Chapter 9

MUSSELS AND ENVIRONMENTAL CONTAMINANTS: MOLECULAR AND CELLULAR ASPECTS

DAVID R. LIVINGSTONE AND RICHARD K. PIPE

INTRODUCTION

Information is presented on the metabolism and toxicity of metal and organic contaminants, so-called xenobiotics (foreign compounds), at the molecular and cellular levels. Data are largely for mussels, but information for other bivalve and molluscan species are included where relevant. Recent major reviews include Viarengo (1989), Renwrantz (1990) and Livingstone (1991a) on, respectively, heavy metals, internal defence systems and organic xenobiotics in molluscs. The uptake and discharge of xenobiotics, including tissue concentrations of metals and organic pollutants accumulated in the field, in particular data from the Mussel Watch monitoring programmes, are described in detail in Chapter 8. However, aspects which are of relevance to the metabolism and effects of xenobiotics are also summarized below.

Organic Xenobiotics

Rates of metabolism of organic xenobiotics are dependent on the tissue concentration of the compound (see p.435–440). Therefore, factors which affect uptake or discharge of the xenobiotic will also affect its metabolism. Lipophilic compounds such as organic xenobiotics are readily taken up into the tissues of bivalves and concentrated to levels greatly above those of the surrounding seawater. The bioaccumulation of a vast range of compounds by mollusc species has been recorded in field and laboratory studies. The xenobiotics include aliphatic hydrocarbons and polynuclear aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans, organochlorines such as 1,1-*bis* [4-chlorophenyl]-2,2,2-trichloroethane (DDT) and aldrin and dieldrin, aromatic amines, nitroaromatics, phthalate esters, organophosphorous pesticides and organometallics such as phenylmercuric acetate and *bis*(tributyltin)oxide (Livingstone, 1991a). Xenobiotics are

bioaccumulated from the water-column, and when associated with either abiotic particulate material, or associated with biota such as detritus and phytoplankton.

The qualitative and quantitative patterns of bioaccumulation and depuration depend on many variables, including bioavailability and route of uptake, exposure time, and various physicochemical and biological factors. The bioaccumulation of most organic xenobiotics involves an initial linear rate of uptake followed by the eventual attainment of a maximal tissue equilibrium concentration, both parameters increasing with the exposure concentration of the xenobiotic. In some cases biphasic or multiphasic uptake has been observed, and this has been interpreted in terms of xenobiotics entering a multicompartment system. Rates of uptake are much lower from sediments, reflecting the lower bioavailability of the associated chemicals. Examples of approximate rates of uptake in relation to xenobiotic exposure concentration, for a range of compounds and species, are given in Livingstone (1991a) and p.435–440.

Uptake is thought to be essentially a passive process, involving movement and equilibrium of the chemical between aqueous (external) and biotic (organism) compartments. The tendency to bioaccumulate increases with increasing hydrophobicity of the chemical, allowing prediction of bioaccumulation from the octanol/water partition coefficient or water solubility of the compound. In some cases the observed bioaccumulation exceeds the predicted, e.g. a x10 excess of *bis*(tributyl)tin oxide taken up by the common mussel, *Mytilus edulis* (Laughlin et al., 1986), whereas in others chemical structure appears to be important, e.g. non-*ortho* coplanar congeners were taken up much more slowly than many other PCB isomers by the mussel *Perna viridis* (Kannan et al., 1989). Possible mechanisms for selective bioaccumulation include membrane penetration phenomena, specific binding sites, macromolecular adduct formation and localization in major subcellular sites of uptake such as the lysosomes (Kurelec and Pivčević, 1989, 1991; Livingstone, 1991a). Important factors in determining tissue distribution and disposition of xenobiotics are lipid levels and route of uptake. Bioaccumulation generally increases with increasing tissue lipid levels, either in individual organisms of the same species, or in different tissues, resulting in the hepatopancreas (digestive gland), which is also high in lysosomes, being a major site of xenobiotic uptake. Gills are an initial site of uptake and can be a major one for particular xenobiotics, e.g. the uptake of *o*-toluidine (Knezovich and Crosby, 1985) and *bis*(tributyl)tin oxide (Laughlin and French, 1988) by *M. edulis*. Differences in disposition occur that are not obviously attributable to either lipid levels or route of uptake, viz. in *M. edulis*, whereas the mantle is a major site of uptake of naphthalene, proportional to lipid levels (Widdows et al., 1983), the bioaccumulation of *bis*(tributyl)tin oxide by this tissue is similar to that of the adductor muscle (Laughlin and French, 1988). Patterns of bioaccumulation are also

Table 9.1. Relationship between exposure time and depuration half-life for the elimination of various hydrocarbons from mussels and other bivalves[1]

Hydrocarbon	Species	Exposure time	Depuration half-life	Reference
N-alkanes	*Mytilus edulis*	2 days	0.2–0.8 days	Farrington et al., 1982
Paraffins/ naphthalene	*M. edulis*	16 days	~6 days	Broman and Ganning, 1986
Naphthalene	*M. edulis*	4h	2.2–5.1h	Widdows et al., 1983
Alkyl-naphthalene	*M. edulis*	2 days	0.9 days	Farrington et al., 1982
Phenanthrene	*Modiolus modiolus*	2 days	~1–2 days	Palmork and Solbakken, 1981
Methyl-phenanthrenes	*M. edulis*	2 days	1.7 days	Farrington et al., 1982
4-ring PAH[2]	*M. edulis*	40 days	14–30 days	Pruell et al., 1986
2–4 ring PAH	*Macoma balthica*	180 days	>60 days	Clement et al., 1980
Benzo[a]pyrene	*Rangia cuneata*	1 day	6–10 days	Neff et al., 1976
	M. edulis	40 days	15.4 days	Pruell et al., 1986
	M. edulis	Field[3]	16 days	Dunn and Stitch, 1976

[1] Half-lives are direct from reference or calculated from body-burden data.

