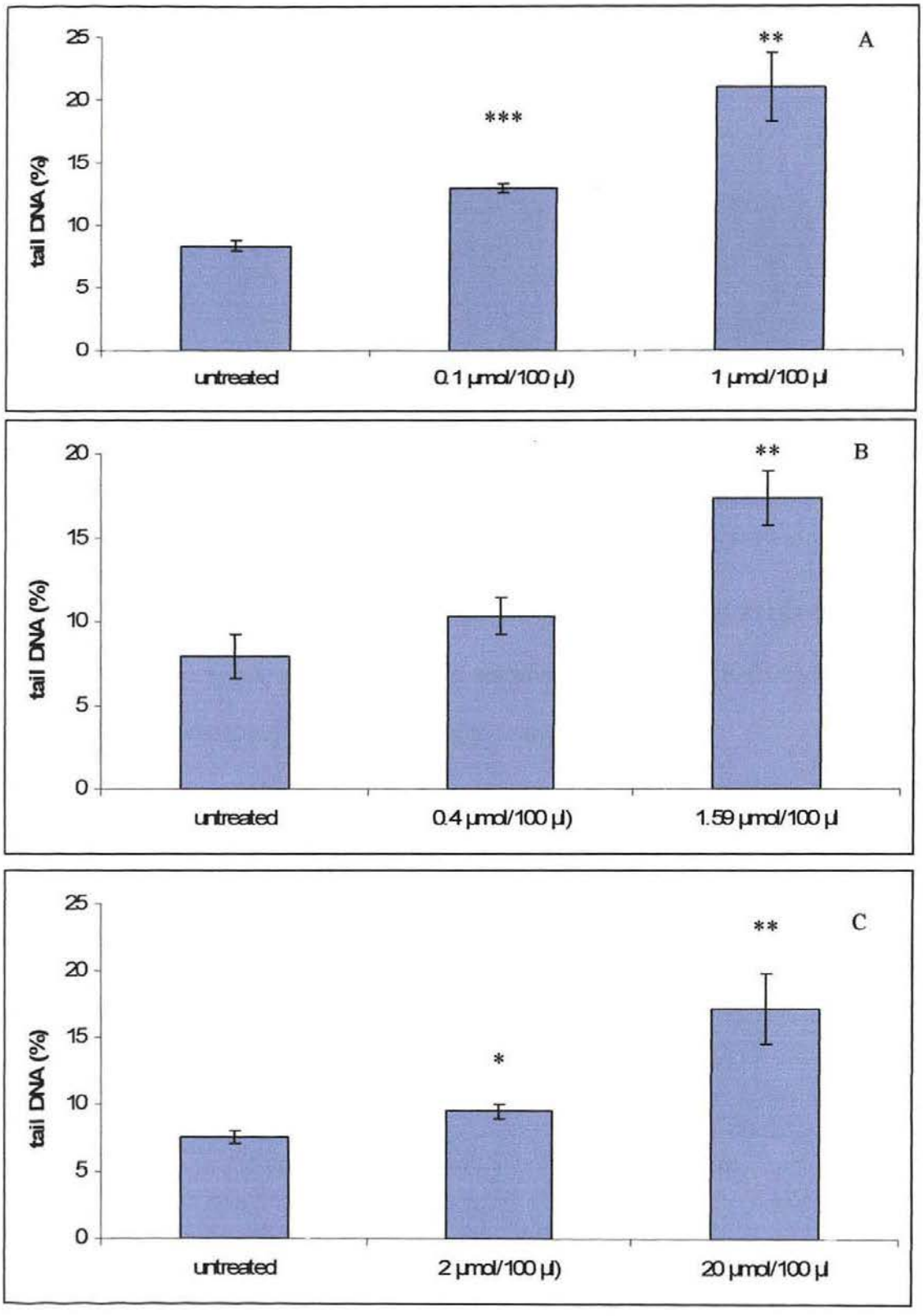


Figure 26 A,B,C: Effect of injection with $Na_2Cr_2O_7$, BaP and deltamethrin respectively 4h post-exposure. * Significantly different ($P<0.05$) compared to controls as assessed using assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test. ** ($P<0.01$), *** ($P<0.001$).

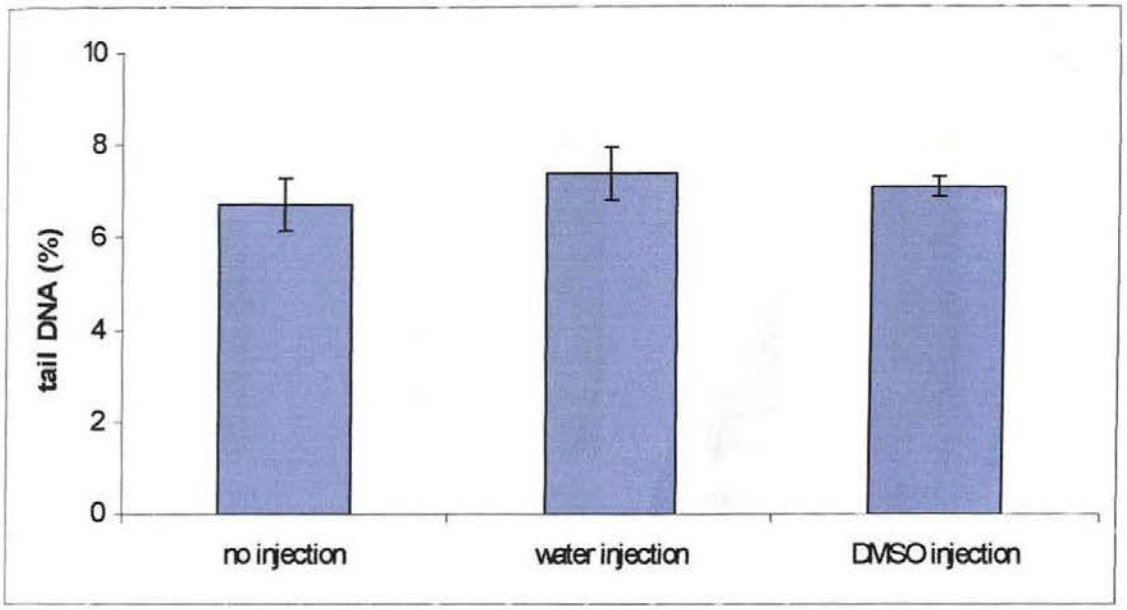


Figure 27: Effect of injection with water (100 µl) or with DMSO (100 µl) on frank SSB in gill cells (n=6 mussels, ± SEM). No significant difference (P>0.05) between treatments as assessed using Student's one-tailed *t*-test for medians.

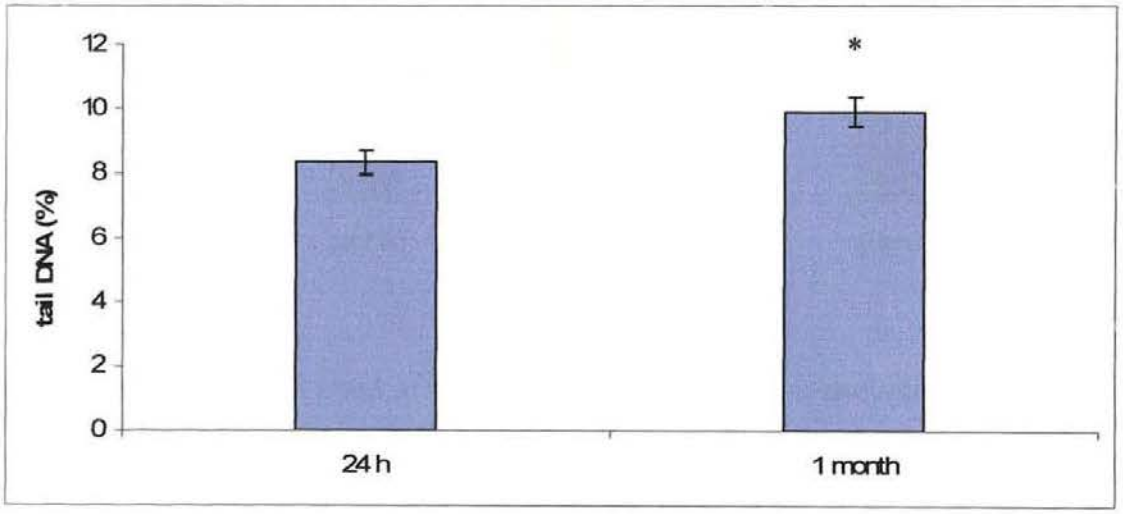


Figure 28: Effect of one-month maintenance in laboratory conditions on frank SSB for NB mussel digestive glands for September 2004. Values are the mean of 12 animals (pooled tissue/two animals, ± SEM) from two separate experiments. * Significantly different (P<0.05) between time-points as assessed using Student's *t*-test for the medians.

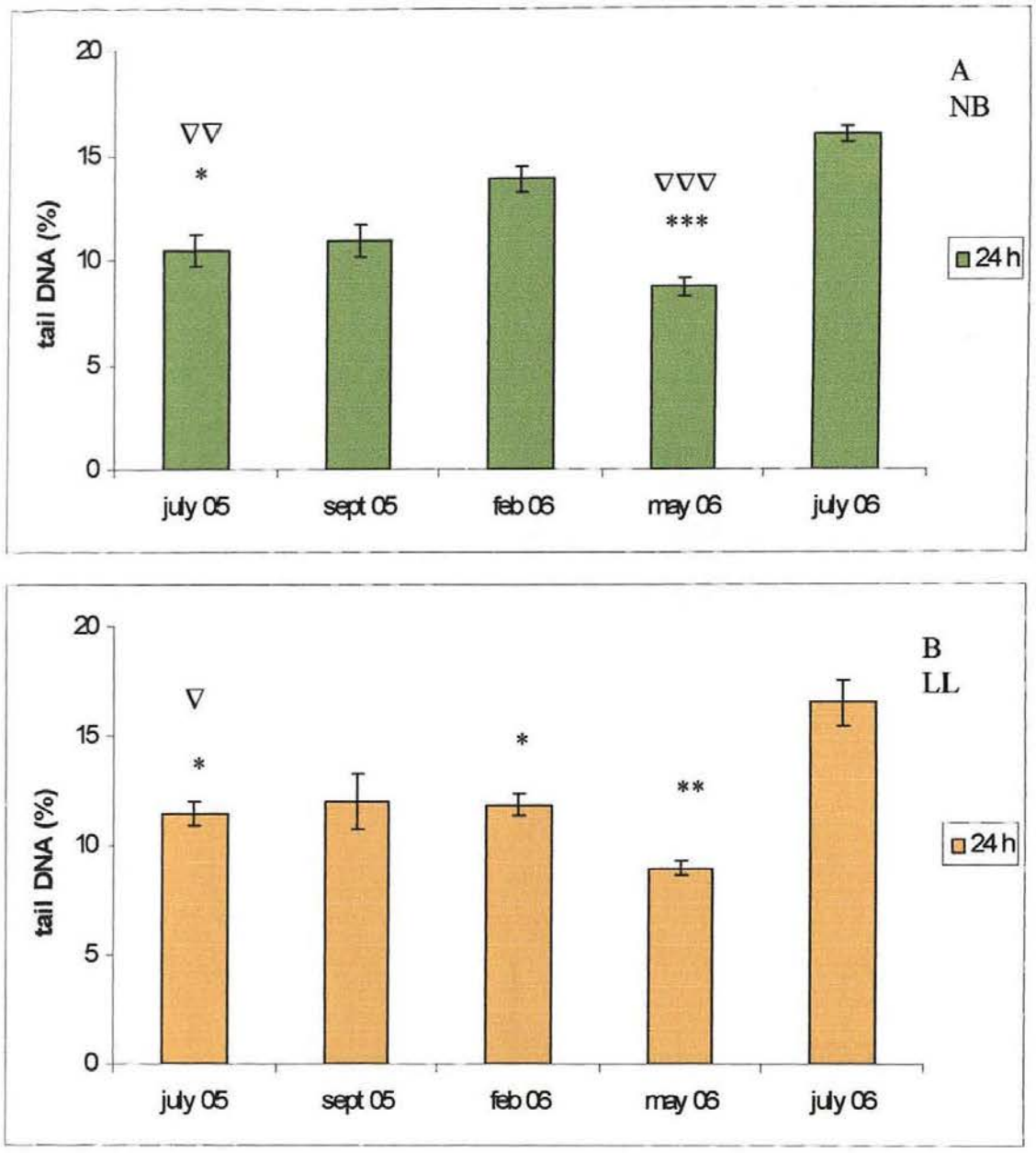


Figure 29A,B: Temporal trend of frank SSB for NB and LL mussel gill respectively (24h samples) Values are the mean of 4 animals (± SEM) except for July 2005 and September 2005 (n=3). **29A**: * Significantly different to February ($P<0.05$), *** Significantly different to February ($P<0.001$), ∇∇ Significantly different to July 06 ($P<0.01$), ∇∇∇ Significantly different to July 06 ($P<0.001$). **29B**: * Significantly different to July 06 ($P<0.05$), ** Significantly different to July 06 ($P<0.01$), ∇ Significantly different to May ($P<0.05$), as assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test

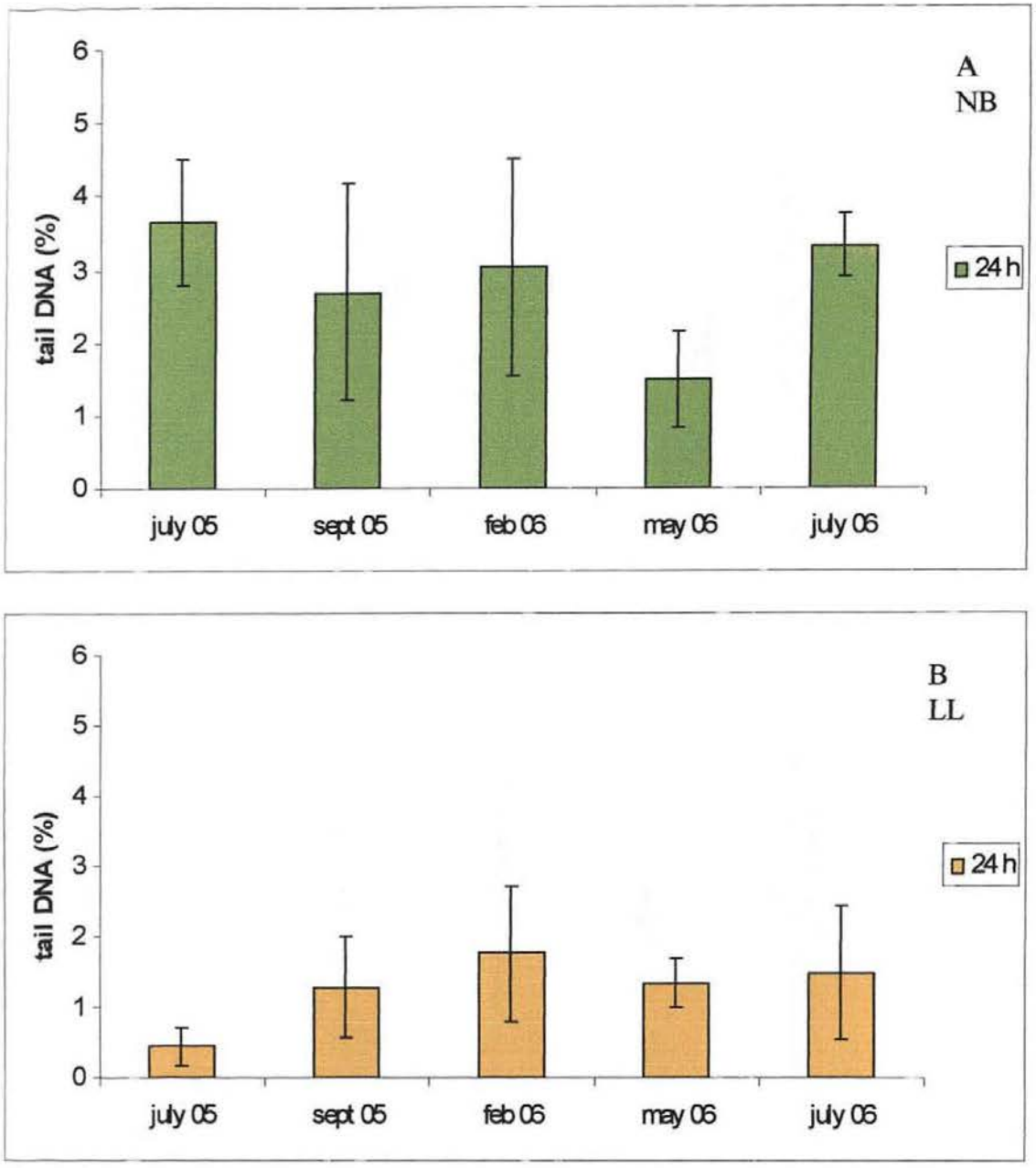


Figure 30A,B: Temporal trend of 8-oxo-dG for NB and LL mussel gill respectively (24h samples) Values are the mean of 4 animals (± SEM) except for July 2005 and September 2005 (n=3). No difference between time points as assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test.

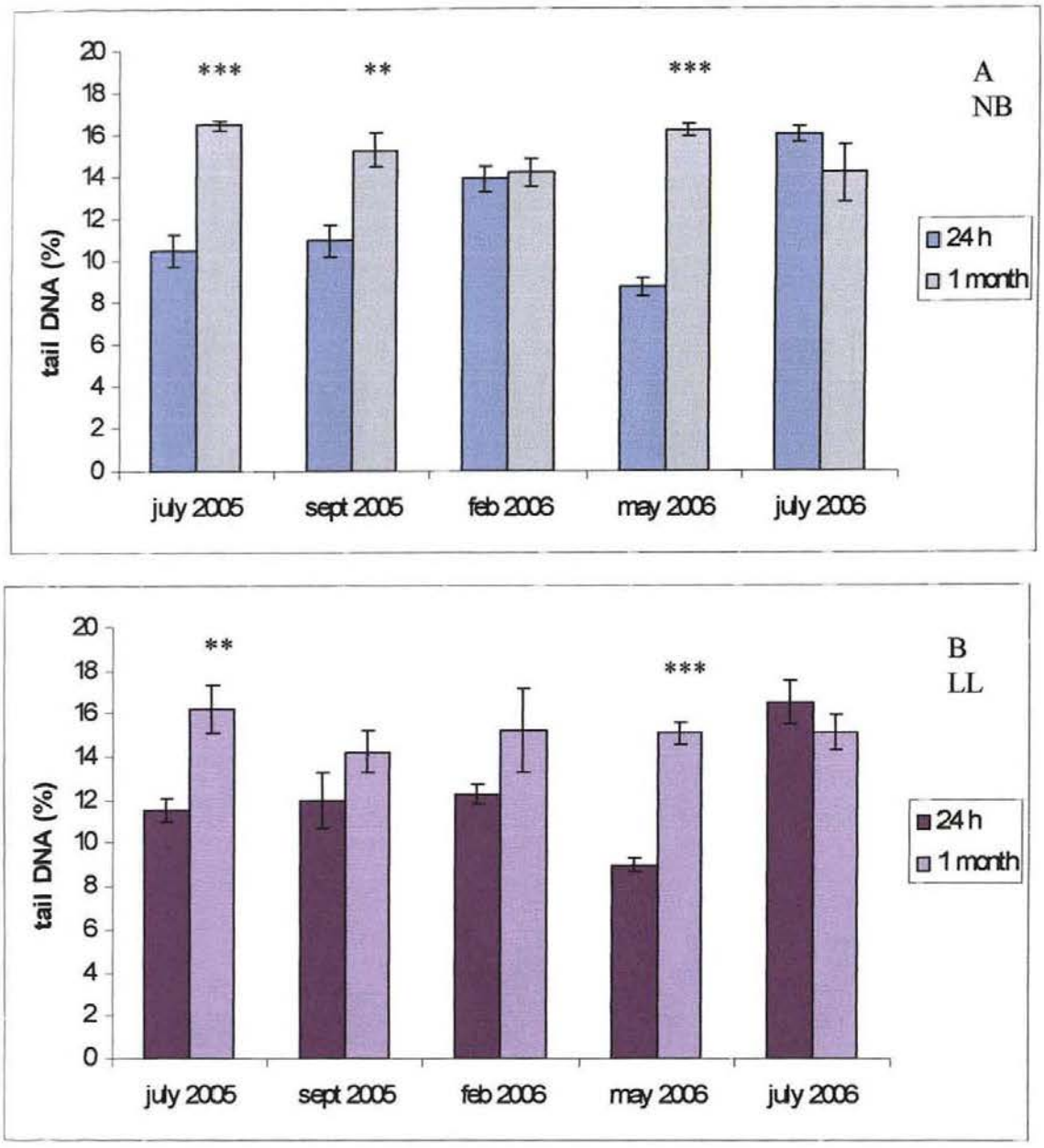


Figure 31A,B: Effect of one-month maintenance in laboratory conditions on frank SSB for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Values are the mean of 4 animals (± SEM) except for July 2005 (24 h), July 2005 (1 month) and September 2005 (24 h) (n=3). ** Significantly different ($P<0.01$) between time-points as assessed using Student's *t*-test for medians. *** ($P<0.001$).

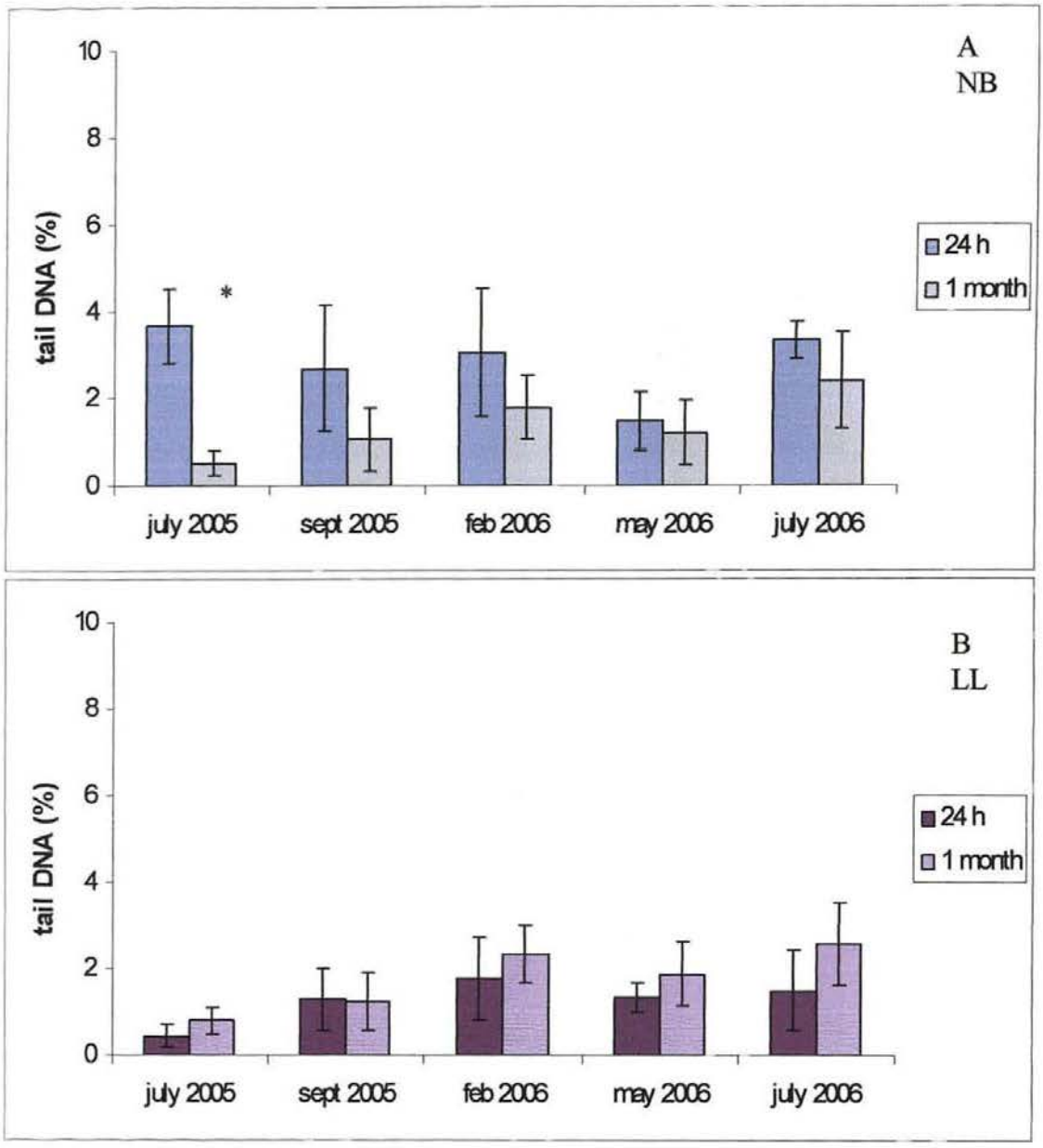


Figure 32A,B: Effect of one-month maintenance in laboratory conditions on Fpg-sensitive sites for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Values are the mean of 4 animals (± SEM) except for July 2005 (24 h), July 2005 (1 month) and September 2005 (24 h) (n=3). * Significantly different ($P<0.05$) between time-points as assessed using Student's *t*-test for medians.

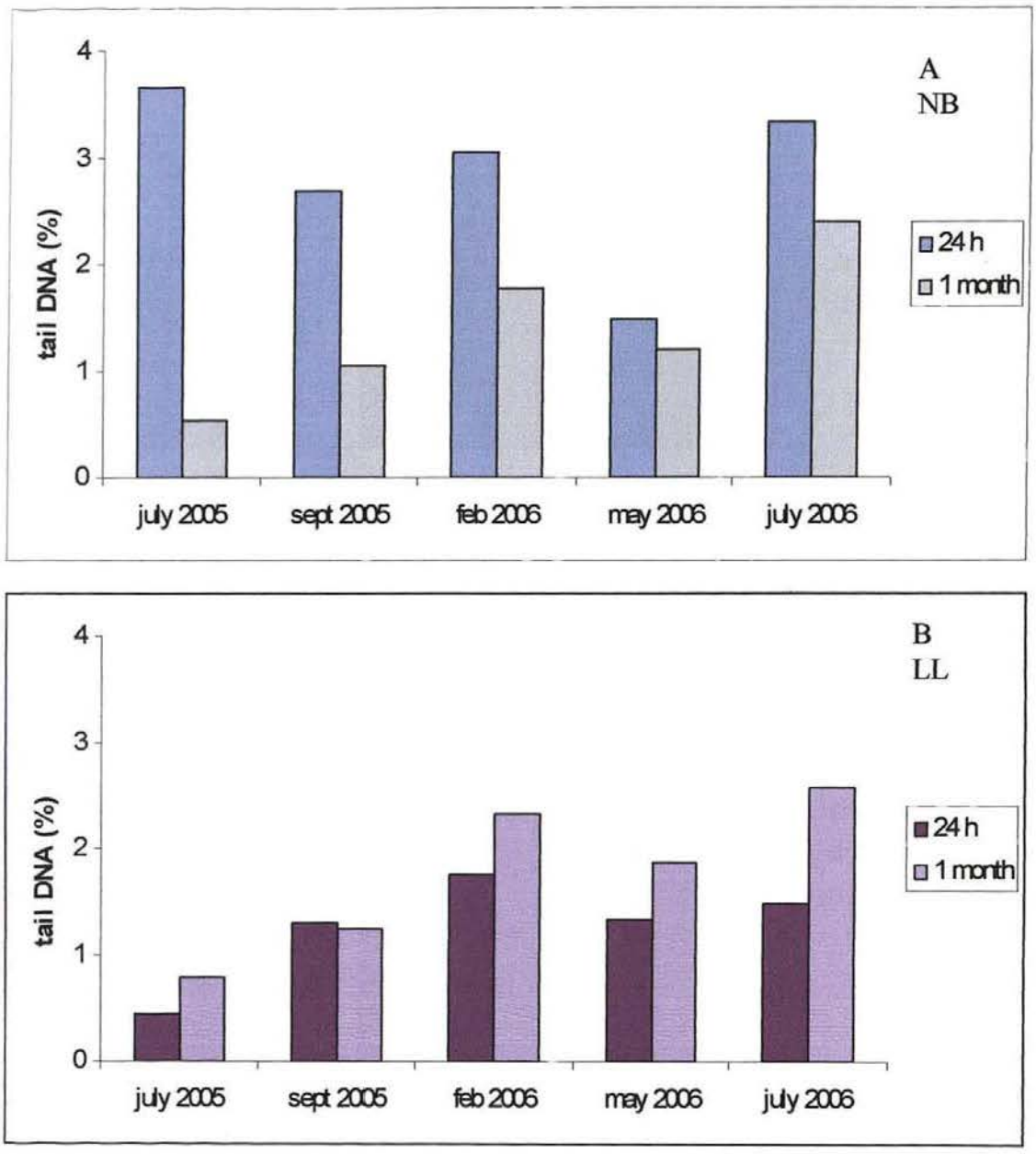


Figure 33 A, B: Effect of one-month maintenance in laboratory conditions on average Fpg-sensitive sites for NB and LL mussel gill (respectively). Values are the mean of 4 animals except for July 2005 (24 h), July 2005 (1 month) and September 2005 (24 h) (n=3). **33A**: Significant overall decrease ($P<0.05$) after one-month maintenance, as assessed by a paired *t*-test. **33B**: No significant overall difference after one-month maintenance.