[2] Polynuclear aromatic hydrocarbons (PAH).

[3] Field animals of unspecified exposure time.

affected directly, or indirectly (e.g. via lipid levels), by reproductive condition and/or seasonality.

Depuration, like uptake, is thought to be essentially a passive process, although some active excretion has been proposed, e.g. loss of naphthalene from gills and kidneys of *M. edulis* (Widdows et al., 1983). Exponential depuration curves are observed, which for many xenobiotics may be markedly affected by the duration of pre-exposure to the chemical. Thus, whereas elimination of the xenobiotic, following short-term exposure, is usually rapid and complete, long-term exposure results in slower and often incomplete elimination. This results in an increase in the half-life of depuration with increasing time of pre-exposure, and the relationship is particularly evident for petroleum hydrocarbons (Table 9.1). The mechanisms underlying such phenomena are unknown, but have been interpreted in terms of the xenobiotic entering a more stable molecular or cellular compartment, with a lower

Table 9. 2. Approximate depuration half-lives of polychlorobiphenyls from mussels and other bivalves[1]

Species	PCB[2]	Exposure time (days)	Depuration half-life (days)	Reference
Cardium edule	Cl_3	10	~7	Langston, 1978
	Cl_4		7–14	
	Cl_5		21	
	Cl_6		>21	
Perna viridis	Cl_2	17	0.5–2.5	Tanabe et al., 1987
	Cl_3		0.5–6.5	
	Cl_5		4.9–8.3	
Mytilus edulis	Cl_3	40	16.3	Pruell et al., 1986
	Cl_5		27.9	
	Cl_6		37–46	
Mytilus edulis	Cl_{2-4}	89	4.6–9.1	Calambokidis et al., 1979
	Cl_{5-7}		20–50	

[1]Half-lives are direct from reference or calculated from body-burden data.

[2]Number of chlorine atoms as subscript.

rate of xenobiotic turnover. Chemical structure, either directly or through hydrophobicity, also affects elimination. Thus, higher molecular weight, less water-soluble compounds are generally eliminated at a lower rate, this being particularly evident for increasingly chlorinated PCB congeners (Table 9.2). The position of chlorine atoms in PCB congeners also affects elimination, those with most *ortho*-substituted chlorines being retained least by the cockle *Cardium edule* and the clam *Macoma balthica* (Langston, 1978). Patterns of elimination are also affected by other factors such as season (probably via lipid levels) and temperature.

Metals

Aspects of the mechanisms of uptake and disposition of metals in bivalves have been the subject of several reviews (Simpkiss et al., 1982; Simpkiss and Mason, 1984; George and Viarengo, 1985; Fowler, 1987; Viarengo, 1989). Mussels are excellent bioaccumulators of both 'essential' metals (e.g. Zn, Cu, Co) and those for which no biological function has yet been discerned (e.g. Cd, Hg). Other metals accumulated include Ag, Al, Cr, Fe, Mn, Ni and Pb, and radionuclides such as uranium and the transuranium elements $^{239,240}Pu$, ^{238}Pu and ^{241}Am. The bioaccumulation of metals is

dependent on chemical speciation, routes and mechanisms of uptake, intracellular compartmentation and other aspects of cellular metal homeostasis.

For most metals uptake is proportional to the concentration of the metal in the external medium, and for the soluble form of heavy metals is indicated to be mainly a passive-transport process. Although the gill is the most important tissue for soluble metal uptake, the digestive gland is the major site for particulate-bound metal via endocytosis, an active-transport mechanism requiring ATP; the endocytotic vesicles subsequently fuse with primary lysosomes. The kidney is another major site of metal accumulation. Major sites of localization of radionuclides in *M. edulis* include the byssal threads, periostracum and pericardial gland. Metal gradients across membranes are maintained, and metals retained within the tissues, by their binding to various specific and nonspecific reactive ligands within the cell (see section on heavy metal metabolism p.442–443). Metal accumulation also varies with many biological and physical factors, including season/reproductive state, salinity and water-depth.

The movement and discharge of metals has been studied mainly in relation to mechanisms of metal homeostasis. Release into the external medium is mainly from lysosomes via exocytosis of residual bodies. Indicated or hypothesized release into the haemolymph is from insoluble granules and possibly also thiol-containing cytosolic compounds.

MOLECULAR ASPECTS

Biotransformation of Organic Xenobiotics

Enzymes

Metabolism of organic xenobiotics by organisms employs a wide range of biotransformation enzymes and is largely divisible into two phases: phase I (functionalization) and phase II (conjugative) metabolism (see Livingstone, 1991a for details of reaction types and enzymes). The enzymes of phase I metabolism catalyze virtually every possible chemical reaction that a compound can undergo, i.e. oxidation, reduction, hydrolysis, hydration and others, and introduce a reactive functional group (–OH, $-NH_2$, –COOH etc.) into the xenobiotic, so preparing it for phase II metabolism. Phase II metabolism attaches a variety of polar molecules to the reactive groups producing water-soluble, excretable conjugates, and is, therefore, the main detoxication pathway. A phase III metabolism is also known in higher organisms, involving hydrolysis of the conjugates and remetabolism of the product. Many of the biotransformation enzymes are also involved in the normal metabolism of endogenous compounds, such as steroids, vitamins and prostaglandins, and the

presence of xenobiotics can alter or interfere with these processes. Of key importance in determining the metabolic fate of a xenobiotic are the levels of the biotransformation enzymes, and their subcellular and tissue distribution, properties (substrate specificity, isozymes) and regulation (induction). Also of importance for mussels are seasonal changes in activities of the biotransformation enzymes, which will affect their capacity to metabolize xenobiotics.

Most known phase I and II enzymes have been detected in vitro, or indicated from in vivo studies, viz. phase I: the cytochrome *P*-450 monooxygenase or mixed-function oxygenase (MFO) system, the flavoprotein monooxygenase system (EC 1.14.13.8), other oxidases, epoxide hydratase (EC 4.2.1.64), reductases, esterases, sulphatases, phosphatases and deacetylases; phase II: glutathione *S*-transferases, uridine diphosphate (UDP)-glucuronytransferases (EC 2.4.1.17), UDP-glucosyltransferases, acetylases, sulphotransferases, methylases and formylases. No information is available on the existence of amino acid conjugases. Specific activities are given in Table 9.3. Not surprisingly, the activities of biotransformation enzymes are generally lower than in fish and other vertebrates, but are in the same proportion to the levels of nonbiotransformation enzymes, e.g. MFO activities and MFO system components compared to enzymes of intermediary metabolism (Livingstone and Farrar, 1984). Depending on the substrates employed, some marine invertebrate biotransformation enzyme activites have been suggested to be particularly high, e.g. glutathione *S*-transferases in molluscs (Kurelec and Pivčević, 1991). The specific activities of molluscan biotransformation enzymes are generally similar to, or possibly slightly lower, than those observed for echinoderms and crustaceans (Livingstone, 1990, 1991a).

MFO system and cytochrome P-450:

The MFO system has been detected or indicated in 23 species of bivalves and other molluscs (reviewed in Livingstone et al., 1989a, 1990a; Livingstone, 1990). Cytochrome *P*-450 and the associated components and oxidative activities of the MFO system (Table 9.3) are localized primarily in the digestive gland, but are also present or indicated in the gills and blood cells. Also found is a 'low wavelength haemoprotein' (so-called '416-' or '418-peak') which is thought either to be derived from cytochrome *P*-450 (equivalent to cytochrome *P*-420, the denatured form of *P*-450), or to be an independent functional protein, possibly a peroxidase.

Most information available on the catalytic nature and functioning of cytochrome *P*-450 and the MFO system is for digestive gland microsomes of *M. edulis* and other mytilids. Substrates binding to any cytochrome *P*-450 give typical difference (binding) spectra, depending upon whether they bind to the apoprotein (type I compounds and type I spectra), or to the haem part (type II compounds and type II spectra) of the

Table. 9.3. Phase I and II biotransformation enzymes in mussels and other molluscs. (From Livingstone, 1991a).

Enzyme	Source and number of species[1]	Units (mg^{-1} protein)	Range
A. PHASE I			
A.1. Mixed function oxidase system	D/M		
Cytochrome *P*-450	(11)	pmol	8–134
Cytochrome b_5	(7)	pmol	26–160
Cytochrome *P*-450 reductase[2]	(8)	nmol min^{-1}	4–22
Benzo[a]pyrene hydroxylase	(4)	pmol min^{-1}	3–35
7-ethoxycoumarin *O*-deethylase	(2)	pmol min^{-1}	0.1–3.2
Aldrin epoxidase	(1)	pmol min^{-1}	1–5
Dimethylaniline *N*-demethylase	(2)	nmol min^{-1}	0.6–3.4
Aminopyrine *N*-demethylase	(2)	nmol min^{-1}	0.05–0.67
A. 2. Others			
Flavoprotein monooxygenase system	D/M (2)	nmol min^{-1}	0.2–1.0
Monoamine oxidase	D/M (1)	nmol min^{-1}	0.1–0.3
Diamine oxidase	D/M (1)	nmol min^{-1}	0.3
Epoxide hydratase	W/M (4)	nmol min^{-1}	0.1–13.2
Azoreductase	D/M (1)	nmol min^{-1}	2.9
	D/C (1)	nmol min^{-1}	0.4
Nitroreductase	D/M (1)	nmol min^{-1}	0.4
	D/C (1)	nmol min^{-1}	0.2
Organophosphate acid anhydrase-glucuronidase	D/C (2)	nmol min^{-1}	20–100
	D/S (1)	nmol min^{-1}	62,000
B. PHASE II			
Glutathione *S*-transferase (epox.)[3]	W/C (4)	nmol min^{-1}	0.1–15.8
Glutathione *S*-transferase (others)	W/C (2)	nmol min^{-1}	21–91
	D/C (3)	µmol min^{-1}	1.2–10.7
UDP-glucuronyl-transferase	D/M (1)	nmol min^{-1}	38
	D/S (1)	nmol min^{-1}	7.6
UDP-glucosyl-transferase	D/M (1)	nmol min^{-1}	0.002