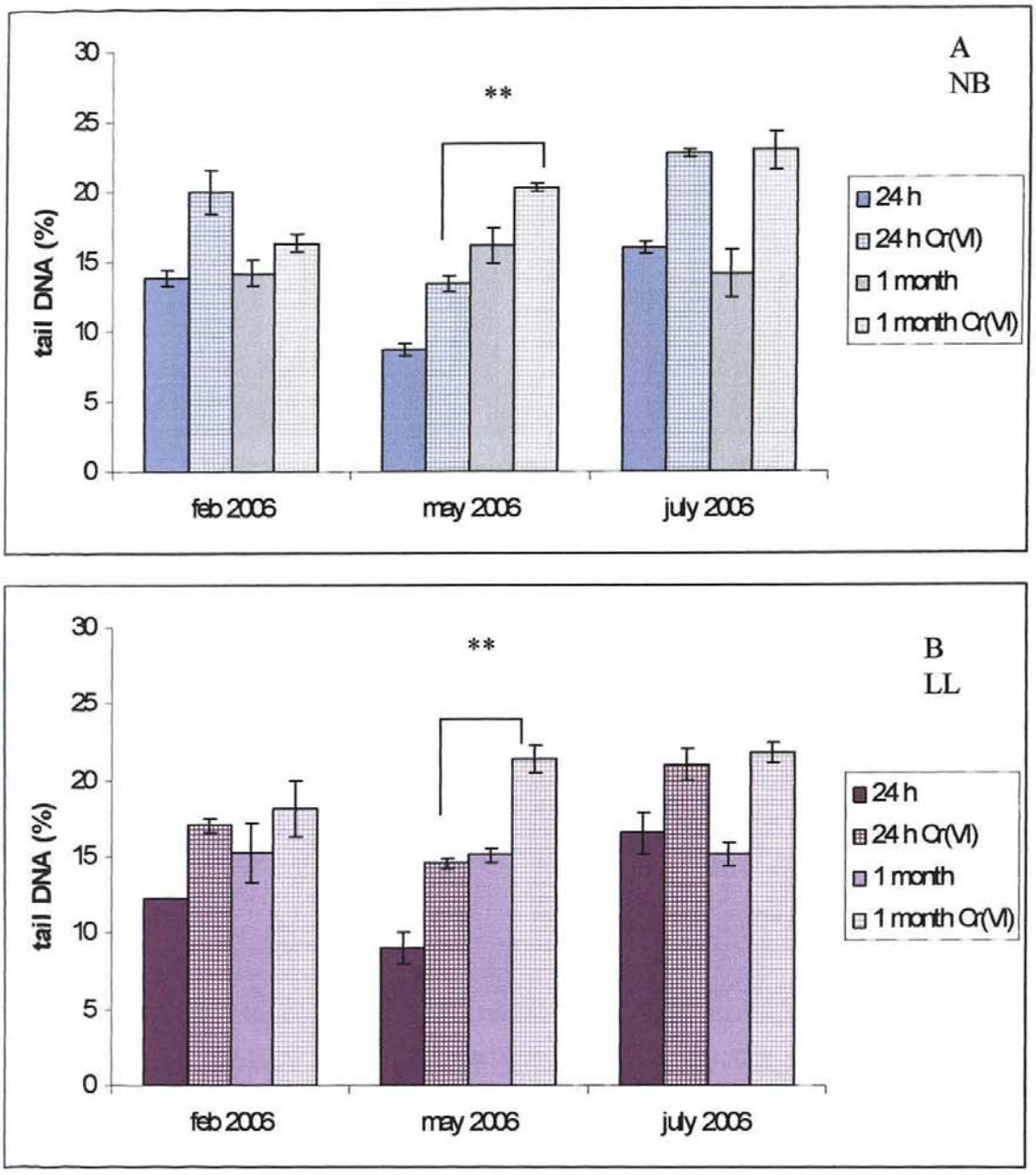


Figure 34A,B: Effect of one-month maintenance in laboratory conditions on frank SSB produced by a single $Na_2Cr_2O_7$ injection (0.1 µmol/ in 100 µl water, 4 h post-exposure) for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Baseline frank SSB are also recorded (based on non-injected animals) Values are the mean of 3 animals (for the $Na_2Cr_2O_7$-injected samples, ± SEM) or of 4 animals (for the non-injected samples, ± SEM). ** Significantly different ($P<0.01$) between time-points as assessed using Student's *t*-test for medians.

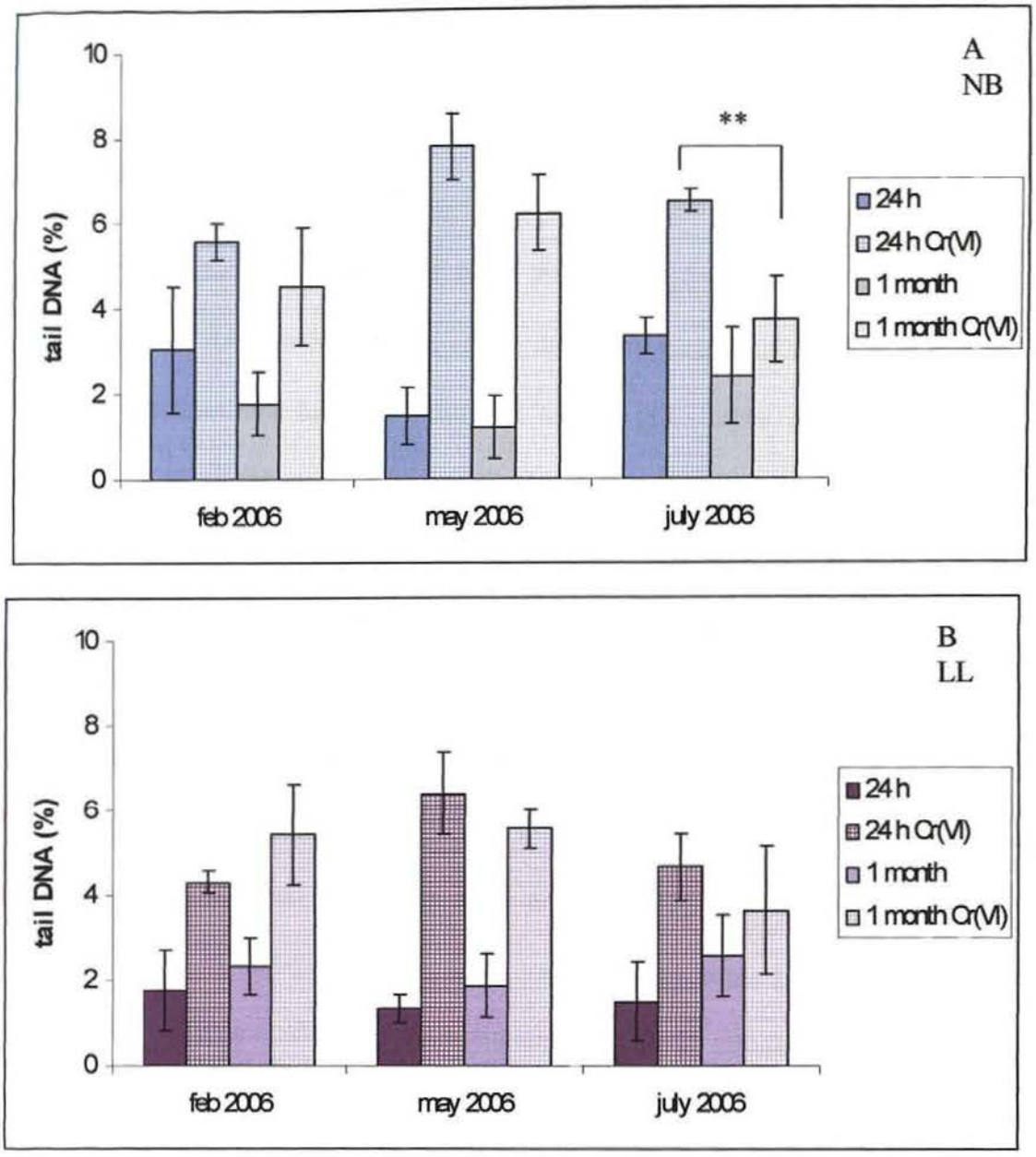


Figure 35A,B: Effect of one-month maintenance in laboratory conditions on 8-oxo-dg produced by a single $Na_2Cr_2O_7$ injection (0.1 µmol/ in 100 µl water, 4 h post-exposure) for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Baseline 8-oxo-dG is also recorded (based on non-injected animals) Values are the mean of 3 animals (for the $Na_2Cr_2O_7$-injected samples, ± SEM) or of 4 animals (for the non-injected samples, ± SEM). * Significantly different ($P<0.05$) between time-points as assessed using Student's *t*-test for medians.

3.4 Discussion

i) Experimental design

The tissue used throughout the present project was mussel gill since it is continuously exposed to pollutants found in water and is the main organ involved in respiration and nutrient uptake (Gomez-Mendikute *et al.*, 2005). A variety of enzymatic and non-enzymatic antioxidants has been found in mussel gill (Power and Sheehan, 1996; Company *et al.*, 2004) as well as metallothionein isoforms (Lemoine *et al.*, 2000). During the experiments presented, we were able to detect differences between time-points or between sites using this particular tissue. This was despite evident interindividual variability, which may be significant in bivalve *in vivo* studies (Wilson *et al.*, 1998). Differences in antioxidant status (minimised by exogenous antioxidant supplementation as proposed by Wilson *et al.*, 1998) or genetic polymorphisms affecting metabolism and repair like these noted on *Daphnia magna* by Depledge, (1994) may be partly responsible for these variable responses. However, the comet assay on gill cells was also able to distinguish genotoxic effects at different stress levels as shown by *in vivo* exposure to chemically unrelated genotoxic chemicals (**Figure 26**). Gills are commonly used in evaluation of biomarkers (e.g. Cossu *et al.*, 1997; Kopecka *et al.*, 2006) and it has been proposed that there is limited seasonal protein variation in this tissue, which decreases the confounding of seasonal changes (Lau *et al.*, 2004). Mussel gills shows higher baseline DNA damage than mussel haemocytes (Akcha *et al.*, 2004b; Halldórsson *et al.*, 2004) but they may be able to detect genotoxic responses with greater sensitivity than the haemocytes (Halldórson *et al.*, 2004; Rank *et al.*, 2005). Digestive gland cells are also commonly used (e.g. Mitchelmore *et al.*, 1998b; Labieniec and Gabryelak, 2006) and many xenobiotic metabolism studies have been performed in such cells (Mitchelmore *et al.*, 1998),

mainly because of the relatively high metabolic and antioxidant enzyme activities recorded for this kind of tissue (Birmelin et al., 1998). Initial experiments performed on digestive gland cells corroborate our subsequent findings on gill cells, namely the increase of SSB after one-month laboratory maintenance (**Figure 28**), which is therefore not a tissue -specific observation. Time and tissue constraints deterred us from using both kinds of cells simultaneously.

The Fpg buffer used did not produce additional DNA damage (in the form of SSB) on gill cells. This was not the standard Fpg buffer for mammalian cell lines [HEPES (40 mM), KCl (0.1M), Na_2EDTA (0.5 mM), BSA (0.2 mg/ml) pH 8.0] since the latter caused high levels of damage in slides characterised by smeared DNA clouds, ghost cells and high nuclear fragmentation. Gielazyn *et al.*, (2003) with oyster haemocytes, encountered similar variable controls. The researchers proposed substitution of KCl with NaCl and we followed the same protocol. Interestingly, the same protocol was applied on grass shrimps (Hook and Lee, 2004) and a variety of Fpg buffers (with NaCl instead of KCl) was also applied on *Unio tumidus* (Labieniec and Gabryelak, 2006). These buffer varieties appear more appropriate for invertebrate tissues.

We have used a single injection of $Na_2Cr_2O_7$ (0.1 μmol in 100 μl distilled water) in the adductor muscle as an effective means of acute oxidative insult. In this set of experiments we were not trying to simulate realistic exposure conditions and we were more interested in assessing responsiveness following known bioavailability of Cr(VI). Thus, injection was chosen instead of water-spiking. Injection through the adductor muscle is a technique commonly used when a predetermined body concentration is desired (e.g. Cancio *et al.*, 1998; Gagné *et al.*, 2001; Marigómez and

Baybay-Villacorta, 2003). The injection and the carrier (water or DMSO) did not cause additional SSB as depicted in **Figure 27**.

The brief overnight acclimatisation post-collection (12 h) whilst submerged was employed. Almeida *et al.*, (2005) observed that air-exposed mussels (*Perna perna*) (such as would occur at low-tide) undergo oxygen deprivation and switch to fermentative metabolism however 4 h post-resubmersion, baseline 8-oxo-dG, lipid peroxidation and antioxidant enzyme activities were restored. Assays were therefore performed within 24 h after the acclimatization period and this protocol was applied for all the mussel collections throughout the year. The bulk of the assays (except GSH evaluation) necessitated freshly dissected tissue since results from frozen extracts were not sufficiently reliable. Indeed Akcha *et al.*, (2004b) noticed an increase in DNA strand breaks in frozen mussel tissues.

ii) Temporal variability

There was a temporal variability in SSB for the year July 2005-July 2006 for both NB and LL mussels as shown in **Figure 29A,B** respectively, however no clear trend of SSB was evident. The positive correlation between bivalve SSB as measured by the comet assay and temperature (Buschini *et al.*, 2003) is therefore not corroborated for July 2005 samples which do not exhibit higher than winter values. However samples from July 2006 exhibit high values, which especially in the case of LL mussels, are significantly different from the majority of the other months tested. The collection at July 2006 was performed on a significantly hotter day than the collection of July 2005, which may explain the differences noted and be indicative of a general cell sufferance (Buschini *et al.*, 2003). In contrast, May sampling gave consistently lower

SSB vales for both sites. Spawning, which might theoretically increased SSB (Hartl *et al.*, 2004) was not evident at collection sites at that time-point, however gametogenesis was complete because when mussels were transported to the aquaria spawning was immediate and continued until autumn, possibly because of food abundance (Gosling, 1992). DNA repair (as measured by 8-oxo-dG excision) (Chapter 4) was minimal on May at both sites, which also minimised the contribution of repair intermediates to the recorded SSB. Finally, condition indices (based on shell and meat volume and/or shell and meat weight) were maximal in May in *Mytilus edulis* from Scotland (Okumuş and Stirling, 1998) and a similar optimal condition status may prevail in mussels from Northwest England resulting in maximal DNA integrity at this particular month.

There was no temporal variability in 8-oxo-dG for the year July 2005-July 2006 for NB or LL mussels as shown in **Figure 30A,B** respectively and no clear trend was evident. There was however a non significant increase in 8-oxo-dG for NB mussels at summer (July 2005 and July 2006), which may be linked to the suppressed DNA repair noted at these time-points and/or the synergistic action of temperature and pro-oxidant chemicals as explained later in this chapter [3.4iii)].

iii) Differences between sites

Despite the indications of different pollution status between NB and LL, especially for PCB congeners, Hg and Cd (Chapter 2), SSB were not significantly different between sites. Only at one time-point (February 24 h) were SSB in NB mussels greater than SSB in LL mussels (two-tailed *t*-test, $P<0.05$), however the biological implications of that slight difference are questionable (13.87 ± 1.04 compared to

11.86 ± 0.83, median % tail DNA) as shown in **Figure 31**. These differences were evident only at one of the highest level of seasonal SSB in accordance to Akcha *et al.*, (2004b) who proposed that low seasonal SSB levels might mask differences between sites. Overall there were no significant differences between the sites. This lack of overall statistically significant difference in SSB is not a unique observation when indigenous mussels are used: gill SSB (% tail DNA) from *Mytilus edulis* from Loch Leven did not differ from SSB from animals from Loch Etive, despite different contamination factors (Large *et al.*, 2002). Actually, SSB (% tail DNA) in *Mytilus* sp. were higher in a reference site (Port Quin) compared to specimens (*Mytilus edulis*) from NB at all the months tested (Shaw *et al.*, 2004). Also, despite a factor of contamination between 2 to 3 between sites, no statistically significant difference (Olive Tail Moment) was found in indigenous *Mytilus* sp. in early spring along the French Atlantic Coast (Akcha *et al.*, 2004). Finally, a large study involving indigenous *Dreissena polymorpha* in Seine showed differences between contaminated and reference sites, but same month the following year these differences did not persist and high damage (tail length) was recorded for all the populations (Rocher *et al.*, 2006). In contrast to the aforementioned studies and our results, other researchers have found an increase in SSB in populations chronically exposed to pollutants: for example indigenous populations (*M. galloprovincialis*) from an impacted area in Italy exhibited higher DNA damage (% DNA in tail) than reference populations (Nigro *et al.*, 2006) and the same pattern (Tail Moment) was observed in mussels sampled from sites from the highly polluted Køge Bay in Denmark, in comparison to mussels from "reference areas" (Rank *et al.*, 2005). Finally mussels in polluted sites in La Spezia also showed higher DNA damage (alkaline elution) compared to clean farmed mussels (Bolognesi *et al.*, 1996). Until today, there is no unanimously accepted

explanation for this discrepancy between studies. Large *et al.*, (2002) suggested that adaptive mechanisms in populations chronically exposed to contaminants might be responsible for lack of increased SSB. These populations may have either been "selected" across generations or adapted via physiological/epigenetic changes in order to cope with their environment. That was the explanation also given by Black *et al.*, (1996) in order to explain the lack of elevated SSB in mussels (*Anodonta grandis*) from a pond contaminated with Pb, Cd and Zn. However, in cases that the pollution levels are extremely high or that the pollutants are specifically toxic for mussels like Cu (Funes *et al.*, 2005) DNA damage (in the form of SSB) may emerge. This "pollution threshold" prerequisite was effectively depicted in the study of Steinert *et al.*, (1998) where comet assay on (transplanted) mussels distinguished between the sites exhibiting the extremes of contaminant exposure (clean versus very polluted). We should also note that increased DNA strand breaks might result not only from direct damage but also from an unknown proportion of DNA excision repair (Nacci *et al.*, 1992; 1996). Translating SSB in comet assays, as a measurement of genotoxicity should be done with caution (Shaw *et al.*, 2004), since they are the sum of direct SSB +/- indirect "transient" SSB due to repair mechanisms. DNA repair is a dynamic process and may contribute positively or negatively to the amount of SSB at any given time. It has been shown that metals like Cd, a common aquatic pollutant, can block DNA repair processes [Chapter 1.8.2.3 iv)]. This is also true for caffeine and drugs of pharmaceutical origin (e.g. *cis*-platin), which are commonly found in municipal effluents (Gagné *et al.*, 2004). Thus pollution may not always lead to higher SSB. As a general conclusion, there is no single predominant reason for the presence of absence of SSB in chronically exposed populations and SSB may be

affected by intensity and duration of exposure, experimental model (species, tissue) as well by the nature of pollutants.

In a similar manner, 8-oxo-dG levels were significantly different only in summer 2005 between sites ($P<0.05$) as shown in **Figure 32**. This difference however was not persistent for the summer of 2006 when a two-tailed *t*-test was used. Differences in 8-oxo-dG repair capacity between sites were evident at both July 2005 and July 2006 (Chapter 4) and may be directly responsible for the higher levels of 8-oxo-dG recorded for NB. Also, higher temperatures can modulate the bivalve response to xenobiotics and their input in water (Buschini *et al.*, 2003) and combined hot temperature (> 30°C) and proxidant treatment (ozone, chlorine) is an effective means of *Dreissena polymorpha* elimination (Harrington *et al.*, 1997). A similar oxidative stress pattern may emerge in *Mytilus edulis*, (which has an upper thermal tolerance of 29°C) dwelling in NB, at warm months.

iv) Differences between time-points

One-month laboratory maintenance increased SSB in gill in most cases (**Figure 31A,B**), an increase that was more pronounced in the NB samples. In the remaining cases the SSB stayed the same. These results, at first sight, are quite surprising since clean conditions would theoretically decrease DNA damage. Temperature may be an important confounding reason: Late September and early May waters probably exhibit lower temperatures than 17° C (laboratory maintenance temperature) so this rise in temperature would have theoretically increased baseline SSB (Buschini *et al.*, 2003) after laboratory maintenance. For May sampling, concomitant spawning (Bayne, 1976) evident by spat recruitment in the aquaria, may have also contributed to % tail

DNA (Hartl *et al.*, 2004). However there was a lack of increase in SSB in the warmer than February aquarium water and a lack of decrease in SSB in the cooler than July aquarium water, which does not corroborate the importance of temperature as a confounding factor. Overall there was a lack of difference between sites or seasons after lab maintenance (two-way ANOVA, $P>0.05$), which shows that there is a stable SSB level peculiar to our maintenance conditions that dominates irrespective of season or genetic background. Oxygen deprivation or high nitrate levels were not noted under our laboratory maintenance. Regarding relevant literature, analysis of DNA damage after chronic laboratory maintenance of mussels without concomitant exposure is not common, however increased SSB were observed by others in lab-maintained *Mytilus edulis* (H.A. Selley, personal communication) and similar results were evident in some *Mytilus galloprovincialis* individuals, during their 7 day depuration from the *Prestige* oil spill (Laffon *et al.*, 2005). Some of the impacted mussels actually showed higher tail length than before, something that the authors explained as additional damage due to recirculation of pollutants from shell oil stains. In a similar manner, Wilson *et al.*, (1998) examined closely the effect of laboratory maintenance on SSB in *Mytilus edulis* gill. Results from both adult and juvenile mussels were extremely variable over time with SSB increasing steadily irrespective of feeding practices and mussel age. Until today we are not aware of any other published studies examining the observations by Wilson *et al.*, (1998). Different repair rates (generally higher after laboratory maintenance) may be a possible reason for the increase: There was a weak positive correlation ($P=0.08$) between SSB levels and excision capacity against 8-oxo-dG (Chapter 4) that highlights the (small) contribution of BER excision repair mechanisms to the recorded by the comet assay SSB. In corroboration of this observation Shaw *et al.*, (2004) noted that mussels from

a reference site (Port Quin) exhibited more SSB than mussels from the polluted NB, possibly because of the higher repair capacities of the former. Furthermore, transplantation of *Elliptio complanata* downstream a primary-treated municipal effluent showed than 1 year after, DNA SSB in gonad tissues were significantly lower compared to the reference populations. Pollutants of pharmaceutical origin and caffeine found in the effluent as well as PAH have been shown to block DNA synthesis and repair and may have debilitated repair mechanisms in gonad tissue (Gagné *et al.*, 2004). In conclusion the reasons for the increase of SSB during laboratory maintenance are not fully understood, however similar increases have been noted after transplantations to cleaner environments and vice versa.

Regarding gill 8-oxo-dG levels, the results showed a statistically significant reduction in the NB mussels after laboratory maintenance at one time point (July 2005) and a similar general trend in all samples (**Figure 32A**). There was no significant reduction in the LL mussels after laboratory maintenance (**Figure 32B**). This finding we consider to be important as the results suggest that DNA oxidation in NB mussels was reduced by depuration and lab-maintenance and the levels after one month were more similar to that of the LL mussels. The reasons for this lowering are not certain but may relate to elimination of genotoxic input and/or to increased repair. There is evidence of the latter (Chapter 4) but only for July and September samplings. Regarding potential pro-oxidant input, PCB and certain metals were elevated in NB (Chapter 2) compared to LL. It has been shown in mammals and fish co-planar PCB bind tightly to CYP1A, and are metabolised slowly by them, which leads to uncoupling of substrate oxidation, oxygen reduction and production of ROS. This uncoupling can be accompanied by slow inactivation of CYP1A *in vitro* (Schlezinger

et al., 2006) and it has been shown that the highly toxic CB77 can denature CYP *in vitro* (Livingstone, 2001). These effects can *in vivo* alter the balance between bioactivation (Phase I) of xenobiotics and detoxification (Phase II). The metabolism of PCB to reactive products is slow and probably does not participate to overall PCB toxicity, however lower halogenated PCB are metabolised to dihydroxymetabolites that, in their turn undergo futile redox semiquinone reactions *in vitro* (Srinivasan *et al.*, 2001). A similar uncoupling and/or inhibition of CYP may also be present in bivalves, thus lead to more intense oxidative stress in mussels from NB. Regarding metal pollution prevailing in NB, Cd can cause oxidative stress via varied and distinct mechanisms (Chapter 1.8.2.3) and *in vivo* exposure to Cd has actually suppressed activity of the mussel *N*-glycosylase responsible for 8-oxo-dG excision (Chapter 7) possibly resulting in elevated oxidative damage. Finally, Hg (found in abundance in Merseyside) may also cause oxidative stress since it suppressed the activity of the bacterial 8-oxo-dG glycosylase Fpg *in vitro* (Asmuß *et al.*, 2000). Thus, alleviation from these pro-oxidant parameters under laboratory conditions may be the main reason for decreased 8-oxo-dG after one-month maintenance for NB samples.

v) Results from Cr(VI)-injection

Regarding frank SSB caused by Cr(VI) injection there was no statistically significant difference between time-points [the higher levels recorded for May 2006 after one-month maintenance are probably due to the higher baseline noted for this particular set of animals (**Figure 34A,B**)]. The SSB were slightly fewer after one-month maintenance for February 2006 (when concomitant decrease in 8-oxo-dG excision capacity was noted at both sites) and also they were slightly more after one-month maintenance for July 2006 (when concomitant increase in 8-oxo-dG excision capacity

for NB was noted), however frank SSB for this tissue and at this Cr(VI) dose are not exclusively due to excision of 8-oxo-dG as noted in other experimental models (Smart *et al.*, 2006). Similar deductions can be reached from **Figure 35A,B** where Fpg-sensitive sites (representing 8-oxo-dG levels) were not different between time-points with the exception of July 2006 for New Brighton, which probably corresponds to a genuine decrease in Fpg-sensitive sites. Increase in 8-oxo-dG excision capacity for NB samples after one-month laboratory maintenance was evident for July 2006 (Chapter 4) and thus higher repair may contribute to the lower 8-oxo-dG levels recorded. However, the (lower) decrease of 8-oxo-dG excision capacity noted for both sites in February 2006 did not alter substantially the corresponding Fpg-sensitive sites for that month.