[1] D: digestive gland; W: whole animal; M: microsomes; C: cytosol; S: supernatant; number of species in brackets.

[2] NADPH-cytochrome c reductase activity.

[3] Epoxide substrate.

enzyme (see Schenkman et al., 1982 for explanation of binding spectra). In the case of *M. edulis*, whereas type II binding compounds (clotrimazole, ketoconazole, miconazole, metyrapone and pyridine) gave type II difference spectra, type I compounds (7-ethoxycoumarin, α-naphthoflavone, SKF-525A and testosterone) gave ap-

parent reverse type I difference spectra. The reason for the latter unusual observation is unknown, but may be due to the presence of endogenous substrates bound to the mussel cytochrome *P*-450, which are subsequently displaced by the added substrates, causing the observed spectral changes. The results differ from those obtained for several crustacean species which showed type I spectra with type I compounds (hexobarbital, phenobarbital, aminopyrine, ethylmorphine, benzphetamine and SKF-525A) (see Livingstone, 1991a).

Other unusual observations, related to the nature and catalytic mechanism of cytochrome *P*-450 action, have also been obtained for mussels compared to crustaceans or echinoderms (Livingstone, 1991a). The typical cytochrome *P*-450-catalyzed MFO reaction requires molecular oxygen and a source of reducing equivalents, usually reduced nicotinamide adenine dinucleotide phosphate (NADPH), the stoichiometry of the reaction being:

$$RH + NADPH + H^+ + O_2 = R\text{–}OH + NADP^+ + H_2O$$

where RH is the xenobiotic or endogenous substrate and R–OH is the hydroxylated (or other) product. The mechanism of this reaction usually proceeds by what is termed two-electron monooxygenation. A second type of reaction can also be catalyzed by cytochrome *P*-450, which utilizes a peroxide, such as cumene hydroperoxide, as the source of activated oxygen (the oxygen has to be activated to react with the organic substrate), and which does not require NADPH or molecular oxygen. The stoichiometry of this peroxidation reaction is:

$$RH + POOH = ROH + POH$$

where POOH is the peroxide. Although this type of reaction has been studied extensively in vitro, the extent to which it has physiological significance and occurs in vivo (employing say a lipid hydroperoxide as substrate) is unknown. Another reaction mechanism that can occur, other than two-electron monooxygenation, once the cytochrome *P*-450/activated oxygen molecular species has been formed, is termed one-electron oxidation. This proceeds via the formation of a cation radical of the xenobiotic substrate, and recent studies in mammalian systems indicate that this mechanism may be of importace in vivo. Only certain PAHs with particular structural features (high ionization potential), such as benzo[a]pyrene (BaP), will react via one-electron oxidation, and their major fate in vitro is covalent binding to DNA, protein and other macromolecules (macromolecular adduct formation), and the formation of quinones, viz. the 1,6-, 3,6- and 6,12-quinones of BaP.

A number of MFO activities catalyzed by digestive gland microsomes of *M. edulis* or *Mytilus galloprovincialis*, in particular BaP hydroxylase (BPH), 7-ethoxycoumarin

O-deethylase (ECOD) and *N*,*N*-dimethylaniline *N*-demethylase (DMAD), occur in vitro in the absence of added NADPH. This contrasts with the situation for vertebrates, echinoderms, arthropods and polychaetes, which have an absolute requirement for NADPH (or NADH) for MFO activity. The source of activated oxygen for the mussel MFO reactions is unknown, but is presumed to be either molecular oxygen and a source of unidentified endogenous reducing equivalents, or a hydroperoxide such as lipid hydroperoxide. The NADPH-independent BPH activity (total metabolites of BaP measured radiometrically using ^{3}H-BaP; N.B. BaP can be metabolized to many products, including dihydrodiols, quinones and phenols), and the ECOD and DMAD activities, are inhibited by reducing agents, including the addition of NADPH itself. The addition of reducing agents could inhibit a one-electron oxidation mechanism by converting the cation radical intermediate of the xenobiotic substrate (lacks an electron) back to the original substrate molecule. The major metabolites of mussel microsomal BaP metabolism are quinones (indicative of one-electron oxidation—see previous paragraph) (contrasted with mainly phenols for crustaceans and vertebrates) and one-electron oxidation has therefore been proposed as a possible mechanism of molluscan cytochrome *P*-450 catalytic action. Additionally, because, unlike quinone formation, the putative formation of phenols from BaP by mussel microsomes (fluorometric assay) is stimulated by NADPH, it is postulated that more than one process may be involved in the microsomal metabolism of BaP, viz. one-electron oxidation and two-electron monooxygenation giving rise to, respectively, quinones and phenols. Putative protein adducts of BaP were formed in incubations with digestive gland microsomes, possibly providing further evidence for one-electron oxidation (Livingstone et al., 1990a). MFO activities have been observed towards a number of other substrates, viz. epoxidase (substrate: aldrin), hydroxylase (biphenyl, antipyrine), *O*-dealkylase (*p*-nitroanisole) and *N*-dealkylase (benzphetamine, aminopyrine, *p*-chloro-*N*-methylaniline) (see also Table 9.3).

Multiple forms of cytochrome *P*-450 are indicated (N.B. a classification system now exists for different *P*-450 gene families and subfamilies based on the degree of similarity of gene and protein sequence information—(e.g. *P*-450 IA1 where I is the family, A is the subfamily and 1 is the individual member)—see Nebert et al., 1989). Partial purification studies revealed at least two forms in digestive gland microsomes of *M. edulis*. Transcript sequences showing similarity to rat cDNA probes to cytochrome *P*-450 IVA have been detected in the total RNA from the digestive gland of *M. edulis*; cytochrome *P*-450 IVA is thought to be an ancient cytochrome *P*-450, involved in fatty acid metabolism, originating some 800 or more million years ago. Activity specifically associated with hydrocarbon-inducible cytochrome *P*-450 IA (7-ethoxyresorufin *O*-deethylase) (involved in the metabolism of many organic xenobiotics, such as PAHs and particular PCB congeners) has only rarely been detected.

Seasonal variations in cytochrome *P*-450 isozyme composition are indicated for digestive gland of *M. edulis* (Kirchin et al., 1992): BPH activity in the tissue varied seasonally and was highest in autumn.

Other phase I enzymes:

The flavoprotein monooxygenase system has been detected or indicated in seven species of mollusc, including *M. edulis* and *M. galloprovincialis* (Livingstone, 1991a; Table 9.3). Metabolism of a wide range of nitrogen- and sulphur-containing xenobiotics has been demonstrated or indicated e.g. *o*- toluidine, *N,N*-dimethylaniline (DMA), aminofluorene (AF), *N*-acetylaminofluorene (AAF), aminoanthracene (AA), 4-amino-*trans*-stilbene, phenylhydrazine, 1,1-dimethylhydrazine and methimazole. Enzyme activity was present in digestive gland microsomes of *M. edulis* (Kurelec, 1985) and indicated in all tissues, but predominantly in the digestive gland of the clam *Mercenaria mercenaria* (Anderson and Döös, 1983). Aspects of reaction stoichiometry have been examined for DMA. Whereas *N*-oxide formation by digestive gland microsomes of *M. edulis* was NADPH-dependent and likely catalyzed by the flavoprotein monooxygenase system, formaldehyde formation was in part, or totally, NADPH-independent and possibly catalyzed by cytochrome *P*-450 (Livingstone et al., 1990a).

Epoxide hydratase activity towards styrene-7,8-oxide, octene-1,2-oxide and BaP-4,5-oxide has been detected variously in four species of bivalves (Table 9.3; Bend et al., 1977; Galli et al., 1988). Activity was similar in whole body and digestive gland microsomes of *M. galloprovincialis* (Suteau and Narbonne, 1988). Azoreductase activity (substrate: 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene) (Hanzel and Carlson, 1974) and nitroreductase activity (substrate: *p*-nitrobenzoic acid) (Carlson, 1972) have been detected in cytosol and microsomes of digestive gland of *M. mercenaria* (Table 9.3). Nitroreductase activity was also found in lower activities in mantle, gill, foot and gonadal tissue. Nitroreductase activity towards 4-nitroquinoline *N*-oxide has recently been demonstrated in cytosol and microsomes of digestive gland of *M. edulis* (Garcia Martinez et al., 1992).

Hydrolases, including esterases, acid- and alkaline-phosphatases (respectively, EC 3.1.3.2 and 3.1.3.1), organophosphate acid anhydrases, β-glucuronidases (EC 3.2.1.31) and hexoseaminidases are widespread in molluscs, and present in a number of tissues (Moore et al., 1989; Livingstone, 1991a; see also Table 9.3). The identified substrates are mostly endogenous, but activities towards xenobiotics and xenobiotic conjugates (phase III metabolism) have been detected or indicated. A number of the enzymes are polymorphic, e.g. nonspecific esterases in *M. edulis*. Esterase activity towards polyethoxylate fatty acid ester-containing commercial oil spill dispersants was present in digestive gland of the scallop *Chlamys islandicus* (Payne, 1982). Putative glucuronides formed from the aromatic amines AA and AAF by postmitochondrial

fractions of digestive gland of *M. galloprovincialis* were hydrolyzed by added mammalian β-glucuronidase (Kurelec et al., 1986). Deacetylase activity towards *N*-hydroxy-AAF was absent in digestive gland microsomes of *M. galloprovincialis* (Kurelec and Krča, 1987), but present in those of *M. edulis* (Marsh et al., 1992). Deaceytlase activity towards AAF and *N*-acetyl-*o*-toluidine has been indicated in *M. edulis* (Knezovich and Crosby, 1985; Knezovich et al., 1988).