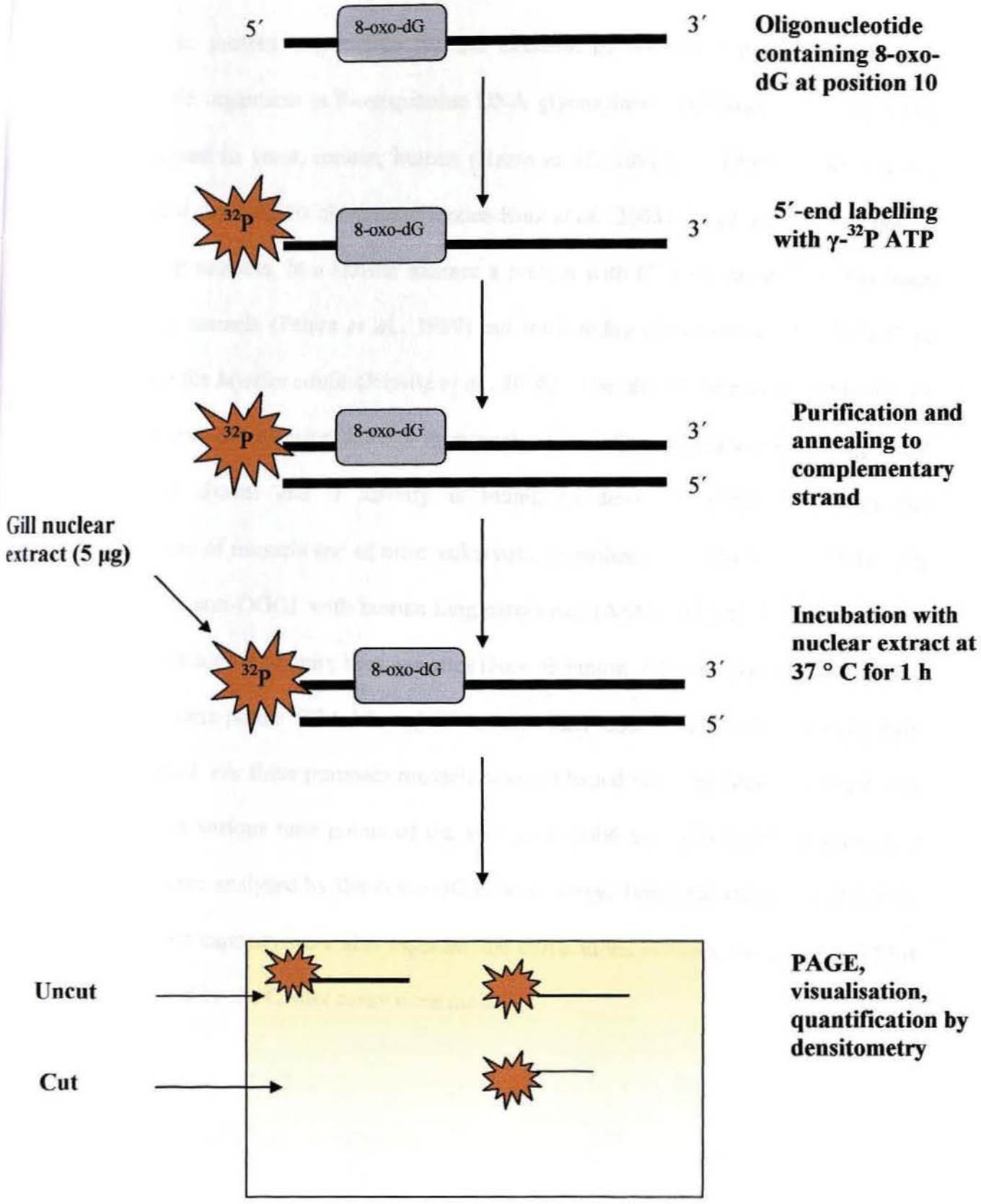
In conclusion, dissimilarities in SSB and 8-oxo-dG between sites emerged at some mussel collections of the year 2005-2006. Differences between time-points ("24 hours", "1 month") were also evident. Differences in repair capacities and their implications to the present results are discussed in the next chapter.

4.0 8-oxo-dG excision capacity of mussel gill from New Brighton and Llandudno populations

4.1 Introduction

The DNA base excision repair pathway rectifies small, non-helix distorting lesions in DNA, which may be alkylated, lost or oxidised bases (Cabelof *et al.*, 2002). BER is highly conserved among organisms from bacteria to mammals (Augusto-Pinto *et al.*, 2003; Didzaroglu, 2005), however very little is known about its existence in mussels. Nonetheless, the idea that DNA repair systems do exist in mussels has been suggested even in the early 1990s (Bihari *et al.*, 1990). Signs of existence of DNA rectifying mechanisms were evident in the study of Akcha *et al.*, (2000): *Mytilus galloprovincialis* exposed to BaP exhibited elevated 8-oxo-dG levels in its digestive gland after 10 days of exposure, which were followed by a significant decrease at day 5 of remediation. However, loss of damage may occur as a result of cell replacement as well as via DNA repair. Black *et al.*, (1996) also noted a decrease in SSB of *Anodonta grandis* after prolonged exposure to Pb, postulated to be due to repair mechanisms, which according to the authors are threshold-dependent.

The monitoring of BER as a whole or the examination of specific steps of the pathway have been greatly facilitated by the application of *in vitro* assays using cell extracts (Hodges and Chipman, 2002; Dianov, 2003). In the case of the latter, an oligonucleotide containing a pre-defined lesion is incubated with cell extracts. "Nicking" of the oligonucleotide signifies the presence of a corresponding glycosylase/endonuclease in the cell. As a result, potential cleaving of an oligonucleotide containing 8-oxo-dG by mussel cell extract signifies the presence of an 8-oxo-dG glycosylase in this species. A schematic representation of the 8-oxo-dG excision assay is given in **Figure 36.**



8-oxo-dG: 8-oxo-7,8-dihydro-2′-deoxyguanosine **PAGE**: polyacrylamide gel

Figure 36: Schematic representation of 8-oxo-dG excision assay

The main protein responsible for the excision of 8-oxo-dG paired with dC in eukaryotic organisms is 8-oxoguanine DNA glycosylase 1 (OGG1) and its gene has been cloned in yeast, mouse, human (Hazra *et al.*, 1998), rat (Prieto-Alamo *et al.*, 1998) and *Arabidopsis thaliana* (Morales-Ruiz *et al.*, 2003). *Ogg1* may or may not be present in mussels. In a similar manner a protein with CYP1A capabilities has been found in mussels (Peters *et al.*, 1999) but until today no sequence of "*CYP1A*" is available for *Mytilus edulis* (Rewitz *et al.*, 2006). The aim of the present study was to detect 8-oxo-dG excision activity in mussel gill (via the endonuclease nicking assay described above) and if activity is found, to detect similarities between the glycosylase of mussels and of other eukaryotic organisms [via immunopositivity with polyclonal anti-OGG1 with human lung carcinoma (A549) cells as positive control]. The differences in activity between sites (New Brighton, NB and Llandudno, LL) and between time-points ("24 h" and "1 month after collection") were subsequently investigated. For these purposes mussels were collected from the sites of interest (NB and LL) at various time points of the year 2005-2006 and gills from representative animals were analysed by the 8-oxo-dG nicking assay. Temporal variations of 8-oxo-dG excision capacity were also assessed and correlations between 8-oxo-dG and SSB as measured by the Comet assay were made.

4.2 Materials and methods

4.2.1 Reagents: see 2.2.1

4.2.2 Buffers

i) for mussel gill cells

(Buffers A, B and C according to Klungland *et al.*, 1999)

Lysis buffer A: Tris-HCl (10 mM) pH 7.5, $MgCl_2$ (0.5 mM), KCl (10 mM), DTT (1 mM) and mammalian protease cocktail inhibitor (10 µl/ml).

Lysis buffer B: Glycerol (25 % v/v), Tris-HCl (20 mM) pH 7.5, $MgCl_2$ (0.5 mM), NaCl (0.42 M), EDTA (0.2 mM), DTT (1 mM) and mammalian protease cocktail inhibitor (10 µl/ml).

Dialysis buffer (C): Tris-HCl (25 mM) pH 7.5, KCl (50 mM) and DTT (2 mM).

ii) for human lung cell carcinoma cells A549

Lysis buffer A1: NP-40 (0.6%), NaCl (150 mM), Tris-HCl (10 mM) pH 8.0, Na_2EDTA (1mM) and mammalian protease cocktail inhibitor (10 µl/ml)

Lysis buffer B1: glycerol (25% v/v), NaCl (0.42 M), Tris-HCl (20 mM) pH 8.0, Na_2EDTA (0.2 mM), $MgCl_2$ (1.5 mM), DTT (0.5 mM) and mammalian protease cocktail inhibitor (10 µl/ml)

iii) for 8-oxo-dG excision assay

1.8 x REC buffer: Tris-HCl (18mM) pH 7.5, KCl (180mM), Na_2EDTA (18mM), and bovine serum albumin (BSA, 0.18 mg/ml)

Formamide loading buffer: Na_2EDTA (0.5 mM), SDS (2.5%) and xylene cyanol/bromophenol blue (XC/BPB) (46.5 µl) up to 1 ml with formamide.

Polyacrylamide gel: Acrylamide: bisacrylamide 30% solution (20%), urea (8M) and 1 x TBE buffer (20%). *N,N,N,N*-tetramethylethyldiamine (TEMED) (6.4 µl per 10 ml)

and 10% w/v ammonium persulphate (APS) (80 µl per 10 ml) added immediately before casting

iv) for western blotting

1 x TBS buffer: Tris base (0.1 M) and NaCl (0.15 M), pH 8.0

1 x TBS –0.05% Tween 20: Tris base (0.1 M), NaCl (0.15 M) and Tween 20 (0.05%), pH 8.0 stored at 4°C 1 h before use.

SDS-PAGE running buffer: Tris base (25 mm), glycine (192 mm) and SDS (0.1%)

Transfer buffer: Tris base (20 mM), glycine (150 mM) and methanol (20% v/v), stored at 4°C 1 h before use.

Blocking buffer: Low-fat powdered milk (Marvel, UK) (5%) in 1 x TBS-0.05% Tween 20, made fresh prior to use

12.5% resolving gel: Acrylamide:Bisacrylamide solution 30% (12.5%), Tris-HCl (375 mM) pH 8.8, SDS (0.1%). TEMED (15 µl per 10 ml) and 10% w/v APS (150 µl per 10 ml) added immediately before casting

4% stacking gel: Acrylamide:Bisacrylamide solution 30% (4%), Tris-HCl (125 mM) pH 6.8, SDS (0.1%). TEMED (10 µl per 10 ml) and 10% w/v APS (100 µl per 10 ml) added immediately before casting

4.2.3 Nuclear extract preparation

i) from gill cells

Mussels (n=3-4) from each of the two sites were randomly selected after overnight acclimatization. The animal valves were opened with a metal scalpel (17 cm) and gill tissues were extracted with small size metal tweezers. The tissue was gently disaggregated in a 25 ml glass beaker (Duran, Mainz Germany) containing lysis buffer A (3ml) and left on ice for 15 min. The solution was then filtered through 2

layers of 150 µm gauze into sterile 1.5 ml microcentrifuge tubes (2 tubes per animal) and subjected to centrifugation (M.S.E., Sanyo, Japan) at 2000 x g for 5 min at room temperature. The pellet was thoroughly resuspended in buffer B (80 µl maximum) and left on ice for 30° C. Following centrifugation at 18 000 x g for 20 min at 4° C (Hawk 15/05 refrigerated bench-top centrifuge, Sanyo, Japan), the supernatant was transferred into a sterilised dialysis tube (Medicell Intl, UK). The samples were dialysed overnight (16 h) against 1000 volumes of buffer C at 4° C with continuous stirring. The next day the samples were transferred into 1.5 ml microcentrifuge tubes and used immediately for the 8-oxo-dG excision assay described in 4.2.6. The same procedure was done with animals (n=3-4), which were kept under laboratory clean conditions for one month.

The samples used for Western blotting were not dialysed and they were stored in 1.5 ml microcentrifuge tubes at –80° C.

ii) from human lung carcinoma cells A549

A549 cells (Dr. N.J. Hodges, European cell culture collection number 86012804) were cultured at 37°C in a humidified chamber (5% CO_2, 95% air, MCO-15AC; Sanyo, Japan) in 25 cm^2 cell culture flasks (Becton Dickinson Falcon™, US) containing Dulbecco's modified Eagles medium (DMEM). Once cells were confluent, growth medium was removed and cold PBS (10 ml) was added. Cells were detached from the flask by vigorous scraping and resuspended in buffer A1 (500 µl). The suspension was transferred to a sterile 1.5 ml microcentrifuge tube and left on ice for 20 min. The solution was then subjected to centrifugation (M.S.E., Sanyo, Japan) at 2000 x g for 5 min at room temperature. The pellet was thoroughly resuspended in buffer B1 (50 µl) and left on ice for 30 min. Following centrifugion at 18 000 x g for 20 min at 4°C (Hawk 15/05 refrigerated bench-top centrifuge, Sanyo, Japan), the

supernatant was transferred into a sterile 1.5 microcentrifuge tube and stored at -20° C prior to Western blotting.

4.2.4 Protein quantification

Protein concentration (in μg/μl) was measured by the Bradford assay (Bradford, 1976) with Bio-Rad dye reagent (Bio-Rad, UK) and bovine serum albumin (0-10 μg) as a standard. Equal masses of protein (5 μg) were used for all the 8-oxo-dG excision assays and higher masses of protein (20-25 μg) were also used for the Western blotting.

4.2.5 Oligonucleotide end-labelling and purification

Essentially as described in Roldán-Arjona *et al.*, (1997). A single stranded oligonucleotide (24 mer) containing 8-oxo-dG at position 10 (R&D Systems, US) was 5′-end labelled with ^{32}phosphorus by incubation with γ-^{32}P-ATP (11 μl, 110 μCi) (Amersham Biosciences, UK), 10 x T4 polynucleotide kinase buffer (5 μl) (New England Biolabs, US), T4 polynucleotide kinase (PNK) (15 units) (New England Biolabs, US) and sterile water (up to 50 μl) at 37° C for 10 min. The reaction was terminated by the addition of 0.5 M Na_2EDTA (2 μl) and heating at 78°C for 1 min. The oligonucleotide was annealed to its complementary strand (18 pmol) (R&D Systems, US) by heating at 95° C for 10 min in a waterbath. The duplex probe was then cooled slowly at room temperature and subsequently purified by the QIAquick Nucleotide Removal Kit (Qiagen, US) according to the manufacturer's instructions. Briefly, 5 volumes of buffer PB were added to 1 volume of the 8-oxo-dG probe and mixed thoroughly. The mixture was transferred onto a QIAquick spin column attached to a provided 2 ml collection tube and subjected to centrifugation (M.S.E.,

Sanyo, Japan) at 8000 x g for 1 min. The flow-through was discarded and the column was attached to a new 2 ml tube. Buffer PE (750 µl) was added to the column, which was centrifuged at 8000 x g for 1 min. The flow-through was discarded and residual PE buffer was removed by an additional centrifugation step. The column was then placed into a sterile 1.5 ml microcentrifuge tube and incubated with buffer EB (10 mm Tris-HCl, pH 8.5) (250 µl) for 1 min. The purified probe was then eluted from the column by centrifugation at 8000 x g for 1 min and used immediately for the excision assay or stored in -20° C until further use.

4.2.6 8-oxo-dG excision assay

Gill cell nuclear protein (5 µg) was added to a sterile 1.5 microcentrifuge tube containing purified ^{32}P-double stranded 8-oxo-dG oligonucleotide (3.5 µl) and 1.8 x REC buffer (5.5 µl) to a final volume of 20 µl. The solution was thoroughly mixed and incubated at 37° C for 1 h. Formamide loading buffer (10 µl) was added to the mixture and the samples was denatured by heating at 100° C for 1 min and subsequently cooled on ice. The samples were centrifuged at 8000 x g for 1 min and then loaded onto a polyacrylamide gel placed in a vertical electrophoresis tank (Gibco BRL V15-17, Invitrogen, US) together with negative and positive controls. Following electrophoresis for 90 min at 300 V, the gel was placed onto blotting paper, wrapped in SaranWrap™ and secured with tape into a Kodak BioMax Cassette (Kodak, UK). The gel was subsequently exposed to X-ray film (Kodak X-omat LS, 18x24 cm) at – 80° C for appropriate time period and the film was developed in an X-ograph machine (X-rograph CompactX2).

4.2.7 Band quantification

The developed films were scanned and the blots were quantified using the densitometry GeneTools™ software (Syngene, US). Results were expressed as % cut probe. Differences between time-points or between sites were assessed by a two-tailed Student's *t*-test. Temporal differences within sites were assessed by a one-way ANOVA accompanied by a *post-hoc* Student's *t*-test.

4.2.8 Western blotting for hOGG1 and putative mussel OGG1.

Nuclear protein extracts (25 μg) were mixed with Laemmli 2 x concentrate loading buffer and heated at 95° C (Minicycler™, MJ Research, UK) for 5 min. Samples were resolved on a 12.5% SDS-polyacrylamide gel together with a pre-stained protein marker (New England Biolabs, US) at 120 V for 90 min. Following electrophoresis, the samples were transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) using a mini trans-blot electrophoresis transfer cell apparatus (BioRad, UK) at 100 V for 60 min at 4°C. The membrane was overnight blocked with blocking buffer at 4° C on a rocking platform (Stuart Scientific STR9, UK). Next day the membrane was incubated with a rabbit anti-hOGG1 polyclonal primary antibody (1:500 dilution, ab204, Abcam, UK) in blocking buffer for 1 h at room temperature on a rocking platform. The membrane was subsequently washed in 1 x TBS-0.05% Tween 20 solution (3 x 10 min) and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1000 dilution, Dako, Denmark) in blocking buffer for 1 h at room temperature on a rocking platform. The membrane was washed in 1 x TBS-0.05% Tween 20 (2 x 10 min) and finally in 1 x TBS (1 x 10 min). Following washing, the membrane was incubated with enhanced chemiluminescence detectors (Geneflow, UK). OGG1 bands were visualized by exposure to ECL hyperfilm

(Amersham Biosciences, UK) and development in an X-ograph machine (X-rograph CompactX2) after 3 min of exposure.

4.3 Results

-8-oxo-dG excision evidence in mussel gill

All gill nuclear protein extracts from mussels from different sites (NB and LL) and at different times of the year were able to excise 8-oxo-dG from the radiolabeled duplex probe as shown in **Figure 37**. A still uncharacterised *N*-glycosylase responsible for 8-oxo-dG excision is therefore present in mussels

-Lack of immunopositivity with anti-OGG1

Gill nuclear protein extract was unable to react with a polyclonal anti-hOGG1 antibody as shown in **Figure 38** (n= 3 experiments). In contrast, the antibody reacted with hOGG1 of lung carcinoma A549 cells.

-Temporal variation in 8-oxo-dG cutting activity in gill of mussels from NB and LL

A graph of temporal comparisons between 8-oxo-dG cutting activities throughout the year is depicted in **Figure 39.** The values recorded for LL comprise the "standard curve" and the values recorded for NB are expressed in relation to this standard curve. Compared to LL values, NB values were always lower ("24 h" samples) ($P<0.05$, two-tailed Student's *t*-test) with the exception of May 2006 when values from both sites are minimal. A trend of temporal differences with minimal values in May and maximal values in February for "24 h" samples was also evident.

-Effect of one-month laboratory maintenance on 8-oxo- dG repair capacity

The % cut probe was recorded for both NB and LL samples at "24 h" and at "1 month" post collection (**Figure 40A, B** respectively) for July and September 2005 and February, May and July 2006. Although not consistent in all the samples, there was a general trend for relatively higher levels 8-oxo-dG excision capacity after one-month laboratory maintenance for NB mussels. This increase was substantial especially in the summer months as shown in **Table 7**. In contrast there was no significant increase of excision capacity in LL mussels as a result of laboratory maintenance. There was a decrease of activity in both populations after laboratory maintenance only for February 2006.

-Effects of one-month laboratory maintenance on Fpg sensitive sites/8-oxo-dG excision activity

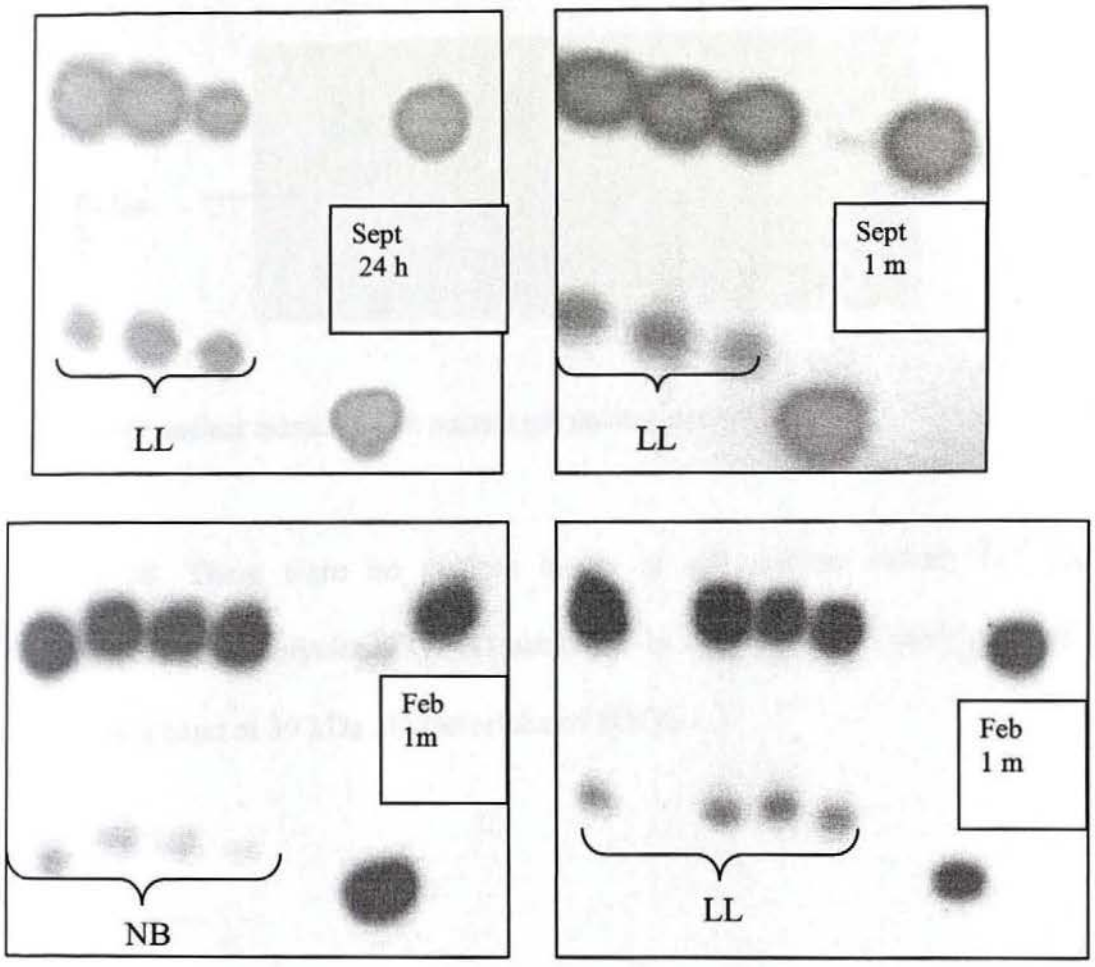
No correlation between baseline Fpg sensitive sites and 8-oxo-dG excision capacity was found for 24 h samples or for one-month samples or as a whole. However, there was a consistent decrease in the ratio "average Fpg sensitive sites/ average 8-oxo-dG excision activity" after lab-maintenance for NB for all the months tested (**Figure 41A**). Furthermore, a paired *t*-test between the ratios ("24 h" versus "1 month") revealed a statistically significant decrease after lab-maintenance ($P<0.01$). There was a slight increase in the ratio "average Fpg sensitive sites/ average 8-oxo-dG excision activity" after lab-maintenance for LL for most of the months tested (**Figure 41B**), however there was no statistically significant difference between the ratios ("24 h" versus "1 month") when a paired *t*-test was performed.

-Relationship between average Fpg sensitive sites after Cr(VI)-treatment and average 8-oxo-dG excision capacity

There was a strong negative correlation between average Fpg sensitive sites after Cr(VI)-injection and average 8-oxo-dG excision capacity ($P<0.01$) (**Figure 42A,B**). 8-oxo-dG levels recorded 4 h post-injection are strongly affected by the 8-oxo-dG excision capacity in mussel gill.

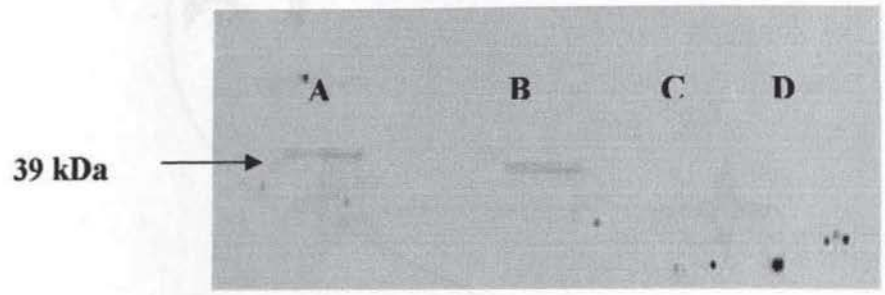
-Relationship between average frank SSB and average 8-oxo-dG excision capacity

There was a weak correlation between average baseline frank SSB and average 8-oxo-dG excision capacity for 24 h samples only (**Figure 43**). The cutting activity of mussel gill towards 8-oxo-dG does not appear to be a major contributor to baseline frank SSB, although it may contribute to the SSB recorded to a certain extent.