Phase II enzymes:
Glutathione *S*-transferases have been detected in 18 species of mollusc (Livingstone, 1991a; Table 9.3). Activity has been observed towards a range of substrates, including 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene, ethacrynic acid, *p*-nitrobenzylchloride, methyl iodide 1,2-epoxy-3-(*p*-nitrophenoxy)propane. The enzyme has a wide tissue distribution, including high activities in the digestive gland, and is mainly cytosolic. Glutathione *S*-transferase isoenzymes have been detected or indicated in a number of gastropod species (Livingstone, 1991a), but as yet no information is available for mussels or other bivalves. Less is known of other phase II enzymes. Glucuronidation of phase I metabolites of AAF, but not of BaP, was indicated in postmitochrondrial supernatants of digestive gland of *M. galloprovincialis* (Kurelec et al., 1986). Sulphotransferase activity (substrate: pentachlorophenol (PCP)) is present in short-necked or 'ascari' clams (*Tapes* or *Ruditapes philippinarum*) and is mainly cytosolic (Kobayashi, 1985). Sulphotransferase activities have also been indicated from mutagenic and in vivo metabolic studies, e.g. in *M. edulis* (substrate: 1-naphthol) and *M. galloprovincialis* (activation of hydroxy-AAF, AAF and AF). Such studies have also indicated the presence of *N,O*-acetyltransferases and paraoxon-sensitive cytosolic enzyme in *M. galloprovincialis*, *N*-acetylases (substrate: *p*-toluidine, aniline and AF) in *M. edulis*, and *N*-methylases and, unusually, *N*-formylases (substrate: *o*-toluidine) in both *M. edulis* and the oyster, *Crassostrea gigas*.

In vivo metabolism
The metabolism of a wide variety of organic xenobiotics by different mollusc species has been observed (Livingstone, 1991a). In the case of mussels and other bivalves, the species and compounds include *p*-toluidine, aniline, AF, *N*-acetyl-*o*-toluidine, AAF, PCP, chlorinated paraffins and 1-naphthol (metabolized in *M. edulis*); *o*-toluidine (*M. edulis* and *C. gigas*); picric acid (2,4,6-trinitrophenol), picramic acid (2-amino-4,6-dinitrophenol), dibutylphthalate, di(2-ethylhexyl)phthalate and *bis*(tributyltin)oxide (the oyster *Crassostrea virginica*); *p*-nitroanisole and antipyrine (*Mytilus californianus*); aldrin (*M. californianus* and the freshwater bivalve, *Anodonta* sp.); dieldrin (the freshwater bivalve, *Sphaerium corneum*); and naphthalene (the clam, *Macoma inquinata* and the oyster, *Ostrea edulis*). Other xenobiotics metabolized by various

gastropods and other molluscs include 2,6-diethylaniline, benzidine, benzoic acid, nitrobenzene, anisole, *p*-nitrophenetole, methoxychlor, ethoxychlor, chlorobenzene, hexachlorobenzene, DDT, vinyl chloride, chlordane, phthalic anhydride, biphenyl and phenylmercuric acetate.

Metabolism of organic xenobiotics usually produces multiple end-products, involving the catalytic action of several phase I and phase II enzymes, e.g. *o*-toluidine is converted to four metabolites in *M. edulis* by methylation, formylation, hydroxylation and oxidation (Knezovich and Crosby, 1985). Most enzymes identified in vitro are indicated to be functional in vivo, although not always on the same substrate, e.g. aromatic ring and alkyl side-chain oxidations were not detected in the metabolism of aromatic amines by *M. edulis* and *C. gigas*, indicating that the observed *N*-oxidations are likely catalyzed by the flavoprotein monooxygenase rather than the MFO system (Knezovich and Crosby, 1985; Knezovich et al., 1988). A number of cytochrome *P*-450-catalyzed reactions are evident, however, e.g. aldrin epoxidation, antipyrine hydroxylation and *p*-nitroanisole *O*-demethylation in *M. californianus* (Krieger et al., 1979), and dealkylation of *bis*(tributyl)tin oxide in *C. virginica* (Lee, 1986). Glucosidic and sulphated conjugates have been identified in vivo, but not as yet those of glucuronic acid or glutathione, e.g. no indication of glucuronide formation from AF or other xenobiotics was obtained in *M. galloprovincialis* (Kurelec and Krča, 1989), and glutathione conjugates of dieldrin could not be detected in the bivalve *Sphaerium corneum* (Boryslawskyj et al., 1988). In contrast, two isomeric methylthioheptachlorostyrenes were isolated from *M. edulis* exposed to octachlorostyrene, which was indicative of the existence of mercapturic acid pathways (Bauer et al., 1989). Conjugates and other metabolites are released by molluscs into the surrounding water, but formation of the former does not particularly appear to predominate as an excretion mechanism, e.g. 1-naphthylsulphate, formed from 1-naphthol, was seen to be retained by *M. edulis* (Ernst, 1979b). Temperature appeared to have little effect on sulphated PCP formation by *M. edulis* (Ernst, 1979a). In some cases, no metabolism has apparently been detected at all e.g. *p*-nitroanisole in *M. edulis* (Landrum and Crosby, 1981).

Information on the metabolism of hydrocarbons, the first step of which would involve oxidation by cytochrome *P*-450 and the MFO system, is limited. Metabolites or conjugates were not detected in the bivalves *M. edulis* (Lee et al., 1972), *Macoma inquinata* (Augenfeld et al., 1982), *Macoma nasuta* (Varanasi et al., 1985) and *Modiolus modiolus* (Palmork and Solbakken, 1981) exposed to variously naphthalene, phenanthrene, BaP and other hydrocarbons. In contrast, 1- and 2-naphthols were formed from naphthalene in the oyster, *Ostrea edulis* (Riley et al., 1981), and unidentified metabolites and conjugates were detected in the freshwater snail, *Physa* sp. exposed to BaP (Lu et al., 1977). A number of possible reasons have been put forward to explain the absence, or low levels, of hydrocarbon metabolism, including

generally or seasonally low MFO activities, and inadequate analytical methodologies for the levels of metabolites produced (Moore et al., 1989). However, given that the levels of microsomal cytochrome *P*-450 and MFO activities such as BPH are not markedly lower in molluscs than in crustaceans or polychaetes (Livingstone, 1990), but that in vivo hydrocarbon metabolism is much more readily detectable in the latter two (see later), another possible explanation, or contributory factor, could be fundamental differences in cytochrome *P*-450 catalytic action (see section on enzymes p.429–435). Specifically, cytochrome *P*-450-catalyzed one-electron oxidation in vivo could result in a major fate of certain PAHs being covalently bound to protein and other macromolecules, rather than to polar metabolite and conjugate formation (Livingstone et al., 1989a, 1990a). Macromolecular adducts of hydrocarbons were indicated in *M. inquinata* (Augenfeld et al., 1982) and *Physa* sp. (Lu et al., 1977), but were not examined for in the other hydrocarbon studies. In digestive gland of *M. edulis*, putative protein adducts of BaP were formed in in vitro incubations with microsomes (Livingstone et al., 1990a), and more recently, in vivo binding of BaP to protein and DNA has been demonstrated (Marsh et al., 1992). Chlorinated paraffins were incorporated into the protein fraction of *M. edulis* (Renberg et al., 1986), which is interesting given that a suggested catalytic function of (rat liver) cytochrome *P*-450 IVA1 is hydroxylation of straight-chain alkanes (Lock et al., 1987). Changes in tissue profiles of accumulated aliphatic hydrocarbons, which could also be due to factors other than metabolism, have been observed for several bivalve species, e.g. in *M. edulis* (Widdows et al., 1982).

The formation of macromolecular adducts could be partly responsible for the observed increase in depuration half-life for hydrocarbons following increased periods of exposure (Table 9.1). The process of adduct formation, and presumably excision and release, could therefore contribute to the more stable cellular compartment, with a lower rate of xenobiotic turnover that has been postulated (Stegeman and Teal, 1973). Similarly, it could contribute to other observations on bioaccumulation, such as aspects of the specific patterns of PCB congener uptake and elution, and the greater than predicted accumulations of xenobiotics such as *bis*(tributyltin)oxide (see section on organic xenobiotics p.425–428 and Table 9.2). Macromolecular adduct formation in bivalves has also been demonstrated for the metabolism of AAF by *M. edulis* (Knezovich et al., 1988) and picric and picramic acids (Burton et al., 1984) and phthalate esters by *C. virginica* (Wofford et al., 1981).

Quantitative analyses of in vivo metabolism have been carried out using pooled literature data involving a large number of xenobiotics and molluscan species (Livingstone, 1991a, 1992). The compounds were divided into two groups: (a) those already containing functional groups (–OH, $-NH_2$ etc.), such as nitroaromatics, aromatic amines, esters and phenols, and termed 'functional group compounds',