LL: Llandudno, **NB**: New Brighton

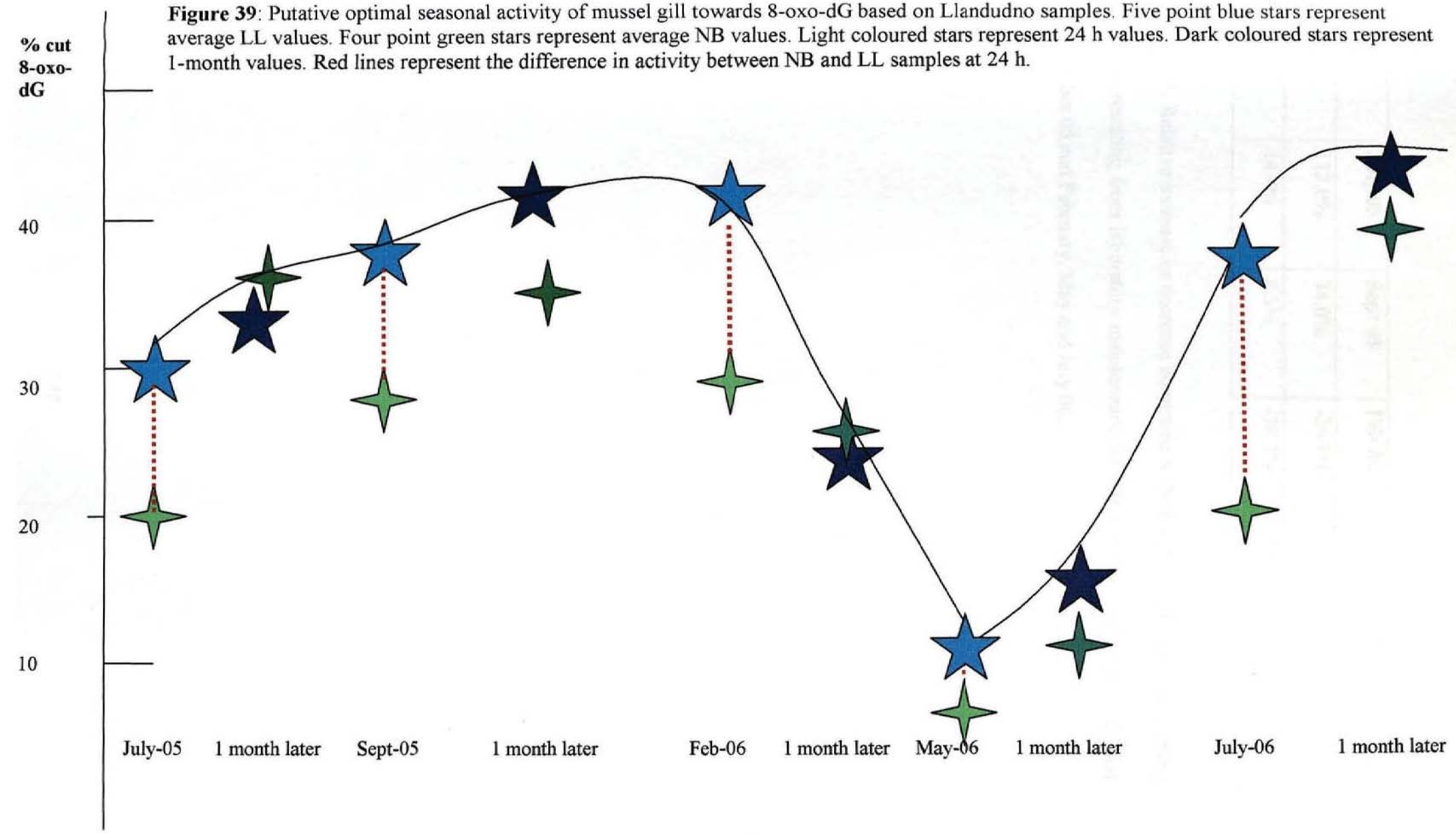
Figure 37: Nuclear gill extract capacity to excise 8-oxo-dG from a radiolabelled duplex probe. Samples were obtained at various times of the year: blots of radiolabelled probe incubated with nuclear protein extract were co-electrophoresed with a negative control (probe only) and a positive control (probe+ 3 units purified Fpg), which will cut 8-oxo-dG. Upper bands represent uncut probe, lower bands represent cut probe: evidence of cutting activity by mussel gill.



A, B: A549 nuclear extract, **C, D**: mussel gill nuclear extract

Figure 38: There were no evident bands of gill nuclear extract (25 μg) after incubation with a polyclonal OGG1 antibody. In contrast, A549 positive controls (20 μg) gave a band at 39 kDa characteristic of hOGG1.

Figure 39: Putative optimal seasonal activity of mussel gill towards 8-oxo-dG based on Llandudno samples. Five point blue stars represent average LL values. Four point green stars represent average NB values. Light coloured stars represent 24 h values. Dark coloured stars represent 1-month values. Red lines represent the difference in activity between NB and LL samples at 24 h.



	July 05	Sept 05	Feb 06	May 06	July 06
NB	112.0%	34.0%	-26.4%	5.6%	51.5%
LL	14.4%	8.3%	-38.7%	6.2%	1.8%

Table 7: Relative increase or decrease compared to 24 h activity of 8-oxo-dG cutting activity resulting from laboratory maintenance for NB and LL samples for July and September 05 and February, May and July 06.

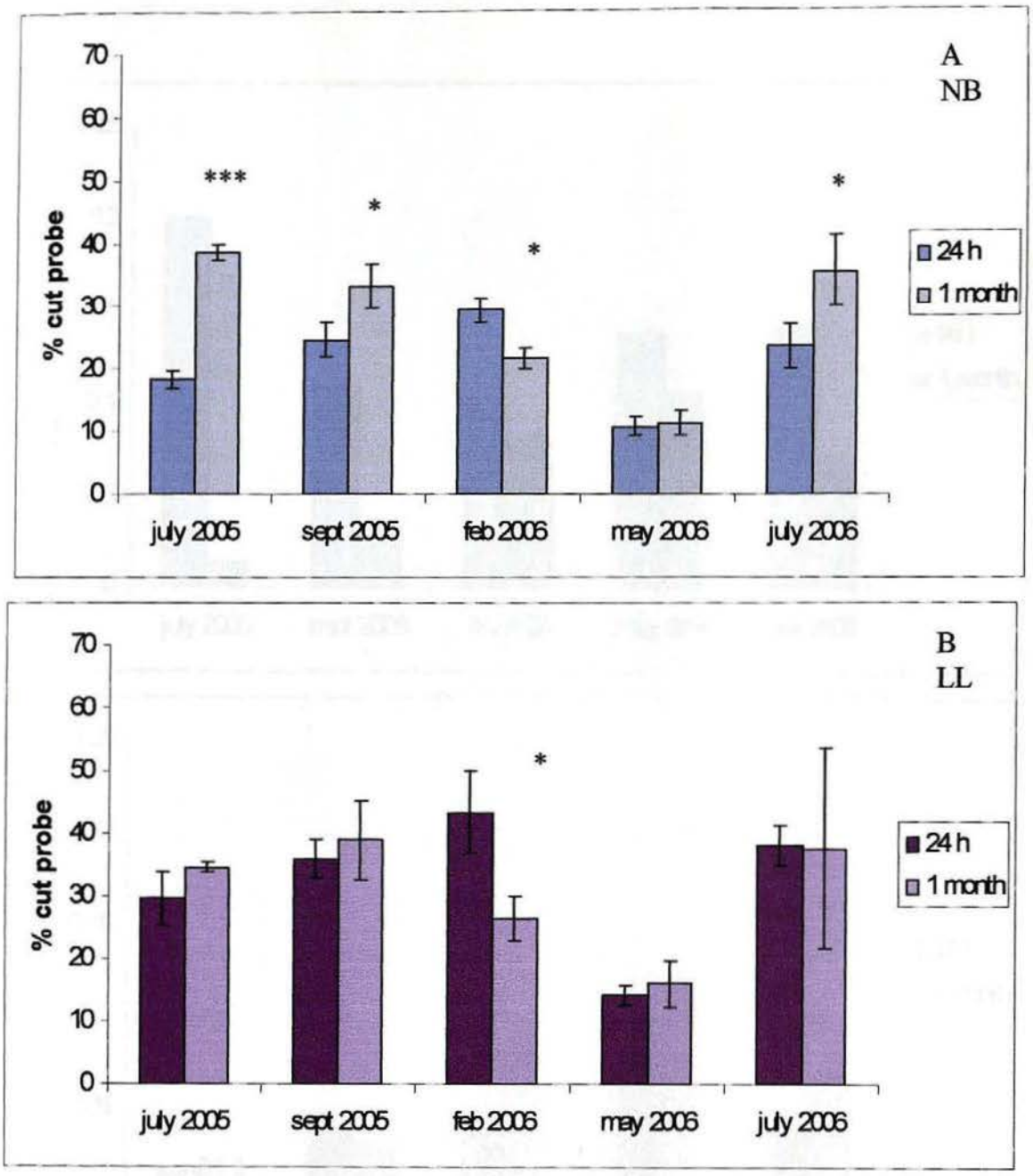


Figure 40A,B: Effect of one-month maintenance in laboratory conditions on 8-oxo-dG excision capacity for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Values are the mean of 4 animals (± SEM) except for July 2005 (24 h), July 2005 (1 month), September 2005 (NB, 24 h), July 2006 (NB, 24 h) and July 2006 (LL, 1 month) where n=3 and for February 2006 (LL, 24 h) where n=2. *: Significantly different ($P<0.05$) between time-points as assessed using Student's *t*-test. *** ($P<0.001$).

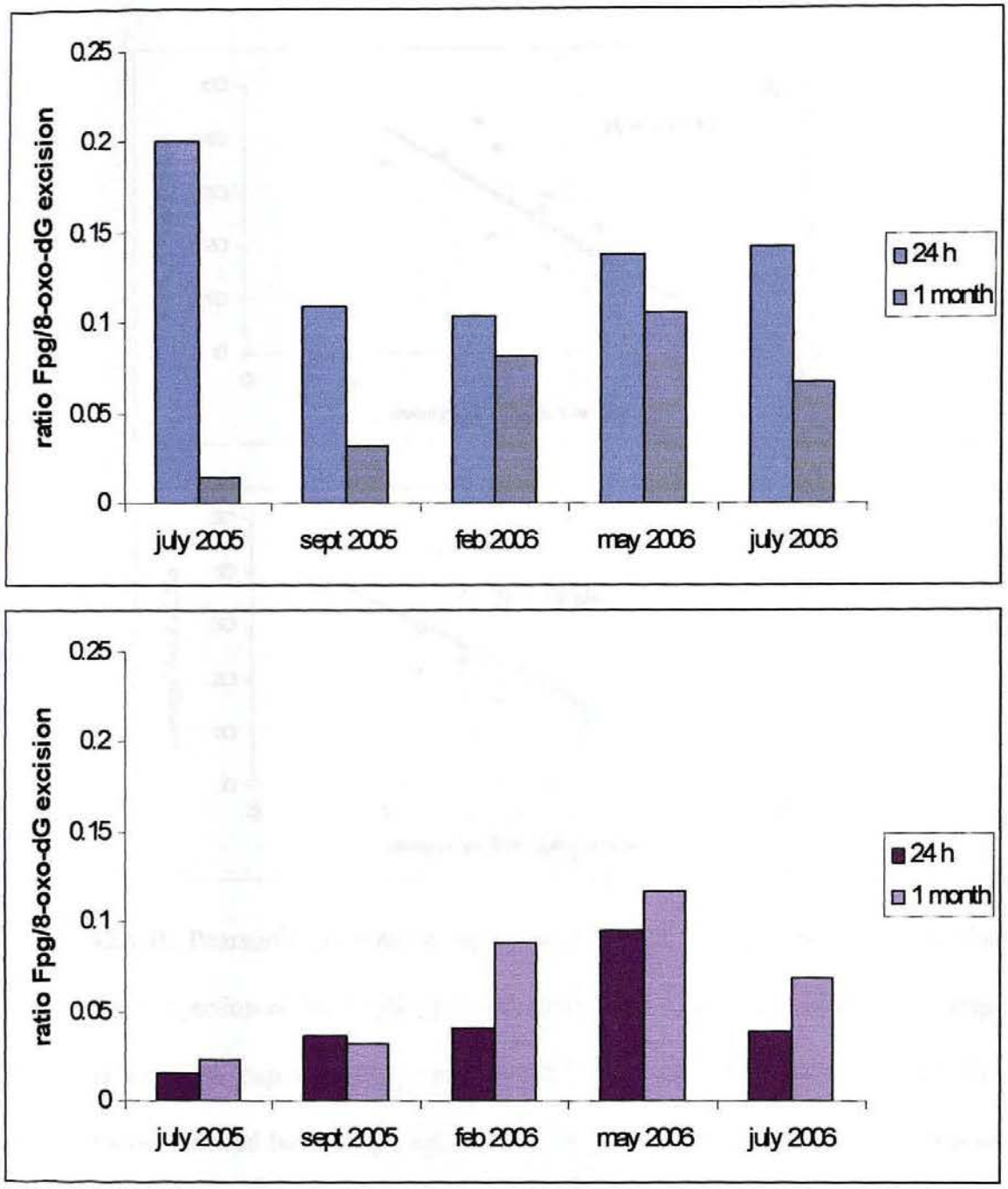


Figure 41A,B: Relationship between average Fpg sensitive sites and average 8-oxo-dG excision capacity for NB (A) and LL (B) mussels respectively. **41A**: Significant overall decrease ($P<0.01$) after one-month maintenance, as assessed by a paired *t*-test. **41B**: No significant overall difference after one-month maintenance.

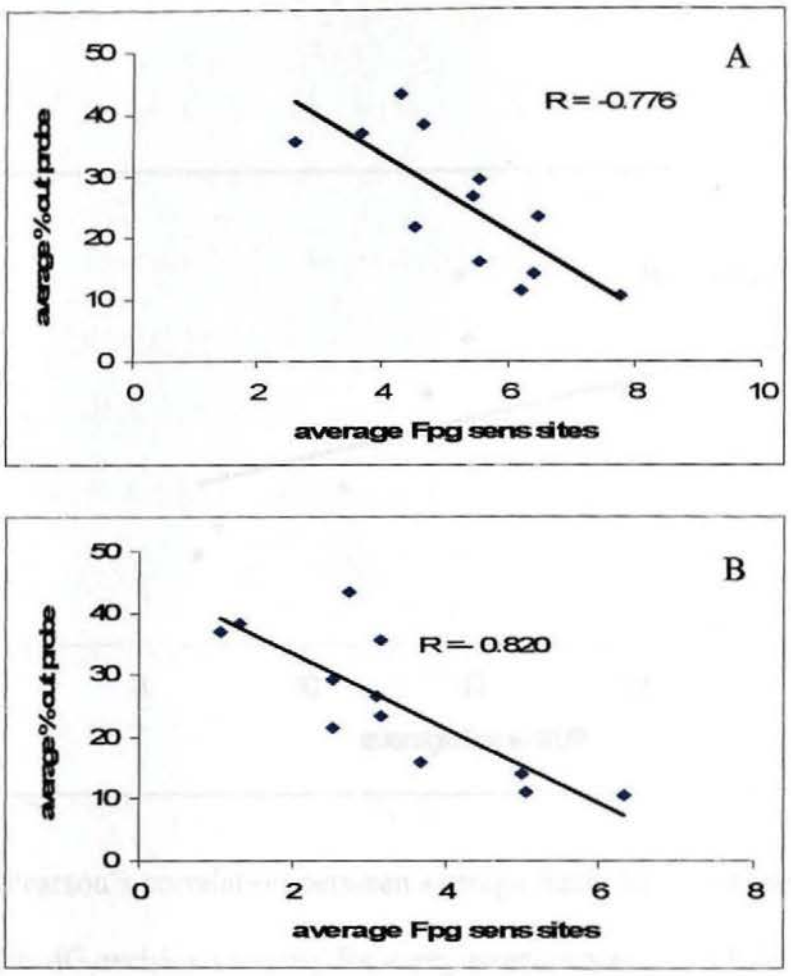


Figure 42A,B: Pearson's correlation between (A): total average Fpg sensitive sites after a single injection of $Na_2Cr_2O_7$ (0.1 μmol/100 μl), 4 h post-injection and average 8-oxo-dG excision capacity for every month tested ($P<0.01$). (B): average Fpg sensitive sites induced by a single injection of $Na_2Cr_2O_7$ (0.1 μmol/100 μl), 4 h post-injection and average 8-oxo-dG excision capacity for every month tested ($P<0.01$).

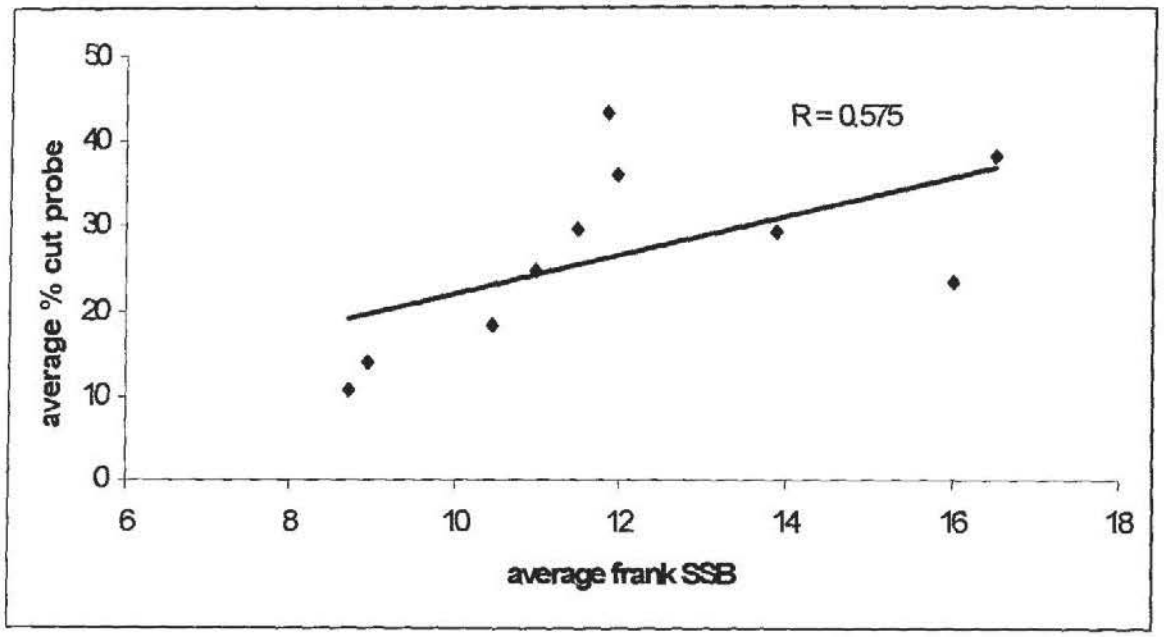


Figure 43: Pearson's correlation between average frank SSB (untreated animals) and average 8-oxo-dG excision capacity for every month tested at 24 h (P=0.08).

4.4 Discussion

i) The mussel 8-oxo-dG *N*-glycosylase

The base excision repair pathway is responsible for removal of damaged DNA bases via the concerted action of many enzymes. The initial step of the pathway is the recognition of the modified base and its excision. This first step is performed by specific DNA glycosylases, which either cleave the *N*-glycosidic bond creating an AP site (type I glycosylase) or create SSB via its 3′endonuclease activity (type II glycosylase) (Christmann *et al.*, 2003). The base excision repair pathway is highly conserved among organisms and its existence in mussels has been inferred by many researchers (Chapters 1.7, 4.1). Here we have shown directly that there is repair capacity against 8-oxo-dG in mussel gill (**Figure 37**).

At this point we can only make assumptions about the *Mytilus edulis* 8-oxo-dG glycosylase because no sequence of the gene is known. Experiments performed in our laboratory with degenerate primers designed against *ogg1* sequences from a wide range of organisms failed to amplify any *ogg1* homologues from a *M.edulis* cDNA library. This result together with the failure of an OGG1 antibody immunopositive for human, rat, mouse, dog and cow to react with mussel nuclear extract (**Figure 38**) suggest that any bivalve 8-oxo-dG glycosylase is quite different from well-known OGG1 homologues. However, there is always the possibility that no OGG1 homologue exists in mussels. This is not impossible since invertebrate genomes have striking differences from the vertebrate even though OGG1 is present in yeast. A good example of substantial differences between vertebrate and invertebrate genomes is the apparent lack of CYP1A enzymes in invertebrates. *CYP1A* is not found in *C. elegans*

or *Drosophila* genome and the partial response of the earthworm *Lumbricus rubellus* to murine CYP1A actually arises from a conserved region among many P450s. Mussel P450s therefore, may belong to the *C.elegans* CYP35 family and not a CYP1A family (Rewitz *et al.*, 2006). In a similar manner, the bivalve 8-oxo-dG glycosylase might be structurally unrelated to OGG1. The presence of a mussel Fpg analogue instead of OGG1 needs to be considered since Fpg has been found in some higher organisms like *Arabidopsis thaliana* (Gros *et al.*, 2002), although this organism also possesses an OGG1 gene (Didzaroglu, 2005) This would explain the decrease of the mussel 8-oxo-dG glycosylase after *in vivo* exposure to Cd (Chapter 6) by means of Zn displacing in the Fpg Zn-finger (Asmuß *et al.*, 2000). Nei eukaryotic homologues are also possible: DNA glycosylases belonging to the Nei/Fpg superfamily have been found in human and in mouse and murine Neil2 and Neil3 also possess Zn-finger motifs (Rosenquist *et al.*, 2003). However, the gel fragments of our assays do not corroborate the latter theories: OGG1 participates in a β-elimination that generates a terminus with a 3′ ring-opened unsaturated sugar whereas a βδ-elimination characteristic for Nei and Fpg produces a 3′-phosphate terminus which runs faster on the polyacrylamide gel (Izumi *et al.*, 2003; Rosenquist *et al.*, 2003; Wiederhold *et al.*, 2004). More detailed investigations in the mussel 8-oxo-dG glycosylase will determine the exact nature of the enzyme.

ii) Experimental design

We have followed the endonuclease nicking assay described in Roldán-Arjona *et al.*, (1997) to detect gill nuclear protein activity against 8-oxo-dG. The initial lysis buffers currently used for cultured cells (Hodges and Chipman, 2002; Smart *et al.*, 2006) did not allow for detection of cutting activity. In contrast, nuclear extracts from wild type

mouse embryonic fibroblasts did show cutting activity against the probe, which proves the validity of the current protocol. Finally substitution of the buffers A,B and C with the corresponding buffers described in Klungland *et al.*, (1999) for mouse organs enabled us to see activity with mussel gill extracts. The reasons for the appropriateness of the second series of buffers is not fully understood, but it may be linked to the uncoupling of glycosylase/endonuclease activity of OGG1: this enzyme is a bifunctional glycosylase, however it spontaneously dissociates from the AP site it created, which is then processed by APE1 thus it acts as a monofunctional glycosylase *in vivo* (Vidal *et al.*, 2001). Magnesium ions further contribute to the uncoupling of glycosylase/endonuclease activity by downregulating the endonuclease activity of OGG1 (Morland *et al.*, 2005). It is therefore possible that the higher Mg^{+2} content of the initial buffers inhibited the endonuclease activity of the mussel gill 8-oxo-dG glycosylase whereas the glycosylase activity remained intact. The high affinity of OGG1 for non-cleaved AP sites may have also attenuated AP removal since APE1 could not interact with them (Vidal *et al.*, 2001; Morland *et al.*, 2005). Why these reactions may be important for one cell type (mussel gill) and not for another (mouse embryonic fibroblasts) has not been addressed.

iii) Temporal variability

There was a temporal pattern in the excision capacity of the mussel 8-oxo-dG glycosylase (based on the LL mussels) (**Figure 39**) with a maximum in cold months and a minimum in May, however it did not follow a strict seasonal pattern with values in July 2006 being higher than values in July 2005 but also with greater inter-individual variability. According to Bayne, 1976, spawning starts in May in Liverpool Bay and in Conway and the intense reproductive activity may have a negative effect

on other biochemical activities including DNA repair. *Mytilus* sp. may show a minimal activity of antioxidant defence systems at winter (Viarengo *et al.*, 1991; Sheehan and Power, 1999) and enhanced DNA repair capacities may act as a compensatory mechanism for this situation. However, there is a dramatic decrease of this capacity pre-spawning, reaching a lowest around spawning period but in the warm months the activity was restored even though there was still concomitant spawning.

iv) Differences between sites

Compared to LL samples NB samples always exhibited lower excision, as shown in **Figure 39** with the exception of May ("24 h" samples). The pollution in NB is more intense than LL (Chapter 2), however no compensatory up-regulation of oxidative damage excision mechanisms (as a result of natural selection) was noted during our experiments. On the contrary, indigenous NB mussels exhibited a "suppressed" activity against 8-oxo-dG compared to LL mussels. A lack of up-regulation of 8-oxo-dG glycosylases under oxidative stress conditions is not uncommon. For example OGG1 repair activity was actually transiently reduced and not increased in rat lungs after intratracheal administration of diesel exhaust particles. However, 7 days after exposure 8-oxo-dG excision activity was restored (Tsurodome *et al.*, 1999). During another experiment exposure of rats to diesel exhaust particles in food did not cause OGG1 mRNA induction even though oxidative stress in the form of Fpg and Endo III-sensitive sites was evident after the exposure (Müller *et al.*, 2004). Finally, Dhénaut *et al.*, (2000) did not notice upregulation of OGG1 in HeLa cells after oxidative stress. These results are indicative of the stable expression levels of OGG1, a typical house-keeping gene, which lacks TATA or CAAT boxes and does not

change expression during cell cycle (Dhénaut *et al.*, 2000). However, there is an Nrf2 binding site in the promoter region of human OGG1 and Nrf2 in association with Jun control the induction of genes of detoxifying enzymes. Enhancing modulation of OGG1 during oxidative stress is therefore possible (Dhénaut *et al.*, 2000). Even though the aforementioned models did not show any up-regulation under oxidative stress on one occasion OGG1 in cells adjacent to carcinomas showed boosted activity in order to cope with the additional oxidative burden (Powell *et al.*, 2005). However, a similar up-regulation of the bivalve 8-oxo-dG glycosylase because of oxidative stress was not noticed for NB populations, at least for the months tested.

On the contrary, a decreased cutting activity was observed for NB specimens compared to LL specimens. This lower value of the bivalve 8-oxo-dG glycosylase activity noted in NB mussels may be linked to specific pollutants, which are able to modulate the transcription, the expression and/or the efficacy of this enzyme. Persistent pollutants of Merseyside like DDT and/or PCB congeners may be implicated in the low activity recorded for these specimens. High doses of DDT (500 ppm) caused a slight decrease in OGG1 mRNA in rats with hepatocarcinomas (and a slight increase in much lower doses) (Kushida *et al.*, 2005). Also the non-mutagenic 2-aminobiphenyl (2-ABP) caused a dose-dependent decrease in OGG1 mRNA and OGG1 levels in HepG2 cells *in vitro* (Wang *et al.*, 2006). Non-organic pollutants and especially heavy metals have been even more strongly incriminated for compromised glycosylase activities, with Cd and Hg levels being elevated in Merseyside (Chapter 2). Ni(II), Cd(II) and especially Hg(II) caused profound inhibition of the *E.coli* 8-oxo-dG glycosylase Fpg at concentrations > 1mM, 50 μM and 0.05 μM respectively (Asmuß *et al.*, 2000). OGG1 is another sensitive target for Cd (Chapter 1.8.2.3) even

though it does not have Zn finger motifs. Cd interferes with its transcription (Youn *et al.*, 2005), its interactions with DNA (Zharkov and Rosenquist, 2002) and/or modulates the glycosylase at the protein level (Potts *et al.*, 2003). Suppression of 8-oxo-dG excision after *in vivo* exposure of mussels to Cd (Chapter 7) also corroborates its possible implication for the results noted for NB and highlights the inhibitory potential of this metal, which is commonly found in contaminated estuaries.

v) Differences between time-points

One-month laboratory maintenance caused an increase in 8-oxo-dG glycosylase activity for the majority of collections from NB as shown in **Figure 40A** and in **Table 7**, whereas there was no significant increase for LL mussels (**Figure 40B** and **Table 7**) throughout the year. These observations also corroborate the previous theory that pollutants and/or other factors in the NB estuary suppressed the activity of this particular glycosylase. After this suppression factor was removed (laboratory maintenance), the levels of activity were comparable to the levels noted in LL mussels [we assumed that the activity of the latter (both "24 h" and "1 month" samples) mirrors the normal, non-suppressed activity expected for *Mytilus edulis* gill with respect to seasonal variation]. The alleviation of oxidative stress after one-month laboratory maintenance for NB populations is also expressed in **Figure 41A**, where laboratory maintenance lowered the ratio "average Fpg sensitive sites/ average 8-oxo-dG excision capacity" whereas a similar difference was not found for the LL populations which were not positively affected by laboratory maintenance, with respect to oxidative stress levels (**Figure 41B**). The alleviation of oxidative stress because of laboratory maintenance is also corroborated by the MDA findings in Chapter 5. Another important outcome from these depuration experiments was that

the low activity noted for NB samples does not persist, thus does not have a genetic component (i.e. is not due to the genetic constitution of the NB mussel population) but is rather a consequence of pollution and/or other conditions of the particular estuary. However, the presence of non-functional genetic variants of key repair enzymes in bivalves in general is possible. The human OGG1 for example, does exhibit single nucleotide polymorphisms and some of them (Cys326Ser) display compromised 8-oxo-dG excision in certain cell models (Nohmi et al., 2005). At this point very little is known about genetic polymorphisms in bivalves and the relevant studies have mostly concentrated on metallothionein variants (e.g. Mackay *et al.*, 1993). Further investigations may reveal genetic polymorphisms regarding oxidative DNA repair in bivalves in the future.

vi) Correlations of 8-oxo-dG excision capacity with Comet assay parameters

There was no significant correlation between 8-oxo-dG excision activity and baseline Fpg sensitive sites for the months tested. A correlation between baseline 8-oxo-dG and the capacity of 8-oxo-dG glycosylase is still possible, however the present baseline levels of 8-oxo-dG were probably too low to bring out the differences in the activity of the glycosylase. An oxidative challenge may be required to reveal a correlation. Indeed there was a clear correlation between average 8-oxo-dG levels caused by oxidative insult [Cr(VI) injection] 4-h post-exposure and average 8-oxo-dG excision capacity, even though the two parameters were not measured on the same animal: the higher the excision capacity the lower the 8-oxo-dG levels. The correlation was valid for both total average Fpg sensitive sites against average 8-oxo-dG excision capacity and for Cr(VI)-related average Fpg sensitive sites (when baseline average Fpg sensitive sites was subtracted from total average Fpg sensitive

sites) against average 8-oxo-dG excision capacity. The strong correlation signifies that the response of mussel gill after *in vivo* challenge by pro-oxidant chemicals is substantially affected by the prevailing repair capacity in mussel gill. Thus, distinct populations or the same population at different season may exhibit different responses after *in vivo* exposures. In corroboration of the theory that a percentage of the SSB recorded in the comet assay reflect a repair intermediate rather than direct damage (Chapter 3) there was a weak correlation between 8-oxo-dG excision capacity and frank baseline SSB (at 24 h only). However we have to note that because of time and tissue constraints the excision capacity was not measured in the same animals that were used for the Comet assay but in other characteristic animals of the same batch. The weak correlation shows that the interference of 8-oxo-dG excision to recorded SSB measured in the comet assay is not substantial, however similar correlations with cutting activities against other lesions (e.g. alkylated DNA bases or bulky DNA adducts) are possible and may also contribute to the SSB noted.

In conclusion, dissimilarities in 8-oxo-dG excision capacity between sites emerged at many of the mussel collections of the year 2005-2006. Differences between time-points ("24 hours", "1 month") were also evident. Differences in non-enzymatic antioxidant status (reduced glutathione), lipid peroxidation and their implications in oxidative stress are discussed in the next chapter.

5.0 Lipid peroxidation and reduced glutathione levels in gill of mussels from New Brighton and Llandudno populations.

5.1 Introduction

ROS are normally produced in organisms as by-products of cell-respiration and other mechanisms. They can also be produced by a variety of pollutants and xenobiotics. ROS can produce numerous damaging effects in cells including lipid peroxidation. Malondialdehyde (MDA) is commonly measured as an indicator of LPO. It is a volatile product which forms adducts with deoxyguanosine, deoxyadenosine and deoxycytosine (Marnett, 2002). Another by-product, 4-HNE, is also implicated in carcinogenesis because it can form miscoding exocyclic DNA adducts (Bartsch and Nair, 2005). It is cytotoxic in high concentrations and it has been implicated in various chronic and inflammatory diseases including chronic obstructive pulmonary disease, Parkinson's disease, alcoholic liver disease and Creutzfeldt-Jakob disease in humans (Iles and Liu, 2005).

A quick and inexpensive method of measuring LPO is based on the reaction of the chromogenic reagent *N*-methyl-2-phenylindole with MDA at 45°C, which produces a chromophore at 586 nm under highly acidic (HCl) environment. This method is more specific for MDA than the TBAR method (Durand *et al.*, 2002) and it has been used in a number of studies concerning lipid peroxidation in mussels (Cavaletto *et al.*, 2002; Shaw *et al.*, 2004; Pampanin *et al.*, 2005). Utilisation of methanosulfonic acid (MSA) as the acid solvent facilitates the detection of both MDA and 4-HNE.

Cells possess a variety of antioxidant mechanisms to combat the deleterious effects of ROS. Enzymatic mechanisms comprise the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Doyotte *et al.*, 1997). One of the most efficient non-enzymatic protective mechanisms is the conjugation of

electrophilic and/or oxidised components with glutathione (GSH). GSH is the most abundant intracellular thiol (Canesi *et al.*, 1995; Iles and Liu, 2005). It is a tripeptide formed via two sequential ATP-consuming reactions: glutamate and cysteine are linked by glutamate cysteine ligase. In a second ATP-dependent reaction glycine is added by glutathione synthetase (Iles and Liu, 2005). Glutathione reductase regenerates the reduced form of glutathione maintaining the optimal levels of GSH pools (Iles and Liu, 2005). GSH has been evaluated in a number of bivalve studies, regarding their exposure to heavy metals (Regoli and Principato, 1995; Canesi *et al.*, 1999), organics (Doyotte *et al.*, 1997; Dafre *et al.*, 2004) or polluted environments (Cossu *et al.*, 2000; Rodriguez-Ortega *et al.*, 2002).

A quick and inexpensive method of GSH evaluation (and oxidised glutathione GSSG) is based on its reaction with the fluorophore *o*-phthalaldehyde; the reagent is not fluorescent until it reacts with a primary amine in the presence of thiol, cyanide or sulfite (Hissin and Hilf, 1976).

Mussels were collected from the sites of interest (NB and LL) at various time points of the year 2005-2006 and gills from representative animals were analysed by spectrophotometric derivatisation with *N*-methyl-2-phenylindole for detection of MDA and 4-HNE. Gills from another set of representative animals per site were analysed by fluorometric derivatisation with *o*-phthalaldehyde for detection of GSH. Comparisons were made between sites (NB and LL) and between time points ("24 h" samples and "1 month" samples, representing different times after collection and laboratory maintenance). Temporal variations for MDA, 4–HNE and GSH were also checked.

5.2 Materials and methods

5.2.1 Reagents: All chemicals unless otherwise stated were of the highest quality available from Sigma-Aldrich, Dorset, UK

5.2.2 Buffers

i) for lipid peroxidation assay

Lysis buffer: 5 mM butylated hydroxyl toluene (BHT) in 1% acetonitrile.

Reagent R1: *N*-methyl-2-phenylindole (10.3 mM) in acetonitrile (3 parts) + methanol 100% (1 part)

MDA stock solution: 1,1,3,3 tetramethoxypropane (TMOP) (20μM) in distilled water just prior to use.

ii) for GSH assay

Protein precipitation buffer: Trichloroacetic acid (TCA) (50% w/v), Na_2EDTA (5mM) and Na_2HPO_4 (100 mM).

Phosphate –EDTA buffer: Na_2EDTA (5mM) and Na_2HPO_4 (100 mM), pH 8.0.

OPT solution: *o*-phthalaldehyde (1 mg/ml) made up with 100% methanol

GSH stock solution: GSH (0.1 mg/ml) in ice-cold phosphate-EDTA buffer just prior to use.

5.2.3 Gill sample preparation

i) for LPO evaluation

Mussels (n=3-4) from each of the two sites were randomly selected after overnight acclimatization. The animal valves were opened with a metal scalpel (17 cm) and gill tissues were extracted with small size metal tweezers. The tissue was dried of excess water, cut in smaller pieces and weighed accurately. The tissue was thoroughly homogenized in lysis buffer (8.0 ml) kept on ice by using the dispersing instrument

Ultra Turrax T8 (IKA Labortechnik, Germany) at setting 12 for 2 min and left on ice until all the samples were completely homogenised (<30 min). The homogenate was used immediately for evaluation of LPO. The procedure was repeated on animals maintained in laboratory conditions for one month.

ii) for GSH evaluation

Mussels (n=3-4) from each of the two sites were randomly selected after overnight acclimatization and gill tissues extracted as above. The tissue was gently disaggregated in a 25 ml glass beaker (Duran, Mainz Germany) containing protein precipitation buffer (3.0 ml) and filtered through 2 layers of 150 μm gauze. The weight of the non-homogenised tissue was subtracted from the initial weight. The filtered solution was collected in sterile 1.5 ml microcentrifuge tubes (2 tubes per animal) and subjected to centrifugion (M.S.E., Sanyo, Japan) at 13 000 x g for 5 min at room temperature. The supernatant was transferred into new 1.5 ml microcentrifuge tubes and used in the assay immediately or stored at -80° C (up to one week). The procedure was repeated on animals maintained in laboratory conditions for one month

5.2.4 Spectrophotometric evaluation of MDA and 4-HNE.

Gill cell suspensions were kept on ice and duplicate fractions/per animal (200 μl) were added to 1.5 microcentrifuge tubes containing reagent R1 (650 μl). Duplicate MDA standard curves (0-4 μM) were plotted from MDA stock solution by adding the appropriate amount of stock solution and distilled water up to 200 μl. Reagent R1 (650 μl) was also added to the standards. Methanosulfonic acid (15.4 M, 150μl) was added to one set of the duplicates and concentrated hydrochloric acid (12 N, 150μl) was added to the remaining set. The solutions were incubated at 45° C for 60 min (Gallenkamp Hotbox oven, UK) and afterwards cooled on ice. Following

centrifugation at 18 000 x g for 5 min at 4°C (Hawk 15/05 refrigerated bench-top centrifuge, Sanyo, Japan), the supernatant was transferred into spectrophotometer cuvettes (Sarsedt AG and Co, Germany; 10x4x45 mm) and absorbance was measured at 586 nm (922 Uvikon spectrophotometer, Kontron Instruments). MDA levels were calculated from the HCl-incubated samples. 4-HNE levels were found by subtracting the MDA reading from the MDA+ 4-HNE reading (from the methanosulfonic acid-incubated samples). Results were normalized to mass of wet tissue. The procedure was repeated on animals maintained in laboratory conditions for one month.

5.2.5 Fluorometric evaluation of GSH.

Gill cell supernatants were defrosted on ice and a fraction (100 µl) was transferred into 3 ml polystyrene fluorescence cuvettes (Sarstedt AG and Co, Germany) containing phosphate-EDTA buffer (1.8 ml). A GSH standard curve (0-2 µg) was plotted from GSH stock solution by adding the appropriate amount to cuvettes containing phosphate-EDTA buffer (1.8 ml) and protein precipitation buffer (100 µl). OPT solution (100 µl) was added to all the cuvettes and thoroughly mixed. The samples were incubated in the dark for 15 min on a rocking platform (Stuart Scientific STR9, UK) and then fluorescence was measured using a fluorometer (Perkin Elmer LS 50B, UK) with excitation at 340 nm (slit width 2.5 nm) and emission at 420 nm (slit width 4.0 nm). Reduced cellular GSH was calibrated against the standard GSH curve and normalized to mass of wet tissue. The procedure was repeated on animals maintained in laboratory conditions for one month.

5.3 Results

-Temporal variation in MDA in gill of mussels from NB and LL

Temporal variation in MDA was recorded for NB and LL samples at "24 h" throughout the year (**Figure 44A,B** respectively). Statistically significant differences between months were noted for both sites. A trend of variability throughout the year was noted with the sampling in July 2005 exhibiting the highest response in mussels from LL and the sampling in both July 2005 and July 2006 exhibiting the highest response in mussels from NB.

-Temporal variation in 4-HNE in gill of mussels from NB and LL

Similarly temporal variation in 4-HNE was recorded at both sites (**Figure 45A,B).** NB populations showed minimal values at winter and maximal at summer. LL populations did not show any clear temporal trend.

-Temporal variation in GSH in gill of mussels from NB and LL

Temporal variation in GSH was recorded for NB and LL samples at "24 h" throughout the year (**Figure 46A,B** respectively). There was a temporal trend with higher values at summer than in February, which in the case of NB were significant (one-way ANOVA for the medians accompanied by a *post-hoc* Student's *t*-test).

-Effect of one-month laboratory maintenance on MDA in gill of mussels from NB and LL

MDA levels were recorded for gill cells of both NB samples (**Figure 47A**) and LL samples (**Figure 47B**) at "24 h" and at "1 month" (post-collection) for July and

September 2005 and February, May and July 2006. Although not consistent in all the samples, there was a general trend for relatively lower levels after one-month laboratory maintenance for mussels of both sites.

-Effect of one-month laboratory maintenance on 4-HNE in gill of mussels from NB and LL

4-HNE levels were recorded for gill cells of both NB samples (**Figure 48A**) and LL samples (**Figure 48B**) at "24 h" and at "1 month" (post-collection) for July and September 2005 and February, May and July 2006. There was a general trend for relatively lower levels after one-month laboratory maintenance for NB with the exception of February. For LL the decreases were less pronounced.

-Effect of one-month laboratory maintenance on GSH in gill of mussels from NB and LL

GSH levels were recorded for gill cells of both NB samples (**Figure 49A**) and Llandudno samples (**Figure 49B**) at "24 h" and at "1 month" (post-collection) for July and September 2005 and February, May and July 2006. No differences were found between time-points for both sites with the exception of July 2005 for NB where an increase was noted ($P < 0.05$, two-tailed *t*-test).

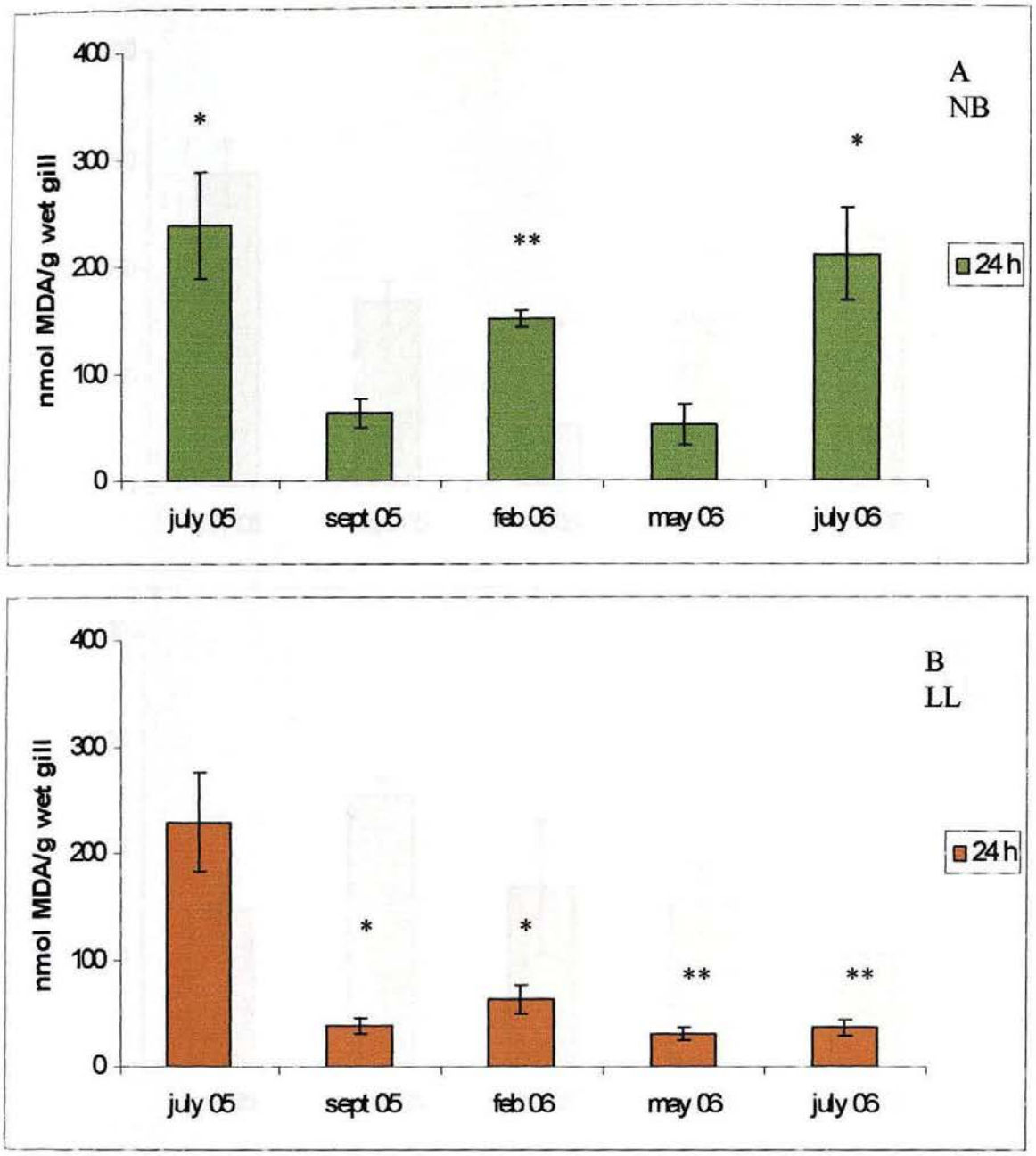


Figure 44 A,B: Temporal trend of MDA for NB and LL mussel gill respectively (24h samples) Values are the mean of 4 animals (± SEM) except for July 2005 and September 2005 (n=3). **44A**: * Significantly different to May ($P<0.05$), ** Significantly different to May ($P<0.01$), **44B**: * Significantly different to July 05 ($P<0.05$), ** Significantly different to July 05 ($P<0.01$), as assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test.

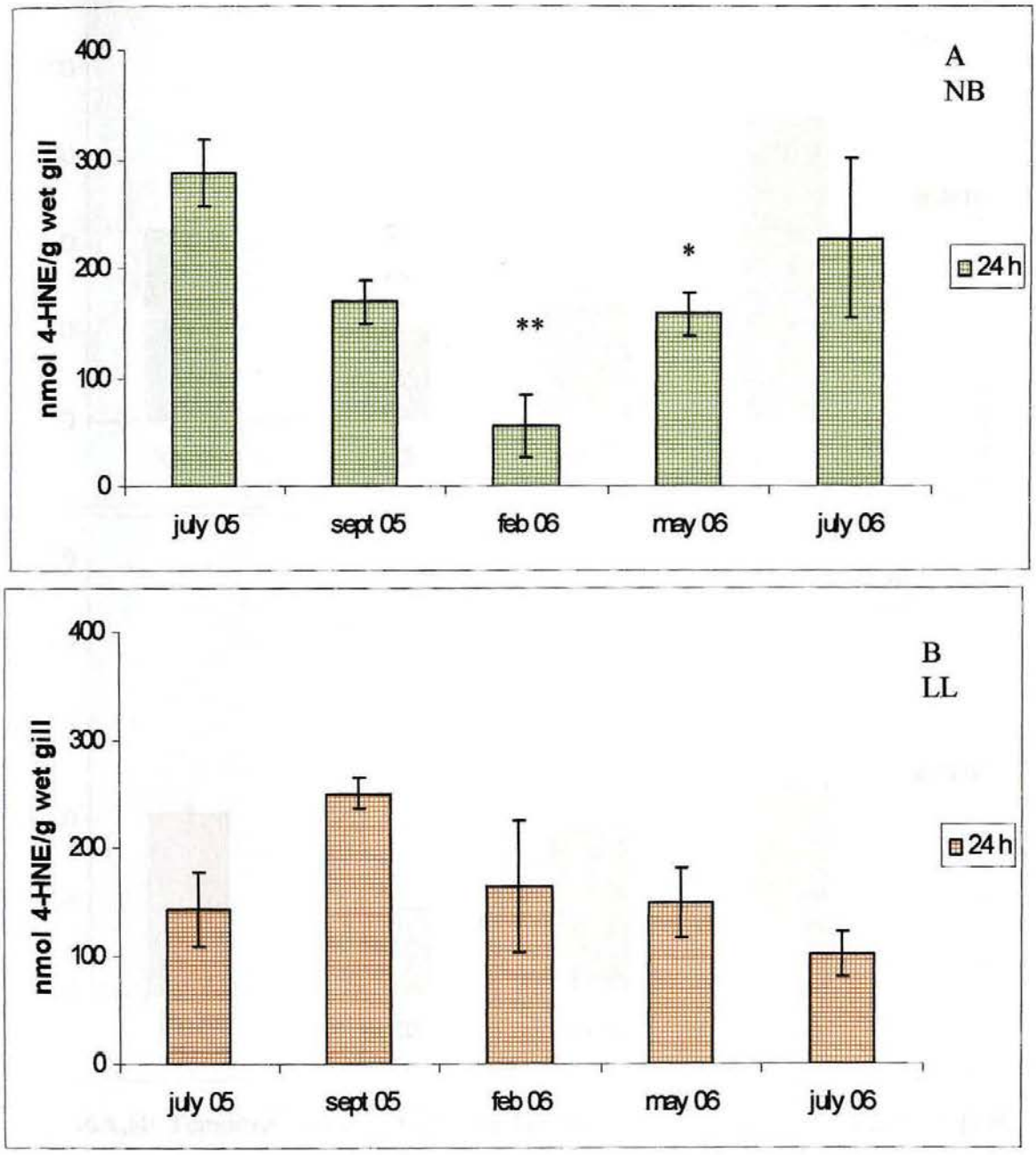


Figure 45A,B: Temporal trend of 4-HNE for NB and LL mussel gill respectively (24h samples) Values are the mean of 4 animals (± SEM) except for July 2005 and September 2005 (n=3). **45A:** * Significantly different to July 05 ($P<0.05$), ** Significantly different to July 05 ($P<0.01$), as assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test

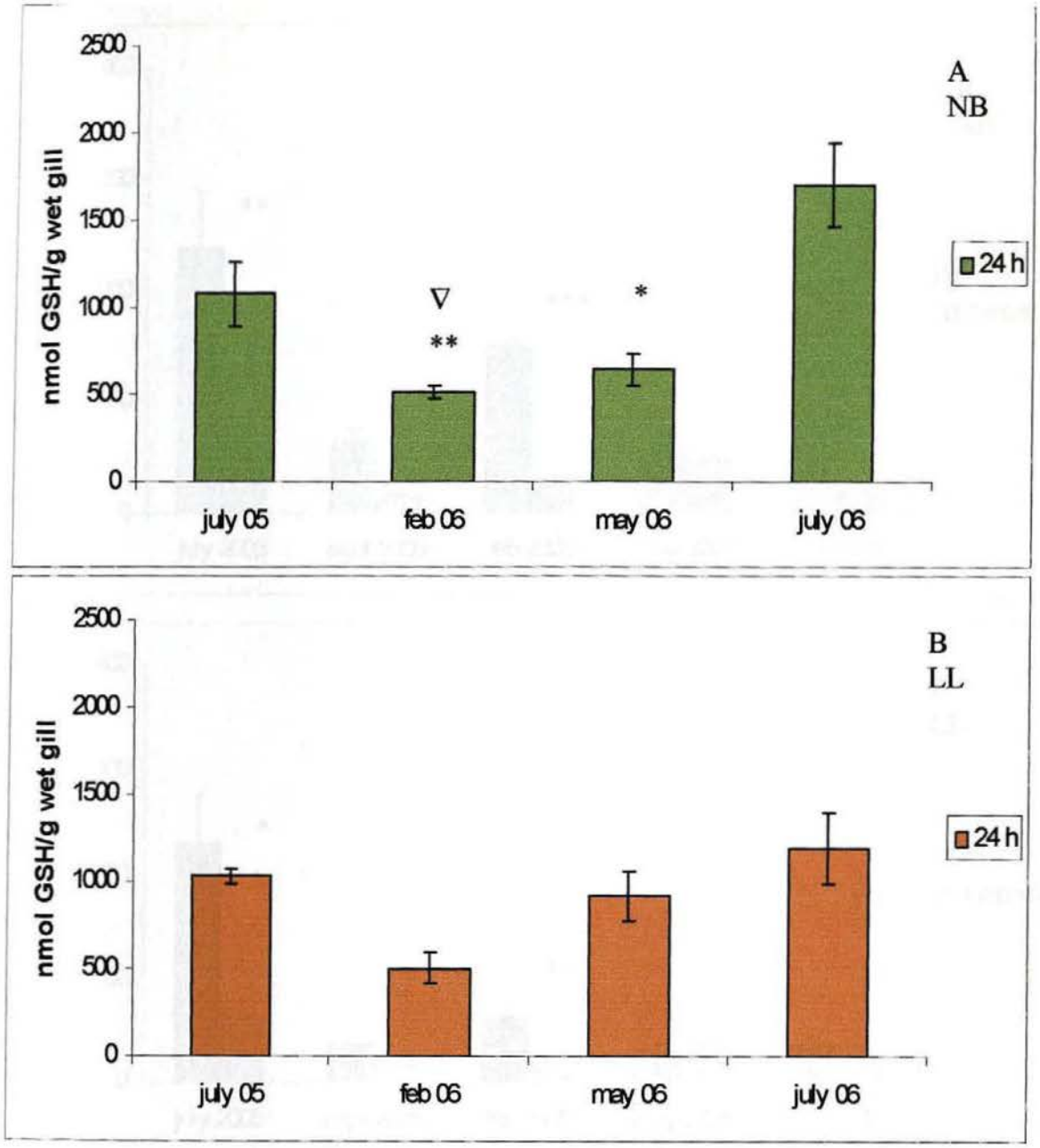


Figure 46A,B: Temporal trend of GSH for NB and LL mussel gill respectively (24h samples) Values are the mean of 4 animals (± SEM) except for May 2006 (n=3). **46A**: * Significantly different to July 06 ($P<0.05$), ** Significantly different to July 06 ($P<0.01$), ∇ Significantly different to July 05 ($P<0.05$), as assessed using a one-way ANOVA followed by a post-hoc Student's *t*-test.

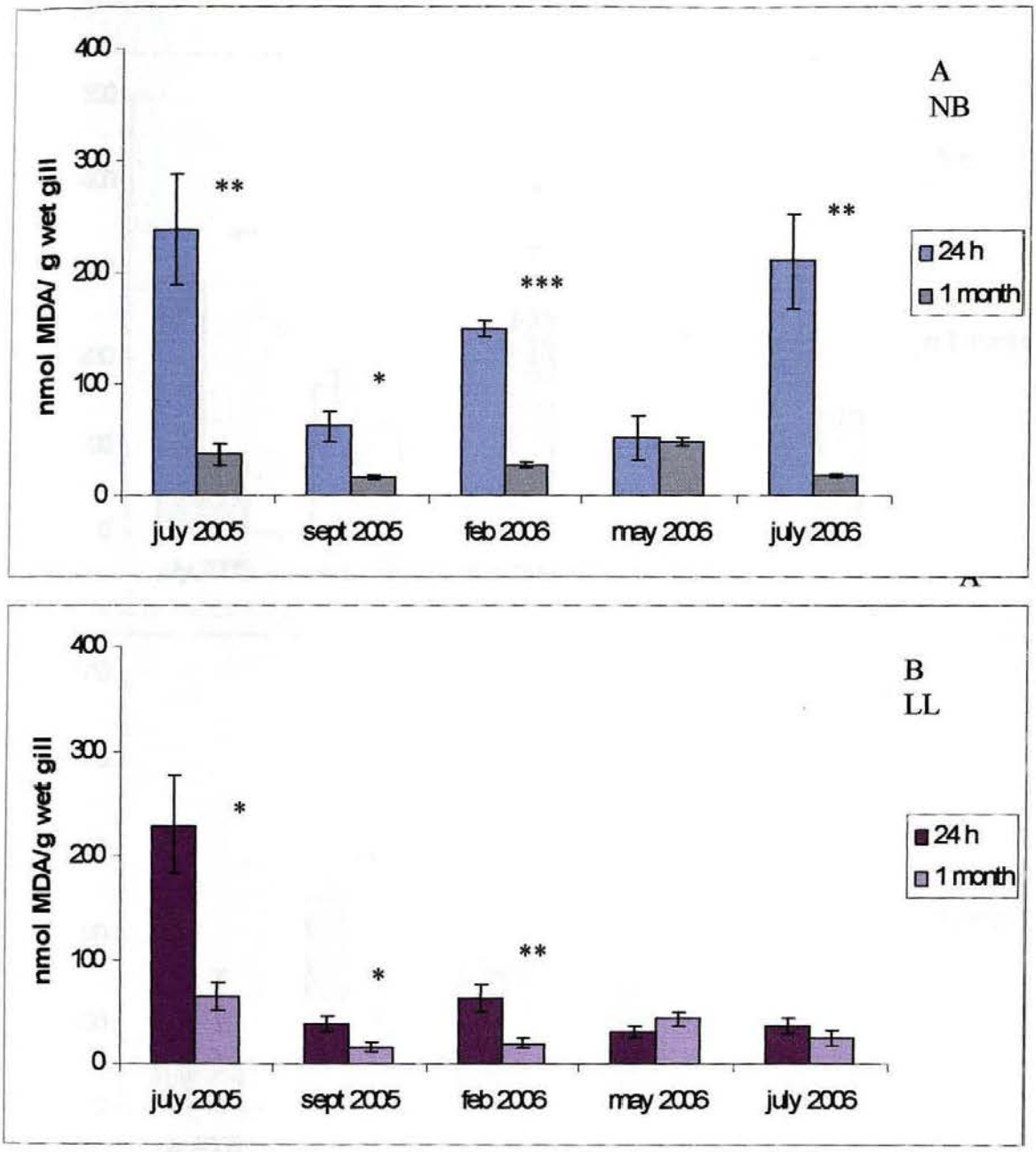


Figure 47A,B: Effect of one-month maintenance in laboratory conditions on MDA for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Values are the mean of 4 animals(± SEM) except for July 2005 (24 h), July 2005 (1 month), September 2005 (24 h) and September 2005 (1 month) (n=3). * Significantly different ($P<0.05$) between time-points as assessed using Student's *t*-test. ** ($P<0.01$), *** ($P<0.001$).

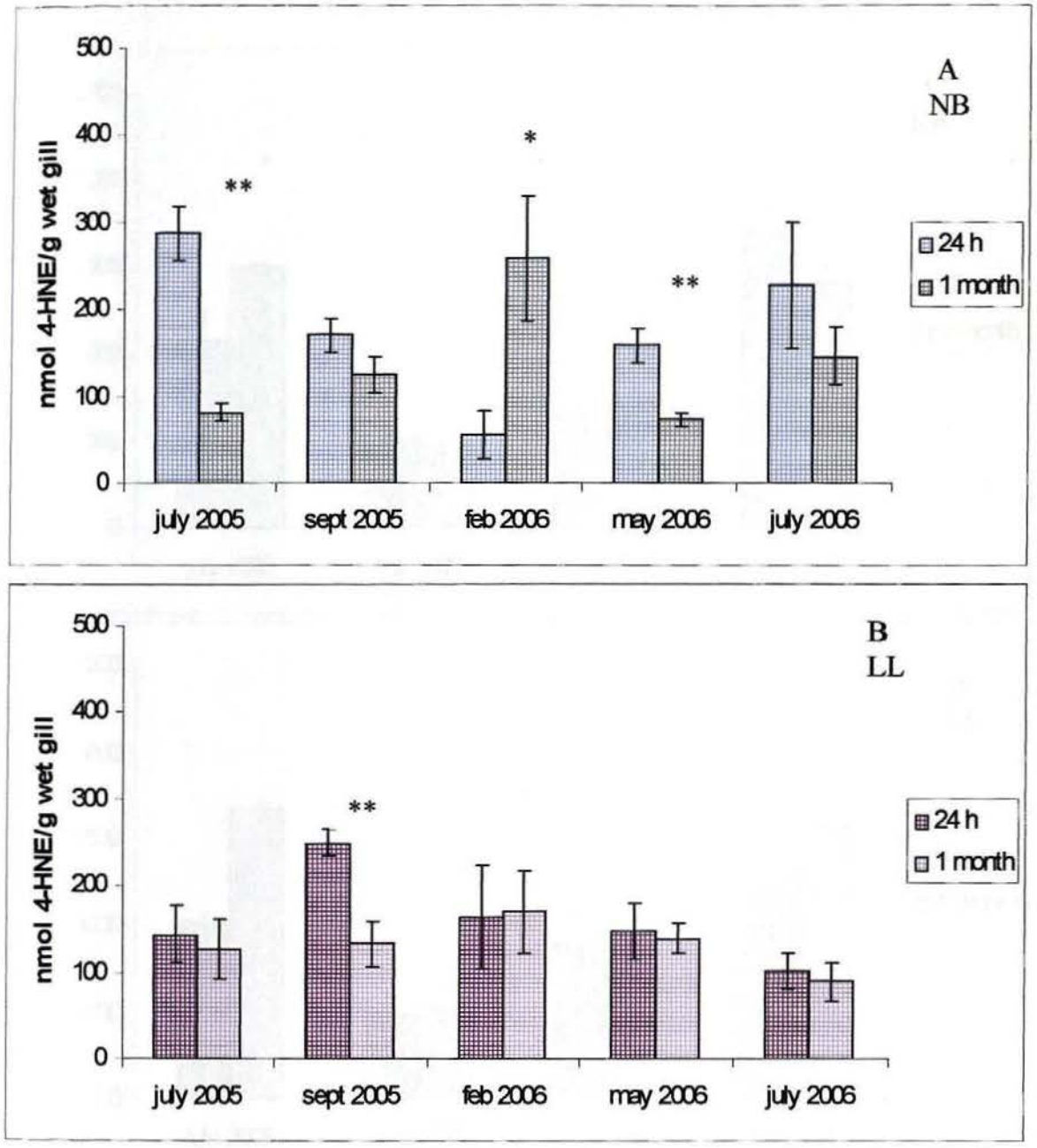


Figure 48A,B: Effect of one-month maintenance in laboratory conditions on 4-HNE for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Values are the mean of 4 animals (± SEM) except for July 2005 (24 h), July 2005 (1 month), September 2005 (24 h) and September 2005 (1 month) (n=3). * Significantly different ($P<0.05$) between time-points as assessed using Student's *t*-test. ** ($P<0.01$), *** ($P<0.001$).

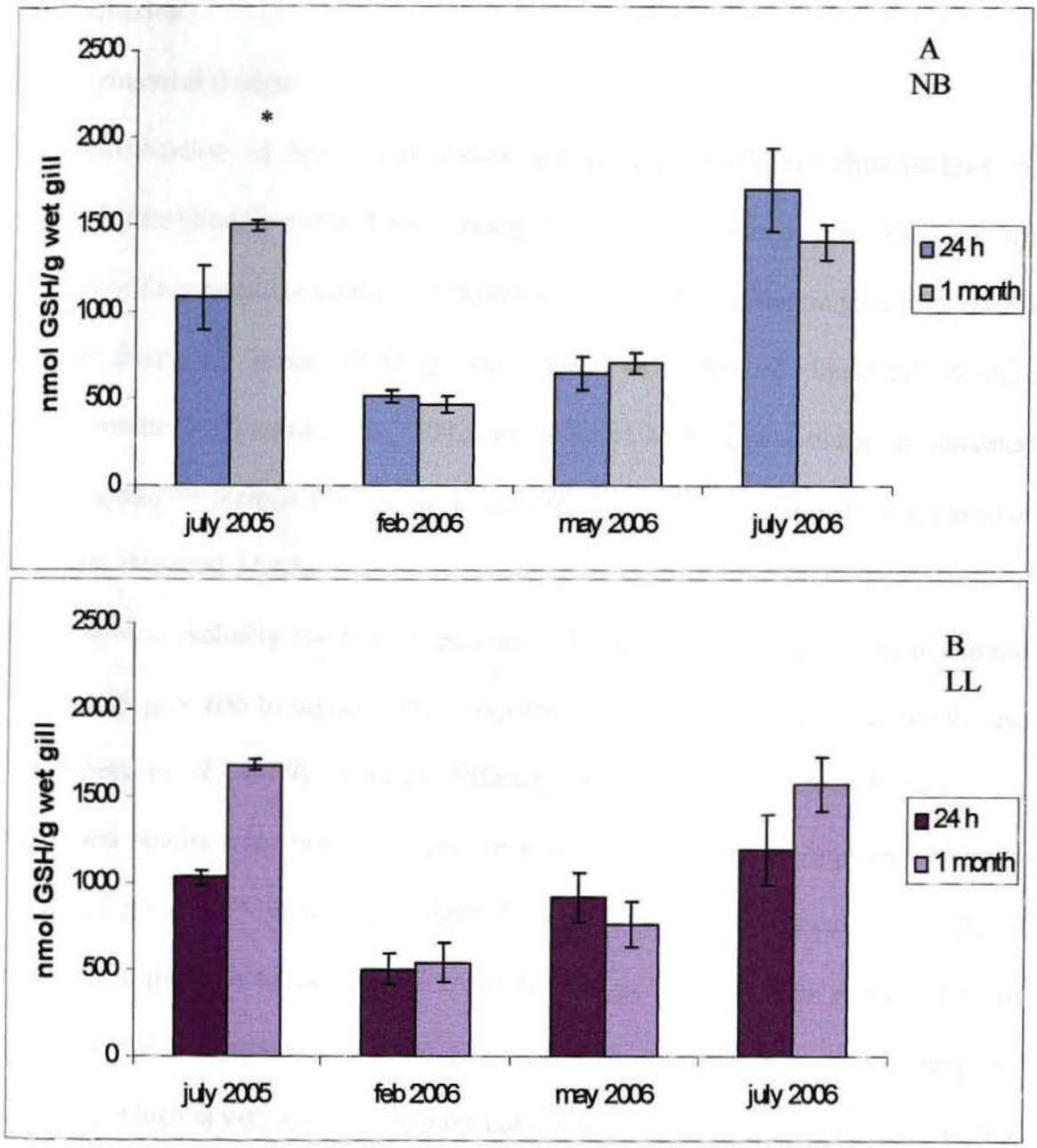


Figure 49A,B: Effect of one-month maintenance in laboratory conditions on GSH for NB and LL mussel gill (respectively) at various collections in the year 2005-2006. Values are the mean of 4 animals (± SEM) except for May 2006 (24 h) (n=3). * Significantly different ($P<0.05$) between time-points as assessed using Student's *t*-test.

5.4 Discussion

i) Experimental design

The derivatisation of lipid peroxidation end-products with the chromophore *N*-methyl-2-phenylindole method has already been cited in other papers. However the majority of them used the method on digestive gland cells and not on gills with results varying from 12 nmol MDA/g wet tissue in untreated intertidal *Mytilus galloprovincialis* (Durand *et al.*, 2002) to 40 nmol MDA/g wet tissue in untreated *Mytilus galloprovincialis* (Pampanin *et al.*, 2005) to 200 nmol MDA/g wet tissue at spring in untreated *Mytilus galloprovincialis* (Cavaletto *et al.*, 2002). The choice of this tissue was probably due to its high xenobiotic metabolising properties, which not surprisingly give rise to higher LPO compared to foot, adductor muscle, mantle and gill (Ribera *et al.*, 1989). Another difficulty regarding comparisons between our results and results from relevant literature was the expression of lipid end-products per mg of protein (Doyotte *et al.*, 1997; Romeo *et al.*, 2003; Gravato *et al.*, 2005) compared to per g of tissue. Gill MDA in *Mytilus galloprovincialis* according to an HPLC method was around 110 nmol/g of wet gill in untreated mussels (Viarengo *et al.*, 1996), which is very close to the average value (~92 nmol/g wet gill) of all of our "24 h" samples. No evaluation of 4-HNE in mussel gill has been found in the literature. Usefully, Shaw *et al.*, 2004, has calculated the total MDA+ 4-HNE burden via the same method, for NB populations and for reference specimens for Port Quin, Cornwall in 1998: The temporal variation was significantly different from ours however total LPO in NB varied from 200 nmol/g wet gill in winter and summer to 450 nmol/g wet gill in May and our results varied from 205 nmol/g wet gill in winter and May to 437 nmol/g wet gill in summer. In a similar way, Port Quin results were around 200 nmol/g wet gill throughout the year and our LL results varied from 136

nmol/g wet gill in summer to 225 nmol/g wet gill in winter (all "24 h" values). In conclusion, despite lack of very relevant literature, our results are within the expected values for the sites, the tissue and the species.

Reduced or total glutathione levels have been cited in a variety of mussel species. Regarding papers expressing the findings per g of tissue our values (500-1500 nmol/g wet gill) were higher than these reported by Power and Sheehan, (1996) for *Mytilus edulis* and by Regoli and Principato, (1995) for *Mytilus galloprovincialis* but there was no accordance between the aforementioned papers also. Our results were comparable to the findings of Regoli and Principato, (1995) for *Mytilus edulis* digestive gland, which in their turn were higher than these reported for *Mytilus galloprovincialis* by Viarengo *et al.*, (1991). Our results were also comparable with total GSH levels in *Perna perna* gill (~ 700 nmol/g wet gill) with oxidised GSH comprising only 15 nmol of the recorded value (Franco *et al.*, 2006). The validity of the OPT method was been verified in a number of studies in our laboratory and the interference of other thiols is minimal (Hisin and Hilf, 1976). However, HPLC quantification (e.g. Doyotte *et al.*, 1997) remains the most accurate method for GSH quantification until today.

ii) Temporal variability

There was a consistent trend of evidence for a temporal change in lipid peroxidation throughout the year in mussels from NB. This was evident from both MDA and 4-HNE measurements. At LL no such trend was evident although the July 2005 sampling gave a relatively high level of MDA which was not seen in July 2006 sampling. This observation may be a direct consequence of the seasonal fluctuations

of pollutant input in the estuary in question: a similar pattern of low TBT mussel burden at winter compared to summer was noted for Tamar estuary where up to eight-fold differences in seasonal water-flow are present between winter and summer (Sheehan and Power, 1999). A synergistic effect between genotoxic pollutants and temperature is also possible: a combination of pro-oxidants and heat is lethal for *Dreissena polymorpha* (Harrington *et al.*, 1997) and chemicals may be more genotoxic for bivalves (causing more SSB) at high temperatures (Buschini *et al.*, 2003) as already mentioned in Chapter 3, facilitating a generalised pro-oxidant situation. The temporal variability in our reference site was less pronounced, in accordance with Bocchetti and Regoli, (2006) results on MDA from reference *Mytilus galloprovincialis* populations and with Shaw et al., (2004) on MDA+ 4-HNE from reference *Mytilus edulis* populations.

Generally a trend for relatively high levels of GSH was found for all samples collected during the summer versus the winter and in only one set of samples did the GSH level alter following laboratory maintenance for one month. The apparent seasonal differences are in accordance with the findings of Power and Sheehan, (1996) on *Mytilus edulis*, Viarengo *et al.* (1989) on *Mytilus galloprovincialis* and Wilhelm Filho *et al.*, (2001) on *Perna perna*, however these differences were not pronounced and in the case of LL were not significant. Gill GSH is thought to follow a seasonal pattern but a less variable one in comparison with digestive gland (Power and Sheehan, 1996), which renders this tissue more appropriate for long-term studies on GSH.

iii) Differences between sites

Generally MDA levels were higher in NB populations compared to LL populations (for February and July 2006). These differences may be related to the different pollution status of the two sites. Metal exposure (Cd) has indeed caused LPO in the gill of *Bathymodiolus azoricus* (Company *et al.*, 2004) and indigenous mussels (*Mytilus galloprovincialis*) from a Cu and Zn polluted site showed higher MDA levels compared to a cleaner one (Funes *et al.*, 2005) Gill LPO was also elevated in *Elliptio complanata* transplanted to a municipal effluent after one year (Gagné *et al.*, 2004) and mussels heavily affected by PAH from the *Aegean Sea* oil spill had higher levels of MDA in digestive gland compared to reference specimens (Solé *et al.*, 1996). However, LPO had a low discriminating power between sites and did not correlate with any metals in indigenous mussel (*Dreissena polymorpha*) tissues (de Lafontaine *et al.*, 2000). In a similar manner, transplanted *Unio tumidus* showed LPO that was somewhat correlated with antioxidant enzyme depletion but not correlated with the metal pollution levels (Cossu *et al.*, 2000). Finally, a short (1 week) transplantation exposure in an effluent from steel and iron industries did not elevate MDA in *Unio tumidus* gills (Doyotte *et al.*, 1997). We have to note that the last two experiments were assessed with transplanted mussels, which does not take into account cumulative effects of pollution, however indigenous mussels may perform in a similar or even better way because of activation of protective mechanisms like metallothionein induction (Cossu *et al.*, 2000).

Overall no distinguishing differences between sites were found with respect to 4-HNE measurements, in accordance with de Lafontaine *et al.*, (2000) who ascribed low

discriminating power to lipid peroxidation together with other non-specific biomarkers of effect like DNA damage.

Regarding GSH levels no differences between sites were noted (two-way ANOVA, $P>0.05$), which suggests that oxidative stress in NB is not intense enough to deplete or up-regulate GSH levels in indigenous populations [see also 5.4 iv)].

iv) Differences between time-points

Laboratory maintenance had a significant effect on both NB and LL mussels lowering MDA levels in most cases, suggesting a decrease in the level of lipid peroxidation. This may signify an alleviation of environmental oxidative stressors like pollution and/or tidal oscillations (Almeida *et al.*, 2005). The latter apart from the anoxic-reoxygenation cycle it creates, brings the mussels closer to the lipid rich surface microlayer of the sea which contains high levels of PAH and other organic pollutants (Skarphéðinsdóttir *et al.*, 2005). Furthermore, intertidal mussels exhibit slower rates of depuration of pro-oxidant pollutants, because of the limited time contact with the seawater (Durand *et al.*, 2002). An alleviation of the combination "tidal oscillations-pollution burden" is probably the main reason for the decrease noted for NB samples whereas the continuous submersion under water may be the main reason for the less dramatic decrease noted for LL samples. However these decreases are not in accordance with the abundance of food in the aquarium, which would favour oxygen consumption (Bocchetti and Regoli, 2006) and thus lead to higher oxidative stress. Simultaneously though, food availability may lead to higher antioxidant supplementation and generally rise in temperature is more detrimental in ROS production than food availability (Bocchetti and Regoli, 2006). Again, rise in

temperature was evident in the laboratory maintenance of the February and maybe the September and May batch, which theoretically would lead to increased LPO. The relationship though is not straightforward because Wilhelm Filho *et al.*, (2001) noticed an increase in antioxidant defences with higher temperature and with more intense spawning activity, as a compensatory mechanism in *Perna perna* and Manduzio *et al.,* (2004) reached at the same conclusions with *Mytilus edulis*. Bocchetti and Regoli, (2006) also noted a higher TOSC towards peroxyl and hydroxyl radical at warmer waters even though some antioxidant parameters (GR) were then suppressed. On a quite different profile, if we take 4-HNE as a marker of lipid peroxidation, the remediation in the laboratory is much less pronounced. A particularly conflicting result was noted in the February 2006 sampling from NB, however a likely reason for this discrepancy is that 4-HNE measurements showed relatively high variability between animals. Thus, the balance among LPO end-products, temperature, food availability and pollution levels is an extremely complex one, especially in cases that (based on the GSH values) severe depletion of antioxidant defences is not present.

At this point we do not have enough information about reasons for the apparent differences of MDA and 4-HNE in pollution/depuration response and in their seasonal control. The pathogenesis of both aldehydes has a common start: the attack of a polyunsaturated fatty acid by ROS: This ultimately gives rise to highly active lipid oxyl radicals (LO·) which fragment to aldehydes and alkyl radicals or rearrange to epoxyallylic radicals. Both MDA and 4-HNE are results of fragmentation; MDA however is more hydrophilic than 4-HNE and it diffuses further (Hoff and O'Neil, 1993) so maybe the different patterns of the peroxides are partly due to a different

source of origin within the gill. Different rates of elevation between MDA and 4-HNE were also observed in plasma from patients with Alzheimer's disease: 4-HNE was particularly elevated compared to reference population whereas no differences were noted for MDA, which was within normal levels (McGrath *et al.*, 2001).

GSH levels were generally not elevated by laboratory maintenance with the exception of NB for July 2005 when a statistically significant elevation was noted ($P<0.05$, Student's *t*-test). This apparent "depletion" in the field of GSH may be a direct consequence of exposure to polluted environments (Cossu *et al.*, 1997; Torres *et al.*, 2004). Although an alternative explanation is that laboratory conditions had "induced" GSH levels this effect was not observed with LL samples under the same conditions. In contrast, De Luca-Abbot *et al.*, (2005) found an increase in GSH of *Perna viridis* gill after transplantation in sites with high PAH and PCB burden. However, this increase did not correlate with the tissue values of the pollutants. Other authors have found a decrease in GSH levels and GR activity after transplantation, as noted in gills of *Unio tumidus* transferred to sites polluted with PAH, PCB and metals (Cossu *et al.*, 1997). Regoli and Principato, (1995) also argued that metal pollution reduces GSH in both native and transplanted *Mytilus galloprovincialis* even though GR activity was reduced only in the latter. As already mentioned, there was no overall difference in GSH levels between sites neither at 24 h post-collection nor after one-month laboratory maintenance for the months tested. At the same time, there is probably a causative relationship between high 8-oxo-dG and 4-HNE and low GSH and 8-oxo-dG excision capacity for NB mussels for July 2005 (see also Chapter 7).

In conclusion, dissimilarities in MDA and 4-HNE levels between sites emerged at some of the mussel collections of the year 2005-2006. Differences between time-points ("24 hours", "1 month") were also evident. In contrast there were no differences in GSH levels between sites and slight differences between time-points. A collective evaluation of an *in vivo* metal exposure on the parameters described in Chapters 3, 4 and 5 is discussed in the next chapter.

6.0 *In vivo* exposure of mussels (Mytilus *edulis*) to Cd and Cr(VI)

6.1 Introduction

Bivalve molluscs are filter feeding organisms which bioaccumulate a variety of pollutants (Gomez-Mendikute and Cajaraville, 2003), including heavy metals. Mussels have been shown to bioaccumulate Ag, Cr, Ni, Se, Ti, Sn, Hg, As, Cu, Pb and Zn (Cantillo *et al.*, 1998; Franco *et al.*, 2002; Kehrig *et al.*, 2002; Yap *et al.*, 2004; Chiffoleau *et al.*, 2005; Orescanin *et al.*, 2006) among others, from their surrounding environments. These metals may have a detrimental effect on bivalves through interference with a range of biological and biochemical pathways. For example, Cd and Cu elicited immunotoxic responses in mussels (Gomez-Mendikute and Cajaraville, 2003; Nicholson, 2003), Cu and Hg lowered heart and filtration rate in bivalves (Stuijfzand *et al.*, 1995) and Cu and organic tin compounds caused apoptosis in mussel haemocytes (Micic *et al.*, 2002). Many of these metals are able to affect DNA integrity by induction of SSB (Black *et al.*, 1996; Bolognesi *et al.*, 1999; Emmanouil *et al.*, 2006), induction of micronuclei (Mersch *et al.*, 1996; Bolognesi *et al.*, 1999) or even gross chromosomal alterations (Chipman and Marsh, 1991). The possibility that metals may affect DNA repair mechanisms in bivalves is still unknown. It has been shown in humans that ROS-induced chronic inflammation may increase the activities and/or the expression of repair enzymes like OGG1, APE1 and AAG (Powell *et al.*, 2005). On the contrary, metals like Ni can dramatically inhibit DNA ligases in CHO cells (Lynn *et al.*, 1997b) and Fpg activity *in vitro* (Hartwig, 1998). Cr(VI) was also shown to inhibit OGG1 activity and expression in human lung carcinoma cells (Hodges and Chipman, 2002) and Cd inhibited Fpg activity *in vitro* (Asmuß *et al.*, 2000) and OGG1 activity in rat epithelial cells (Potts *et al.*, 2001). Thus implication of Cd or Hg (which were found to be elevated in Merseyside) to the

suppressed 8-oxo-dG excision activity noted for New Brighton samples is an interesting possibility. The aim of the present studies was to investigate whether metals can cause oxidative macromolecule damage in mussel gill and if interference with repair mechanisms for oxidative DNA damage contributes to the aforementioned damage.

Cadmium was an ideal candidate for *in vivo* experiments because of it is widely used (Waisberg *et al.*, 2003), it is not an essential metal and it exhibits an extremely long half-life (McMurray and Tainer, 2003). Also there are indications of interference with repair of oxidative damage (Hartwig, 1998; Zharkov and Rosenquist, 2002; Potts *et al.*, 2003). Furthermore, mussels are able to bioaccumulate Cd (Serra *et al.*, 1999; Erk *et al.*, 2005).

Chromium is another widespread metal in the environment because of its various uses in industry (Dana Devi *et al.*, 2001). Chromium is a well-known human carcinogen and a model genotoxic agent for oxidative DNA damage. There are also indications of interference with removal of oxidative damage since chromium decreased hOGG1 activity, OGG1 mRNA and protein expression in human cells (Hodges and Chipman, 2002) and OGG1 activity in rat lungs (Maeng *et al.*, 2003). Cr(VI) is accumulated in mussel tissue (Walsh and O'Halloran, 1997) and it is not readily reduced to its trivalent form in aquatic environments unless large sediment concentrations are present (Mayer and Schick, 1981; Wang *et al.*, 1997).

In this study groups of 25 animals (after overnight acclimatisation) were exposed to two concentrations of Cd or Cr(VI) for 10 and 7 days respectively. Animals were

examined for DNA oxidative damage and 8-oxo-dG and ethenoadenosine repair capacity. Lipid peroxidation end-products were also measured as an indicator of general oxidative stress. Metal accumulation was verified by inductively-coupled plasma mass spectrometry (ICP-MS) in foot and digestive gland tissue. Assessment of intracellular ATP was also performed to verify lack of metal-mediated cytotoxicity.

6.2 Materials and methods

6.2.1 Reagents: see 2.2.1

6.2.2 Buffers

Cd stock solution for 200 μg/l: 6.53 mg/ml $CdCl_2$

Cd stock solution for 10 μg/l: 0.33 mg/ml $CdCl_2$

Cr(VI) stock solution for 200 μg/l: 11.32 mg/ml $K_2Cr_2O_7$

Cr(VI) stock solution for 10 μg/l: 0.57 mg/ml $K_2Cr_2O_7$

Comet assay buffers: see 3.2.2

Endonuclease nicking assay buffers: see 4.2.2

LPO evaluation buffers: see 5.2.2

6.2.3 Animal husbandry: see 2.2.3.

6.2.4 Soft tissue metal determination

At least 3 animals from each exposure group (control, 10 μg/l and 200 μg/l) were chosen after the end of the exposure period and sacrificed for chemical analyses. Digestive glands and foot tissue were collected with plastic tweezers and snap frozen in liquid nitrogen. Additional samples from the 24-h acclimatisation non-treated group were analysed in order to establish background levels of the metals.

Measurement of accumulation of metals in mussel soft tissue was performed by Mr T.M.T. Sheehan (Regional Toxicology Laboratory, Birmingham City Hospital, Birmingham, UK).

6.2.5 Assessment of intracellular ATP

Lack of cytotoxicity was verified by intracellular ATP assessment (Bioluminescent Assay Kit, Sigma, UK) according to the manufacturer's instructions. Briefly, the animal valves were opened with a metal scalpel (17 cm) and gill tissues were extracted with small size metal tweezers. The tissue was dried of excess water, cut in smaller pieces and weighed accurately. The tissue was thoroughly homogenised in somatic ATP releasing agent (2.0 ml) at 0° C by using the dispersing instrument Ultra Turrax T8 (IKA Labortechnik, Germany) at setting 12 for 2 min and left on ice for 1 h. The homogenate (100 µl) was added to an opaque 96-well plate (Costar, UK) and firefly luciferase enzyme (100 µl, 1:25 dilution) was added to the well. Light emitted was immediately measured with a luminometer (Spectra Fluor Plus, Tecan, UK). Results were normalized to mass of wet tissue.

6.2.6 Comet assay: see 3.2.3, 3.2.6-3.2.8.

6.2.7 Lipid peroxidation evaluation: see 5.2.3-5.2.4.

6.2.8 8-oxo-dG excision assay: see 4.2.3-4.2.7.

6.2.9 εA excision assay: As described in 6.2.8 with a ^{32}P-radiolabeled (21 mer) duplex probe containing ethenoadenosine at position 13 (R&D Systems, US).

6.3 Results

-Heavy metals analyses

Control mussels showed relatively low metal concentrations in their tissues at both time points: within 24 hours acclimatisation and after 7 and 10 days. Concentrations of both cadmium and chromium increased in soft tissues of *Mytilus edulis* (digestive gland) in a dose dependent manner following exposure (**Table 8**). As further support of exposure, cadmium concentrations were also measured in mussel foot tissues.

-Assessment of toxicity

Cadmium did not cause a statistically significant decrease in intracellular ATP for any treatment group (one-way ANOVA) as depicted in **Figure 50**. Chromium also did not cause a statistically significant decrease in intracellular ATP as depicted in **Figure 50.** However there were some responses noted in treatment groups such as discharge of profuse amounts of mucus and minimal secretion of byssus threads (Nicholson, 2003), reduced feeding rates and an increase in occasional observation of closed shells especially in the Cr(VI) treated groups.

- Comet assay

Cadmium caused a dose-related increase in levels of frank SSB (**Figure 51A**), which was statistically significant at the high dose ($P<0.01$, one-way ANOVA for the medians accompanied by a *post-hoc* Student's *t*-test), whereas there was not a significant increase in Fpg-sensitive sites (indicative of oxidative damage) (**Figure 51A**) in both treatment groups. Chromium also caused a dose-related increase in levels of frank SSB (**Figure 51B**), which was statistically significant both in the low

dose and in the high dose ($P<0.01$, $P<0.001$ respectively). Again there was not a significant increase in Fpg-sensitive sites (**Figure 51B**) in both treatment groups.

-Lipid peroxidation evaluation

Cadmium caused a statistically significant increase in the high dose group (**Figure 52A**) of both MDA ($P<0.05$, one-way ANOVA accompanied by a *post-hoc* Student's *t*-test) and 4-HNE ($P<0.01$), compared to control animals. Chromium in contrast caused only a slight and not significant increase (**Figure 52B**) in the high dose group for both MDA and 4-HNE compared to control animals ($P=0.052$ for MDA).

-8 –oxo-dG and εA excision evidence in mussel gill

Nuclear extracts from Cd or Cr(VI) treated animals were able to excise an 8-oxo-dG probe or an εA probe as shown in Figure **53A,B,C** and **D** respectively.

-Endonuclease nicking assay

Cadmium caused a statistically significant decrease of the high dose in 8-oxo-dG excision capacity (**Figure 54A**) compared to control animals ($P<0.05$, one-way ANOVA accompanied by a *post-hoc* Student's *t*-test) whereas chromium did not cause a significant decrease in 8-oxo-dG excision capacity (**Figure 54B**) in both treatment groups. Regarding εA cutting capacity cadmium did not cause a statistically significant decrease (**Figure 55A**) in both treatment groups whereas chromium did cause a marked and statistically significant decrease (**Figure 55B**) in the high dose compared to control animals ($P<0.05$).

Mussel no.	24 hrs control	10 days control	10 days 10 µg/l	10 days 200 µg/l
Cd				
1	0.40	0.26	1.42	54.9
2	0.14	0.19	1.65	32.7
3	0.17	0.22	1.69	30.5
4		0.20	2.42	36.7
Mean	*0.24 ± 0.14*	*0.22 ± 0.03*	*1.80 ± 0.43*	*38.70 ± 11.10*
	24 hrs control	7 days control	7 days 10 µg/l	7 days 200 µg/l
Cr				
1	0.45	0.31	0.32	14.93
2	0.62	0.32	0.53	15.05
3	0.44	0.37	4.38	15.09
4	0.45	0.24	1.27	12.91
5			6.98	15.22
Mean	*0.49 ± 0.09*	*0.31 ± 0.05*	*2.70 ± 2.90*	*14.64 ± 0.97*

Table 8: Cadmium and chromium accumulation (µg/g wet tissue) in mussel digestive gland at beginning of experiment (24 h) and after 10 days exposure to 10 µg/l and 200 µg/l Cd or after 7 days exposure to 10 µg/l and 200 µg/l Cr(VI). Additional data for foot tissue after exposure to Cd are as following: (0.10 ± 0.08, 0.69 ± 0.26 and 14.03 ± 3.38 µg Cd/g wet tissue for 10 day controls, 10 µg/l and 200 µg/l respectively.

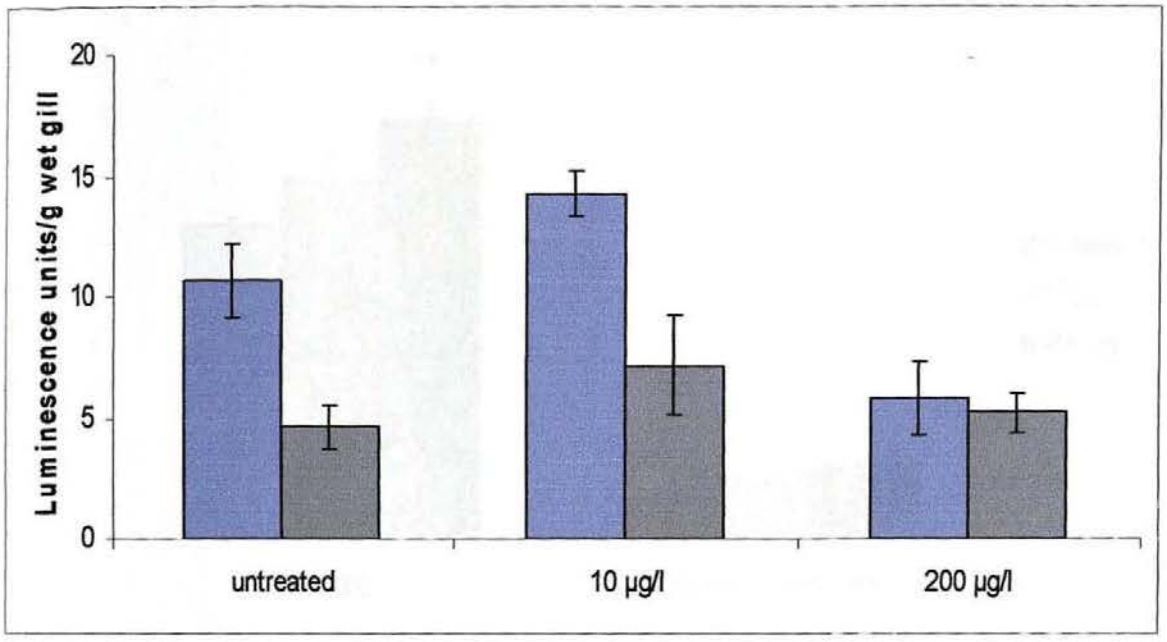


Figure 50: Levels of intracellular ATP in mussel gill. Blue bars-cadmium exposures, grey bars-chromium exposures. Values are the mean of 4 animals (± SEM). No statistically significant difference between control and treated groups.

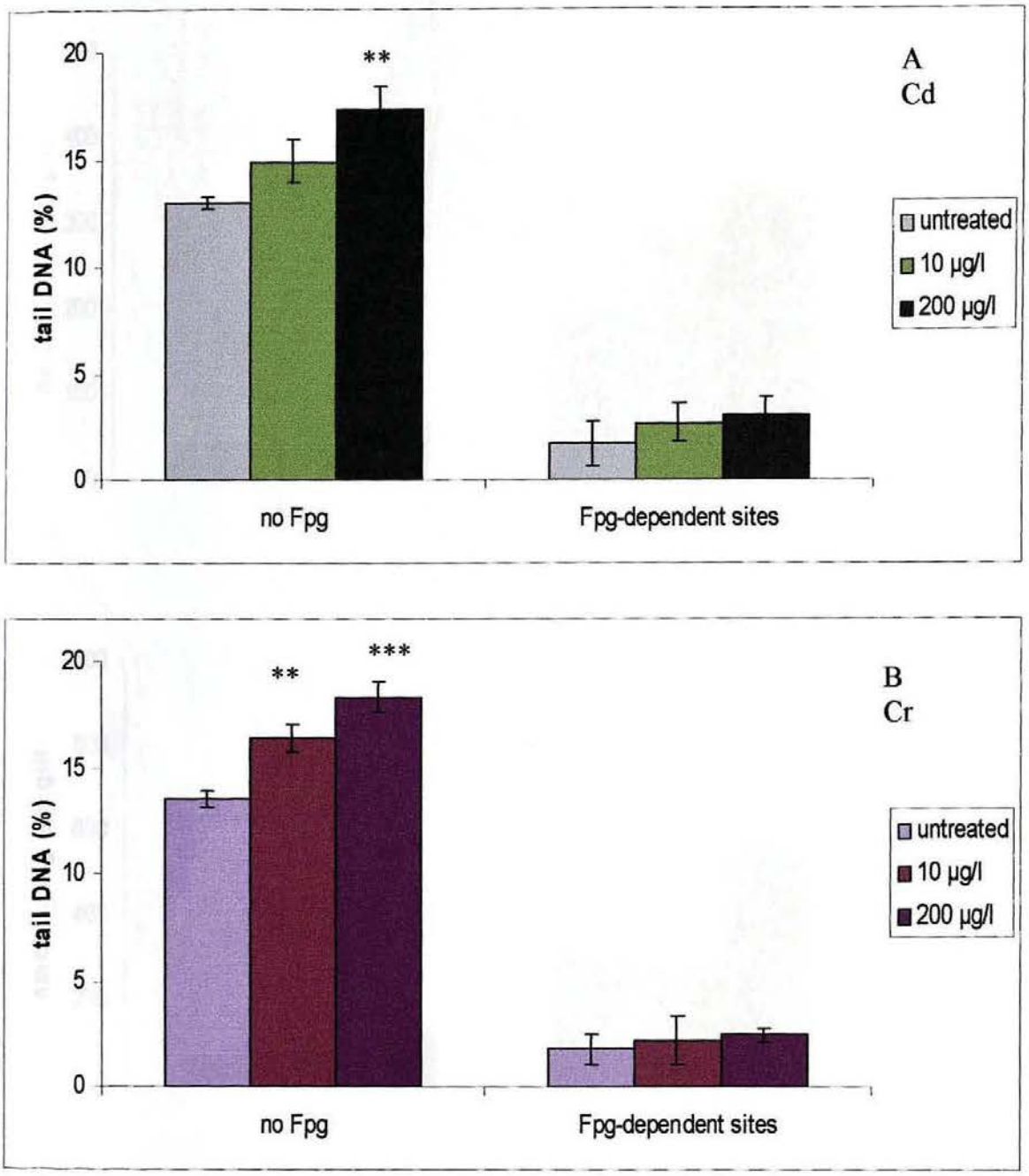


Figure 51A,B: Effect of Cd (A) and Cr(VI) (B) exposure on frank SSB and Fpg-sensitive sites after 10 or 7 days of exposure respectively. Values are the mean of 4 animals (± SEM). **Significantly different ($P<0.01$) compared to corresponding control values as assessed using a one-way ANOVA for median values followed by a *post-hoc* Student's *t*-test. *** ($P<0.001$).

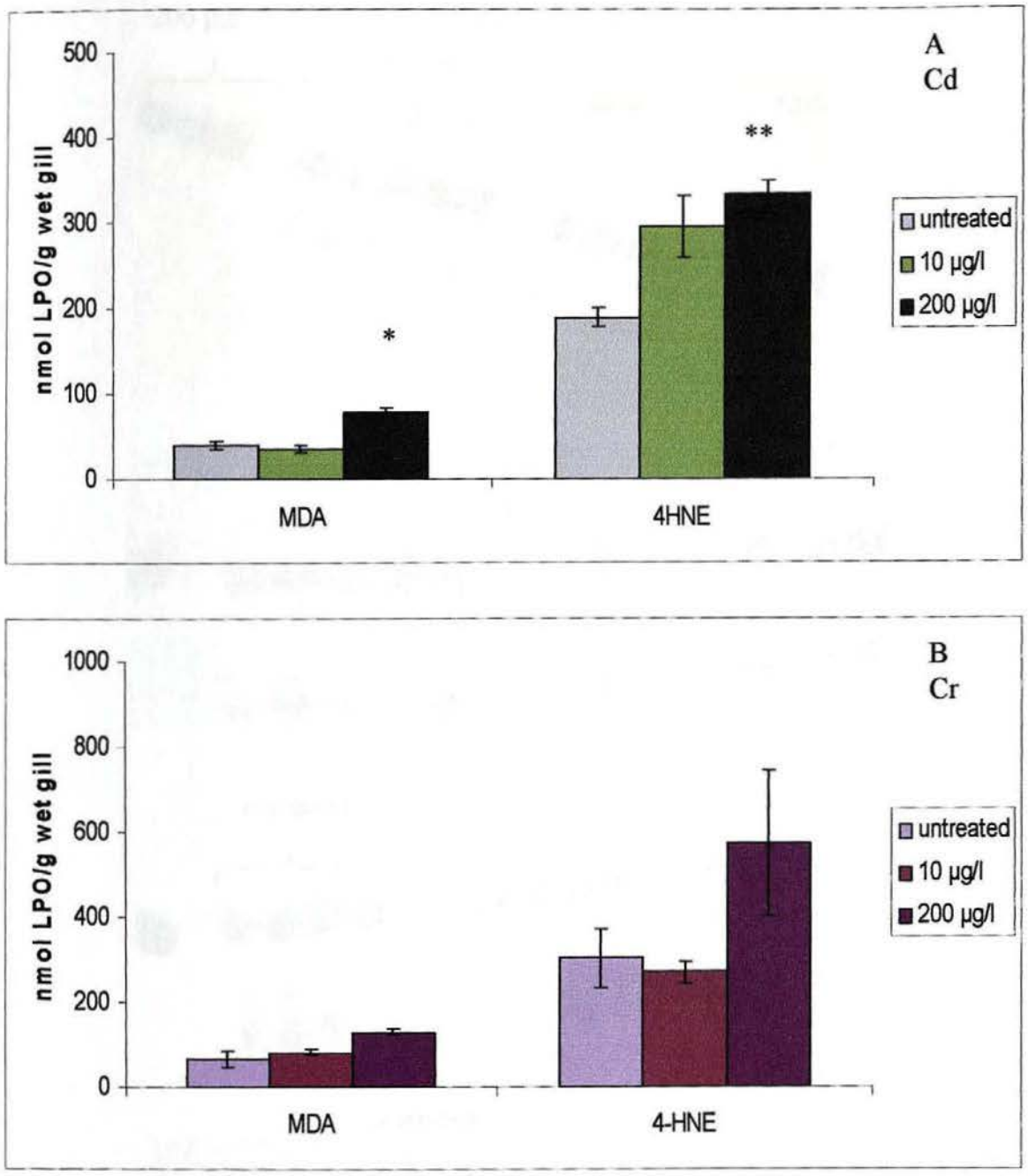


Figure 52A,B: Effect of Cd (A) and Cr(VI) (B) exposure MDA and 4-HNE after 10 or 7 days of exposure respectively. Values are the mean of 4 animals (± SEM) for Cd exposures and of 3 animals (± SEM) for Cr(VI) exposures. * Significantly different ($P<0.05$) compared to corresponding control values as assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test. ** ($P<0.01$).

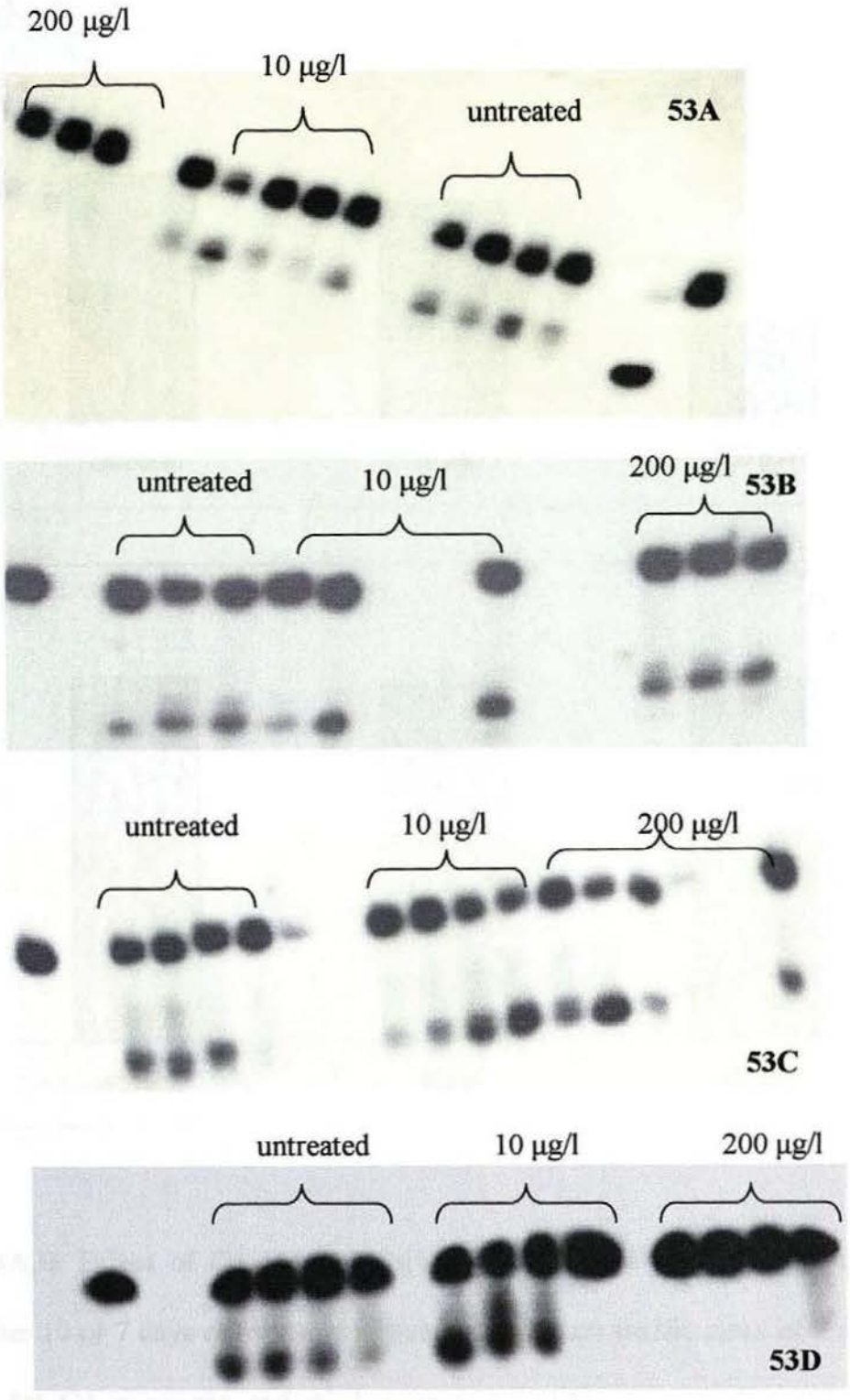


Figure **53A,B**: Blots of excised ^{32}P-labeled 8-oxo-dG probe after incubation with Cd and Cr(VI) treated mussel extracts respectively. **53C,D**: Blots of excised ^{32}P-labeled εA probe after incubation with Cd and Cr(VI) treated mussel extracts respectively.

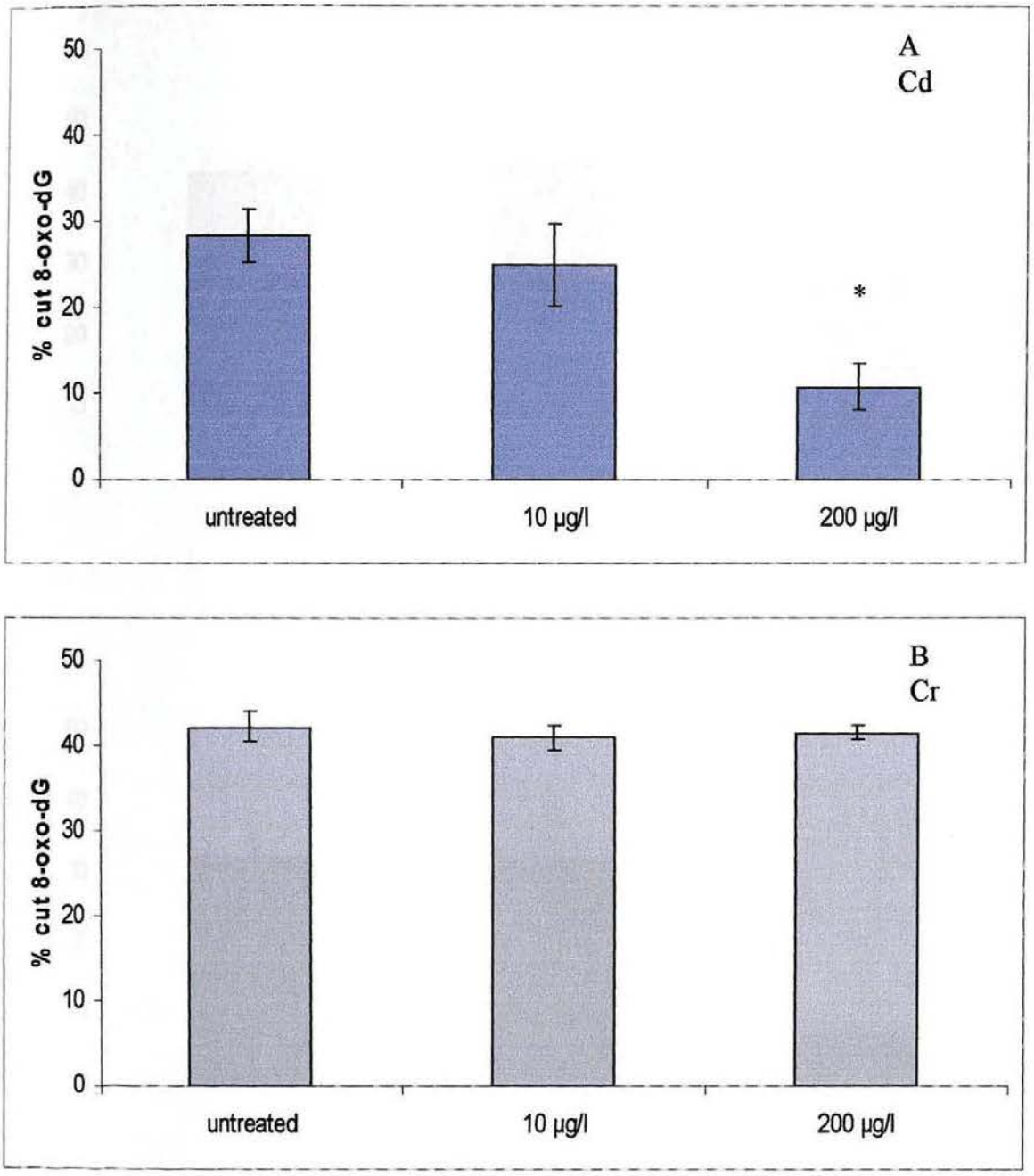


Figure 54A,B: Effect of Cd (A) and Cr(VI) (B) exposure on 8-oxo-dG excision activity after 10 or 7 days of exposure respectively. Values are the mean of 4 animals (± SEM) for Cd exposures and of 3 animals (± SEM) for Cr(VI) exposures. * Significantly different ($P<0.05$) compared to control values as assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test.

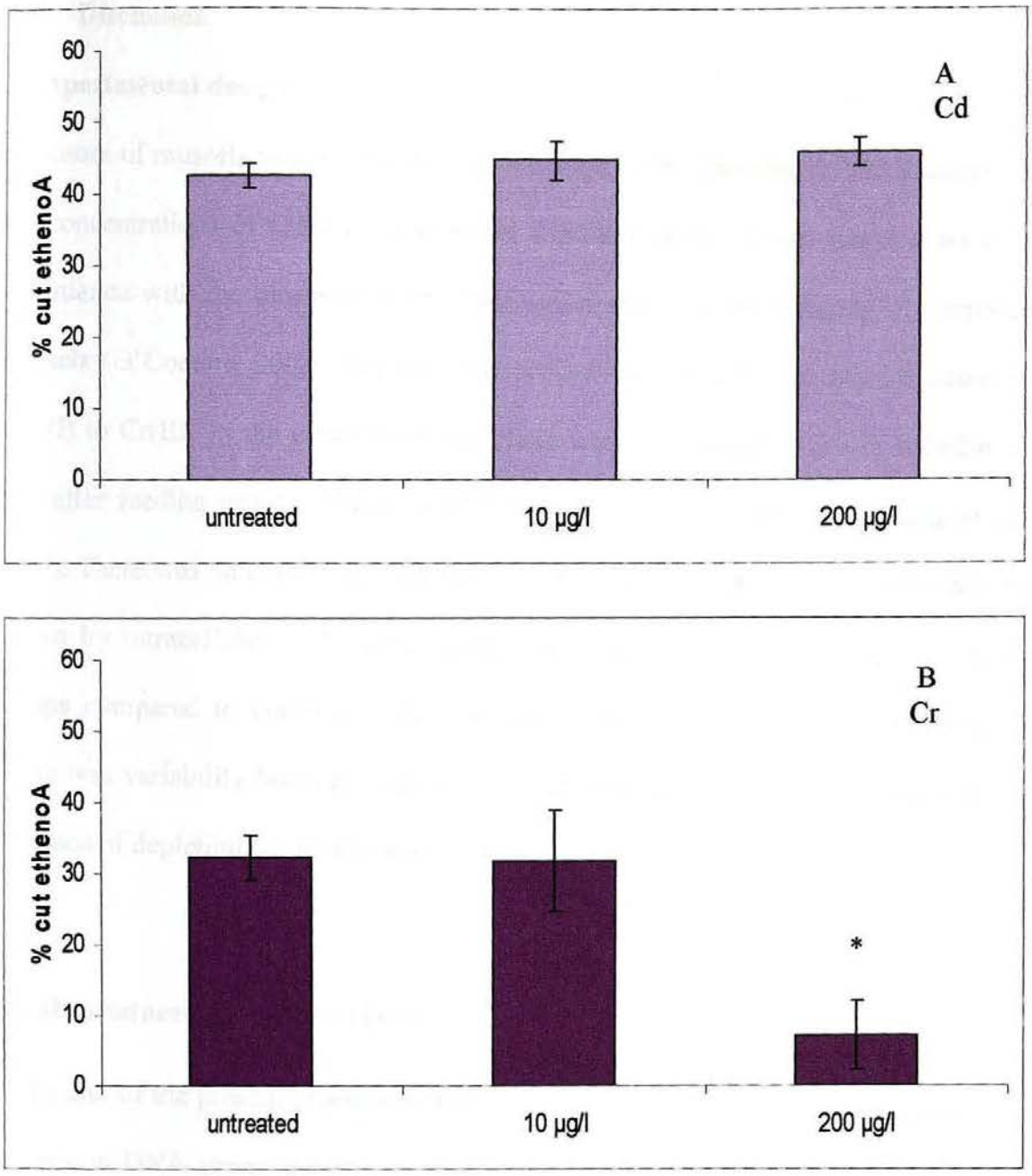


Figure 55A,B: Effect of Cd (A) and Cr(VI) (B) exposure on A excision activity after 10 or 7 days of exposure respectively. Values are the mean of 4 animals for Cd and of 3 animals for Cr(VI). * Significantly different ($P<0.05$) compared to control values as assessed using a one-way ANOVA followed by a *post-hoc* Student's *t*-test.

6.4 Discussion

i) Experimental design

Exposure of mussels to both metals was indicated by the dose-dependent increases in the concentrations of Cd and Cr in either digestive gland or foot tissue. This is in accordance with the observation of metal accumulation in environmentally exposed mussels (O'Connor, 2002). We took the precautions to minimise any reduction of Cr(VI) to Cr(III) in the exposure water (daily water change, addition of solution 30 min after feeding time) and thus, Cr(VI) bioavailability was enabled (Wang *et al.*, 1997). There was no cytotoxic confounding for exposure to cadmium or chromium as shown by intracellular ATP levels, which were not statistically reduced in treated groups compared to controls. GSH was also measured but data are not included. There was variability between samples that hampered comparisons but there was no evidence of depletion by the metal exposures.

ii) SSB produced by Cd or Cr(VI)

At the end of the present exposures, both metals produced a concentration dependent increase in DNA strand breaks in gill cells. SSB (as measured by the Comet assay) may have many origins: they are frank SSB associated with DNA damage, alkali labile sites transformed into SSB in alkaline environment and transient SSB associated with incomplete excision repair of lesions rectified by a variety of repair pathways in the cell (Tice *et al.*, 2000). Even though this uncertainty of origin exists, the Comet assay has been extensively used as a non-specific measure of genotoxic damage in aquatic organisms (Mitchelmore and Chipman, 1998). Cadmium is regarded as a weak mutagen [Lynn *et al.*, 1997, Chapter 1.8.2.3i)]. Regarding Cd-produced SSB in mammalian cells, these occurred only at high, cytotoxic

concentrations (Beyersmann and Hechtenberg, 1997; Filipic et al., 2006). Cadmium did however cause significant SSB in HeLa cells (and most interestingly without concomitant increase in 8-oxo-dG) (Hartwig, 1998) and in the haemolymph of *Mytilus galloprovinciallis* after 5 days of exposure at 112 μg Cd/l (Bolognesi *et al.*, 1999). On the contrary, Pruski and Dixon, (2002) failed to observe increase in SSB in mussels even after 4 weeks of exposure at 200 μg Cd/l but they noted that Cd caused an increase in SSB after co-exposure with H_2O_2. H_2O_2 is a pro-oxidant, which can produce hydroxyl radicals by the Fenton reaction. The authors assumed that the accumulation of SSB evident in their (unmodified) Comet was due to the inhibition of the final step of ligation of BER after the excision of the oxidized lesions. It is true that Cd can interfere with the final ligation step of BER by inactivation of the enzyme(s) taking part (Lynn *et al.*, 1997) thus leading to an accumulation of the intermediate SSB. Bivalve DNA is subjected to considerable amounts of oxidative damage evident by levels of 8-oxo-dG, which generally are higher than those commonly observed in mammals (de Almeida *et al.*, 2003). Suppression of the final ligation step of BER therefore could enhance the accumulation of intermediate SSB even without additional oxidative insult. Since the suppression of ligation by Cd has been proven to be more sensitive than the suppression of excision (Lynn *et al.*, 1997), a simultaneous accumulation of 8-oxo-dG is not a requirement. This may be the case in the present experiment where the suppression of 8-oxo-dG evident in our *in vitro* excision assay was not accompanied by a statistically significant increase in 8-oxo-dG levels *in vivo* evident by the Fpg-modified comet assay. The ratio of direct SSB versus "frozen" repair-intermediates caused by Cd still needs to be addressed. The effects of Cd on DNA repair are discussed later in this section.

Cr(VI) produced a profound increase in SSB both at the low and the high dose. Cr(VI) is a model oxidative genotoxicant which produces a suite of DNA lesions during its intracellular reduction to Cr(III) mainly via low molecular weight thiols (O'Brien et al., 2003). SSB are mostly created by the hydrogen abstraction from C4′ of the sugar moiety which ultimately gives rise either to an alkali labile site or to SSB with a 3′-phosphoglycolate and a 5′-phosphate end (Casadevall *et al.*, 1999). This direct oxidation is performed by high valency oxochromium species and does not involve pseudo-Fenton reactions (Kortenkamp *et al.*, 1996). GSH exhibits separate and conflicting characteristics in the toxicity of Cr(VI) compounds because of its role in the reductive activation of Cr(VI) and as an antioxidant (Chipman *et al.*, 2006).

iii) 8-oxo-dG produced by Cd or Cr(VI)

Given the oxidative potential of Cd (Filipic *et al.*, 2006) and especially Cr(VI) it is rather surprising that no increase in Fpg-sensitive sites was recorded following long-term exposure since this enzyme is active against 8-oxo-dG, its reduced form formamidopyrimidine, other damaged purines and ring opened N^7 guanine adducts (Speit *et al.*, 2004). Acute Cr(VI) insult on the other hand, in the form of injection with 10.4 μg Cr(VI) to NB and LL mussels produced a profound increase both in SSB and 8-oxo-dG just 4 h post-injection, with extensive Fpg-sensitive sites (Chapter 3). This acute experiment proves that Cr(VI) can indeed cause oxidative damage in mussel gill. However, oxidative damage from prolonged exposure at lower concentrations of water-borne Cr(VI) may have adequately been repaired or adaptive protective systems may have been induced during that time. It is interesting to note that 5 μg of gill nuclear protein had a (maximum) activity during the course of these experiments comparable to 1.35 units of purified Fpg in cleaving an 8-oxo-dG

containing probe. Furthermore, when the glycosylase was suppressed, as in the case of high Cd group, a small but statistically not significant increase in 8-oxo-dG was noted (1.75 ± 1.81 compared to 3.01 ± 1.57 % tail DNA respectively).

iii) MDA and 4-HNE produced by Cd or Cr(VI)

Even though there was no clear evidence of DNA oxidative damage from Cd, there was an increase in lipid peroxidation end products in the high Cd group. The mechanism of ROS formation by Cd is not clear, however Cd suppresses the activity of many antioxidant enzymes and displaces Fe and Cu from cytoplasmic and membrane proteins which may then participate in Fenton reactions (Filipic *et al.*, 2006). Other *in vivo* exposure of mussels to Cd for different time periods has been reported to cause lipid peroxidation (Geret *et al.*, 2002). Cr(VI) also caused only a slight (non-significant) increase in MDA and 4-HNE (the latter being a variable parameter), which again does not mirror the high oxidative potential of this metal species. The lack of correlation between the oxidative biomarkers, 8-oxo-dG and MDA in mussel gill is not surprising: Charissou *et al.*, (2004) noted that gills were rapidly subjected to lipid peroxidation whereas oxidative DNA damage was less pronounced and it occurred later than the lipid damage. Thus, a correlation between 8-oxo-dG and MDA was only achieved after 21 days of exposure, which supports the theory that DNA and lipid peroxidation occur independently. Furthermore, a direct action of lipid peroxidation products on DNA would produce predominantly propano, etheno and MDA adducts (Bartsch and Nair, 2005).

v) Interference of Cd or Cr(VI) with excision of 8-oxo-dG and A

We tested the hypothesis that DNA damage produced by Cd or Cr(VI) may be partly attributed to inhibition of certain DNA repair systems. 8-oxo-dG excision was compromised by Cd at the high dose group after 10 days of exposure. It has been shown that Cd attenuates the removal of 8-oxo-dG created by H_2O_2 both in alveolar epithelial cells (Filipic *et al.*, 2006) and in GSH-depleted rats (Hirano *et al.*, 1997) but the actual mechanism of inhibition by Cd is not clear. It is known that the common motif Zn-finger is a potential target for this metal: Cd competitively displaces Zn from the motif (Hartwig, 1998) and Fpg, the bacterial functional homologue of the mammalian 8-oxo-dG *N*-glycosylase OGG1, possesses Cd-inhibited Zn-finger motifs (Asmuß *et al.*, 2000). However the mussel *N*-glycosylase responsible is probably non-Fpg or Nei related (Chapter 4). We should note however that Zn post-exposure completely reversed the damage (SSB) noted in Cd-treated mussels (Pruski and Dixon, 2002), which is a classic Zn-mediated salvation mechanism for Cd-inhibited Fpg (Asmuß *et al.*, 2000) and is suggestive of inhibition at the protein level.

Should the mussel enzyme be an OGG1 homologue, the affinity of Cd for thiols may be a more important reason for inactivation (Potts *et al.*, 2001). Cd may also interfere with the DNA-OGG1 catalytically competent complex (Zharkov and Rosenquist, 2002). Finally, several putative sites for binding of transcription factors have been identified on the human *ogg1* promoter and it is possible that Cd affects this regulation (Potts et al., 2003). Youn *et al.*, (2005) have shown that Cd down-regulates the binding of the transcription factor Sp1 to the promoter region of human OGG1, because of its interference with the Zn-finger-containing Sp1. Watkin *et al.*, (2003) also verified the inhibition in rat cell lines and but they argued that the interference of Cd with the Zn-finger-rich Sp1 may be facilitated by cysteine oxidation, Sp1

phosphorylation and finally Sp1 proteolysis. Until the mussel glycosylase is characterised the mechanism is unlikely to be revealed.

Cd did not, however, substantially suppress excision of εA in any of the doses tested. In contrast the human *N*-methylpurine glycosylase (AAG), which excises εA was strongly inhibited *in vitro* by Cd (Wang *et al.*, 2006), suggesting a major difference between human and mussel AAG.

Cr(VI) did not suppress 8-oxo-dG excision in mussel gill under our experimental conditions, even though Cr(VI) is implicated in down-regulation of human OGG1. Sodium dichromate reduced both OGG1 mRNA and 8-oxo-dG nicking capacity in human A549 lung carcinoma cells (Hodges and Chipman, 2002) and murine OGG1 activity in the early phase of exposure (Maeng *et al.*, 2003). Cr(VI) can directly oxidize guanine in the Sp1 transcription factor binding region of *ogg1* (GC box) and compromise the binding of Sp1 (Ghosh and Mitchell, 1999). In this case the implication of Sp1-mediated inhibition in mussels is not corroborated. Nevertheless, the oxidation potential of the water-borne Cr(VI) exposure was low as shown by the minimal 8-oxo-dG and lipid end-products increase, thus the probability of promoter region oxidation was also low. Longer duration of exposure or higher acute exposures could possibly lead to an accumulative effect of oxidation and raise the probabilities of an Sp1-mediated inhibition of OGG1.

Quite surprisingly, Cr(VI) dramatically inhibited AAG activity with some mussel extracts exhibiting non-measurable activity towards εA in the high dose group. Since AAG is the sole mammalian BER glycosylase for alkylating damage excision and

since it cleaves a variety of different substrates, its inactivation could lead to higher morbidity. Besides Sp1 a variety of other transcription factors are implicated in AAG control including CBP (Bouziane *et al.*, 2000) whose binding may be modulated by Cr(III) (Shumilla *et al.*, 1999). Cloning of the mussel glycosylases responsible for oxidative and alkylating damage excision in the future will elucidate not only their mechanisms of regulation but will also predict their possible suppression or induction by common pollutants in realistic environmental conditions.

7.0 General discussion

The blue mussel *Mytilus edulis* is a mytilid species commonly found in marine waters of the Northern hemisphere. Separate and distinct manifestations of DNA damage [e.g. single strand breaks (SSB), modifications of nitrogenous bases, bulky DNA adducts, micronuclei or gross chromosomal alterations and deletions] have a prominent place among the biomarkers used on mussels, since genotoxicity is a frequent characteristic of numerous pollutants. A common and non-specific sign of genotoxicity is the formation of SSB (Mitchelmore and Chipman, 1998). These lesions do not necessarily lead to mutagenicity (Speit *et al.*, 1996), however they are indicative of damaging interference with the genome. SSB are routinely measured in mussel species as depicted in Table 2, p.22. Modified purines or pyrimidines are other useful biomarkers of DNA damage and 8-oxo-dG (a C8′ oxidised form of guanosine) is a widely accepted marker of cellular oxidative stress (Kasai, 1997). Exhibiting high rates of formation, 8-oxo-dG is of particular concern since it also possesses a high mutagenic potential (Kawanishi *et al.*, 2001). Besides SSB, 8-oxo-dG levels have also been measured in mussel tissues (e.g. de Almeida *et al.*, 2003; Charissou *et al.*, 2004; Machella *et al.*, 2005; Labieniec and Gabryelak, 2006) but to a lesser extent than the former.

Given the irreplaceable role of DNA, detailed mechanisms of its repair have been evolved in organisms. Some of the repair pathways discovered include the Base Excision Repair, the Nucleotide Excision Repair and the Mismatch Repair. In mammals 8-oxo-dG is mainly rectified through BER with the aid of numerous *N*-glycosylases namely OGG1, NEIL, MTH1, MYH and OGG2. Furthermore, other repair pathways like NER and MMR are also implicated in 8-oxo-dG excision from

the genome (Slupphaug *et al.*, 2003), however bivalve BER has not yet been studied in detail.

A plethora of organic and inorganic aquatic pollutants exhibit genotoxic capabilities towards mussels. Some of these are metals [Cd, Cu, Cr(VI), Pb] or persistent organic pollutants [PAH]. Worth mentioning is the fact that their genotoxicity is not only direct (attack of the DNA double helix by their active intermediates) but may also be facilitated by modulation of metabolic and/or detoxifying enzymes, inactivation of repair enzymes or interference with cellular antioxidant status (see also1.8-1.9).

The estuaries we have chosen to examine are both facing the northern Irish Sea so are characterized by comparable salinities and tidal oscillations. They also contain pure *Mytilus edulis* populations as verified by a PCR method. Studies on mussels from Merseyside (Thompson *et al*, 1996; Widdows *et al*, 2002; Shaw *et al*, 2002, 2004; Harino *et al* 2005) and in North Wales (Widdows *et al*, 2002) and governmental reports for the same sites (Defra Quality Status Report of the Marine and Coastal Areas of the Irish Sea and Bristol Channel, 2000; DEFRA Status of the Seas Report, 2005) corroborate the differences in the pollution status of the aforementioned estuaries, with New Brighton exhibiting a higher degree of pollution. These differences are depicted on some but not all the biomarkers examined during the course of our experiments as collectively shown in the following table.

Parameter	Significant differences between sites
Direct assessment within 24 h	
Frank SSB	No significant differences in all but one (February 2006) time-points (NB higher)
Fpg-sensitive sites	Significant differences confined to summer (July) samplings (NB higher)
MDA	Variable differences depending on samplings time-point (When differences significant, NB higher)
4-HNE	Variable differences depending on samplings time-point (NB higher in July 2005, LL higher in September 2005)
8-oxo-dG excision	Consistent differences in all but one (May 2006) time-points (NB lower)
GSH concentration	No significant differences
After depuration (1 month)	
Frank SSB	No significant differences (*compared to 24 h sampling, higher values for both sites*)
Fpg-sensitive sites	No significant differences (*compared to 24 h sampling, the same or lower values*)
MDA	No significant differences (*compared to 24 h sampling, lower values for both sites*)
4-HNE	No significant differences in all but one

	(May 2006) time-points (*compared to 24 h sampling the same or lower values*)
8-oxo-dG excision	No significant differences (*compared to 24 h sampling higher values with the exception of February 2006 for both sites*)
GSH concentration	No significant differences (*compared to 24 h sampling the same values with one exception for July 2005 for NB*)

Table 9: Summarised differences between NB and LL for the parameters tested

Findings presented in Chapter 3 show that direct SSB had a low discriminating power between sites (24 h samples). Possible reasons for the overall lack of difference in SSB may include adaptive mechanisms of chronically exposed populations (Large *et al.*, 2002), pollution differences that were not intense enough to bring about significantly elevated DNA damage (Steinert *et al.*, 1998) or existence of chemicals in NB municipal wastewaters which block DNA repair pathways (Gagne *et al.*, 2004). Finally a periodic input of chemicals like the one noted for Tamar estuary (Sheehan and Power, 1999), might also modulate the genotoxic insult that especially animals from more polluted estuaries (like NB) undergo. Relevant studies on indigenous mussel populations have also highlighted the temporal confounding factors and the lack of persistent differences throughout the year or during the course of subsequent years. A large-scale study by Rocher *et al.*, (2006) using *Dreissena polymorpha* revealed substantial differences (more SSB) in polluted sites of Seine compared to a

clean site in June 2002 but a lack of differences was noted for June 2003. According to authors, high water temperatures and substantial decrease of river flow reported for summer 2003 have affected DNA integrity in all the sites tested, masking possible differences between sites. A similar study was also conducted by Rank *et al.*, (2005) with blue mussels at various coastal sites in Denmark. Even though there was a clear difference in SSB in all the polluted sites tested, 12 months later SSB were substantially lower in almost all the polluted sites tested, with summer values being indistinguishable from the reference site values. Differences in genetoxic input during the years of monitoring may be directly responsible for this observation however, according to the authors, at least non-genotoxic contaminants had actually increased and not decreased in later years.

8-oxo-dG showed a slightly higher discriminating capacity between sites with higher levels in NB in July 2005 and July 2006 (the latter being statistically non-significant) as shown in Chapter 3. The aforementioned reason of periodical input of pollutants may be implicated in the present seasonal pattern also. Other possible reasons for the higher values recorded at summer (but not in winter) for NB include the synergistic effect of temperature and pollutants on damage caused by chemicals to bivalves (Harrington *et al.*, 1997; Buschini *et al.*, 2003) and the relatively low 8-oxo-dG excision capacities noted for NB especially at summer. Regarding relevant literature, generally, measurements of 8-oxo-dG in mussels are relatively few (approximately 9 years of studies) compared to studies of bivalve SSB. According to de Almeida *et al.*, (2007) indigenous mussels from polluted estuaries or mussels chronically exposed to pollutants exhibit higher levels of 8-oxo-dG in their tissues. Seasonal variability may

or may not be an important confounding factor since results are still ambiguous (Charissou *et al.*, 2004; de Almeida *et al.*, 2007).

4-HNE also exhibited low discriminating power between sites as highlighted in Chapter 5 whereas MDA had a slightly better distinguishing ability (24 h samples). It is interesting to note that the differences were under strong seasonal influence (NB exhibited more MDA in February and July 2006 and more 4-HNE in July 2005 whereas LL exhibited more 4-HNE in September 2005). Again, these differences may be related to the periodical input of pollutants to NB (and LL) estuary whereas an additional confounding factor of a seasonal difference in the relative ratio MDA/4-HNE cannot be excluded: 4-HNE is the product of ω6-polyunsaturated fatty acids in mammals whereas MDA was mainly produced by oxidative degradation of polyunsaturated fatty acids with more than two methylene-interrupted double bonds in mammalian models (Esterbauer *et al.*, 1991). Seasonal differences in fatty acids and antioxidants intake through the diet may directly modulate lipids and their susceptibility to oxidation. Regarding relevant literature, lipid end-products exhibited different levels according to season for the site in question (New Brighton) in 1998, however at one time-point significant differences with reference sites were revealed for the sum of lipid products MDA+ 4-HNE (Shaw *et al.*, 2004).

Chapter 4 underlines the differences between the sites in question regarding the excision activity of mussel gill against 8-oxo-dG. The discriminating power of this biomarker was quite high (NB exhibited lower capacities than LL) however we are still not aware of its *in vivo* biological consequences since the baseline 8-oxo-dG levels did not inversely correlate with the cutting activity observed unless under

conditions of forced oxidative challenge. This was also evident in Chapter 6 where the suppression of 8-oxo-dG glycosylase activity by cadmium did not cause statistically significant increases in Fpg-sensitive sites *in vivo*. However, an acute oxidative insult [Cr(VI) injection] caused oxidative DNA damage (Fpg-sensitive sites) which was inversely correlated to the efficiency of DNA repair (8-oxo-dG excision capacity). The apparent suppression of activity noted in NB samples and its lack of persistence (apparent recovery) after one-month depuration points towards a pollution-related effect. Indeed, *in vivo* exposure of mussels to selected metals in Chapter 6 showed that interference with BER stages is possible. Mussels have evolved to combat oxidative stress efficiently since in most cases their environment (tidal oscillations, polluted estuaries, slow depuration) favors oxidative burden. However, studies have mostly concentrated on enzymatic and non-enzymatic defense systems. For example, superoxide dismutase which decomposes $O_2^{\cdot -}$ to H_2O_2, catalase which decomposes H_2O_2 to water and molecular oxygen, glutathione peroxidase which also reduces H_2O_2, glutathione reductase which regenerates oxidized GSH and metallothioneins which trap heavy metals have been studied extensively in bivalves. Actual DNA repair mechanisms are much less studied despite the manifestation of β polymerase activity in *Mytilus galloprovincialis* digestive gland more than ten years ago (Accomando *et al.*, 1990). Another BER enzyme, a functional 8-oxo-dG glycosylase is also present in mussel gill. This was evident via an *in vitro* nicking assay of a radiolabelled DNA duplex probe containing the oxidised base as described in Chapter 4. However there are serious gaps in our knowledge on bivalve DNA repair systems and the molecular basis of the activities noted for 8-oxo-dG and alkyl-adenine glycosylase are yet uncharacterised. Progress in this domain in the future would

elucidate many of the responses that mussels show after genotoxic exposure and would determine to some extent their susceptibility to pollutants.

As a general deduction differences between sites were evident on some on the biomarkers tested whereas they were subtler for others. For some biomarkers (reduced GSH) no significant differences between sites were noted for the time-points tested. Even though the physicochemical characteristics of the estuaries may be a confounding factor for the current results, the dominant pollution profile of Merseyside can also be incriminated. An important observation during the course of our studies was the lack of persistence of differences in the biomarkers examined when mussels from the estuaries in question were transferred to identical, unpolluted conditions. Mixing of gametes (LL towards NB) between sites is not impossible and is in accordance with the northern flow of Irish Sea currents (Widdows *et al.*, 1999) however NB and LL samples are basically two distinct allopatric *Mytilus edulis* populations. Thus, even their genetic composition is not the same, transfer to identical conditions for just one month leads to indistinguishable responses [including results from the Cr(VI)-injection experiments]. In other words, a forced selection of polymorphic alleles for certain enzymes (e.g. GSH cysteine ligase and cysteine synthetase, glutathione reductase or 8-oxo-dG glycosylase) because of the pollution in NB was not verified by our experiments. Different frequencies of specific alleles may still be presented in NB compared to LL however their performances seem to be comparable.

The present studies underline some of the difficulties linked to pollution monitoring based on bivalve biomarkers. As a general strategy, frequent and repetitive samplings

for more than one year highlight the possible confounding factors (temperature, adverse weather conditions, spawning season, water and pollutant inputs) and minimize the chance of fortuitous results. A significant difference between sites noted during a particular sampling may not be representative; a repetition of the result in subsequent samplings will not only strengthen the statistical power of the study but will also give insights on the actual biological significance of the biomarker. At the same time, extreme weather conditions or large-scale contamination incidents (e.g. oil-spills) may skew the results of repetitive sampling and should be treated with caution. Simultaneous studies on indigenous and transplanted mussels may additionally help on distinguishing effects of acute exposure from cumulative effects due to chronic exposure to pollution (Nigro *et al.*, 2006). Depuration studies are not frequent for mussel specimens however they can be proven highly indicative as shown here. The way mussels respond to a controlled environment reveals the genetic or physiological basis of their initial responses in their natural environment. Under the same light, *in vivo* exposures of mussels to selected genotoxic substances elucidates the mechanisms involved in the responses noted (e.g. why some populations are more susceptible to oxidative damage than others).

The ultimate question however is to what extent are the measured biomarkers predictive of an effect on the population level. An integrated risk assessment should take into account many parameters, for example the intensity and the duration of the exposure, which will determine to some extent the internal dose of the genotoxic agent. Early biological effects (mainly represented by biomarkers of exposure), altered structure and function of key cellular components and morbidity may follow. Also the susceptibility of the species and of the individual are of paramount

importance for the expression of the aforementioned damage and possible disease (Galloway, 2006). Exposure to genotoxic agents also affects the genotypic makeup of populations by exerting selective pressure against individuals ill-fitted for the particular pollution profile (Depledge, 1996). Furthermore it can affect population size (e.g. by increasing energy demands in expense to reproductive maturation) (Soetaert *et al.*, 2006). However, the detrimental effects of pollution on population size and constitution are the result of a multi-step procedure. In the case of DNA modifications like the ones described in this work, risk is elevated only when these perturbations become persistent and the affected cells start showing altered function and uncontrollable proliferation. Said perturbations in somatic cells may lead to neoplasia and morbidity whereas if the damage is expressed in gametes alterations in the following generation may follow (Monserrat *et al.*, 2006). Biomarkers of DNA damage have been proven to be valuable tools in assessing acute or chronic exposure to genotoxic substances (Shugart and Theodorakis, 1994; Depledge, 1996), which later may lead to carcinogenesis. For the same reason, suppression of DNA repair pathways or creation of DNA-reactive intermediates (e.g. lipid peroxidation products) may also be predictive of a pre-neoplastic condition. To conclude, biomarkers should not only be used to characterize impacted areas but their value as predictive tools of population size and constitution should be enforced and encouraged in the future.

8.0 References

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- **List of publications and abstracts**

Papers

- Emmanouil C., and Chipman J.K.: Variability in 8-oxo-deoxyguanosine levels and its excision capacity in *Mytilus edulis* populations from two sites in UK. (Manuscript in preparation).

- Emmanouil C., Green R.M., Willey F.R, and Chipman J.K: Oxidative macromolecule damage in gill of *Mytilus edulis* from Merseyside, UK and reversibility after depuration. *Environmental Pollution*. In press.

- Emmanouil C, Sheehan T.M.T. and Chipman J.K: Macromolecule oxidation and DNA repair in mussel (*Mytilus edulis* L.) gill following exposure to Cd and Cr(VI).*Aquatic Toxicology* 82(1):27-35.

- Emmanouil C., Smart D.J., Hodges N.J., and Chipman J.K., (2006) Oxidative damage produced by Cr(VI) and repair in mussel (*Mytilus edulis* L.) gill. *Marine Environmental Research* 62(1):292-296.

Abstracts

- C.Emmanouil and J.K. Chipman: 8-oxo-dG by Cr(VI) and its repair in mussel (*Mytilus edulis*) gill. *1st International Conference of Environmental Toxicology*, 11-13/9/2006, Mykonos, Greece.

- C.Emmanouil and J.K. Chipman: Differences in 8-oxo-deoxyguanine excision repair capacity in mussel (*Mytilus edulis*) gill from two sites within UK. *SETAC-UK Annual Meeting*, 4-6/9/2006, Liverpool, UK.

- C. Emmanouil and J.K. Chipman: Oxidative macromolecule damage in gill of *Mytilus edulis* L. and reversibility after depuration. *Protection and Restoration of the Environment VII*, 3-7/6/2006, Crete, Greece.

- C.Emmanouil and J.K. Chipman: Molecular basis of variability of susceptibility to genotoxic chemicals in mussel (*Mytilus edulis*) populations. *Postgraduate Conference, School of Biosciences*, University of Birmingham 24-25/4/ 2006

- C.Emmanouil and J.K. Chipman: Oxidative damage produced by Cr (VI) and repair in mussel (*Mytilus edulis* L.) gill.*PRIMO 13*, Alessandria, Italy, 19-22/6/2005

- C.Emmanouil and J.K. Chipman Oxidative damage and repair induced in mussel (*Mytilus edulis* L.) gill from a contaminated site. *12th International Symposium on Toxicity Assessment*, 12-17/6/2005, Skiathos, Greece