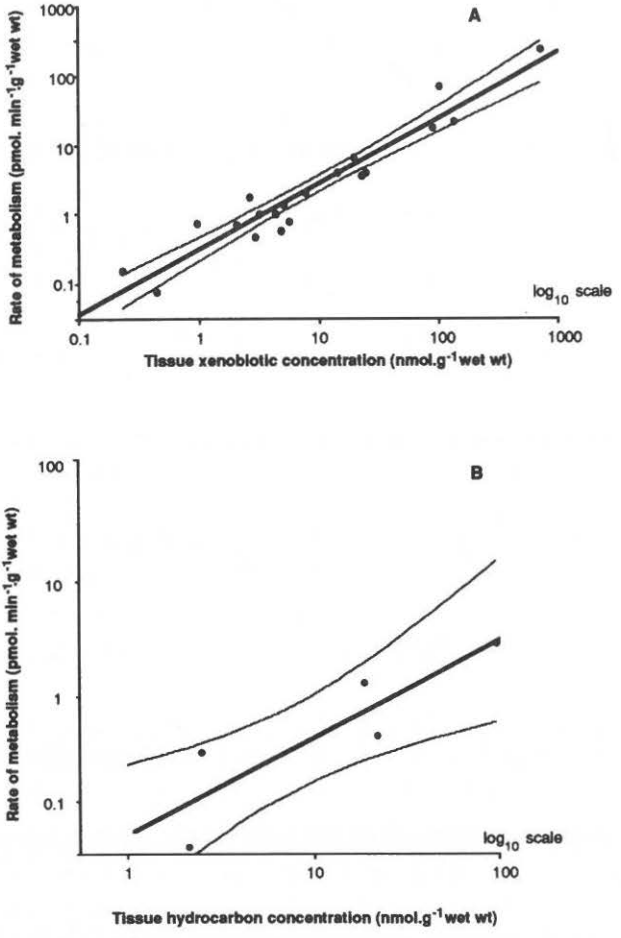

Fig. 9.1. Relationship between rate of in vivo metabolism and tissue xenobiotic concentration for the pooled data of a variety of functional group compounds (A) and hydrocarbons (B) and molluscan species. The former include nitroaromatics, aromatic amines, phenols, phthalate esters and others. The latter include naphthalene, benzo[a]pyrene, chlorinated paraffins and hexachlorobenzene, but not studies in which metabolism was not detected. The species include *Mytilus edulis, Crassostrea gigas, Crassostrea virginica, Macoma inquinata, Ostrea edulis, Cryptochiton stelleri* and *Physa* sp. The correlation coefficients for (A) and (B) were respectively 0.96 (n=20) and 0.87 (n=5) and the 95% confidence limits are shown on the plots. (Figures from Livingstone, 1992 with permission; data from Livingstone, 1991a, 1992).

Table 9.4. Theoretical comparative rates of in vivo organic xenobiotic metabolism for molluscs and crustaceans calculated for a tissue xenobiotic concentration of 10 nmol per gram wet weight[1]

Animal group	Type of compound	Regression equation[2]	Rate of metabolism (pmol min^{-1} g^{-1} wet wt)[3]
Crustacean	Functional group	$\log_{10}R = -0.46 + 1.01 \log_{10}T$	3.55 (2.82 – 4.47)
Mollusc	Functional group	$\log_{10}R = -0.51 + 0.94 \log_{10}T$	2.69 (1.91 – 3.80)
Crustacean	Hydrocarbon	$\log_{10}R = -0.90 + 0.93 \log_{10}T$	1.01 (0.83 – 1.38)
Mollusc	Hydrocarbon	$\log_{10}R = 1.31 + 0.92 \log_{10}T$	0.41 (0.09 – 1.91)

[1] From Livingstone (1991a, 1992).

[2] The regression equations relate rate of in vivo metabolism to tissue substrate concentration for the pooled data of a large number of organic xenobiotic metabolism studies in the literature; R and T are, respectively, rate of metabolism of xenobiotic in pmol per min per gram wet weight, and tissue xenobiotic concentration in nmol per gram wet weight.

[3] Mean plus, in parenthesis, the range for 2 standard errors.

Crustaceans include species of crabs, lobsters, crayfish, copepods, amphipods and zooplankton. Functional group compounds include aromatic amines, nitroaromatics, phthalate esters, phenols and others. Hydrocarbons include aliphatic and aromatic compounds. Mollusc equations are derived from Figure 9.1.

which can be directly metabolized by both phase I and II enzymes; and (b) hydrocarbons which require oxidation by the MFO system, before phase II enzymes can act on them. For functional group compounds, calculated whole body rates of xenobiotic metabolism linearly increased with tissue concentrations of parent compound, over four orders of magnitude of the two parameters (Fig. 9.1A). A similar relationship was indicated for the much smaller data set for hydrocarbons (Fig. 9.1B). However, in both cases it is important to realize that the treatments represent generalizations, and exceptions which fall outside the derived relationships will exist with respect to both particular compounds and species. Increased rates of metabolism with increased tissue concentrations of xenobiotic have also been seen in studies on single compounds, viz. sulphated PCP formation by *M. edulis* (Ernst, 1979a). Using the regression equations of Figures 9.1A and 9.1B, and data similarly derived for crustaceans (Livingstone, 1991a, 1992), it is possible to calculate rates of in vivo metabolism for particular tissue concentrations of xenobiotics, allowing comparison

between types of compounds and animals. Thus for molluscs, rates of metabolism are lower for hydrocarbons than for functional group compounds (Table 9.4), presumably reflecting either that several enzymes can act on the latter substrates at once, or that the action of the MFO system is rate-limiting. A similar difference is observed between functional group compounds and hydrocarbons for crustaceans, and the rates of metabolism of both groups of compounds are higher than in molluscs (Table 9.4).

The rates of uptake and final tissue equilibrium concentrations of organic xenobiotics, including hydrocarbons, are both mainly determined by the exposure concentration of xenobiotic in the water-column (see section on organic xenobiotics p.. 425–428). A relationship therefore exists between the former two parameters, allowing determination of one from the other. The tissue xenobiotic concentration determines the in vivo rate of metabolism (see above), and therefore, it is possible to compare rates of uptake and metabolism for a given tissue concentration of xenobiotic. Thus, using pooled literature data on the uptake of hydrocarbons by molluscs, it has been shown that rates of uptake generally exceed rates of metabolism by an order of magnitude or more (Livingstone, 1991a, 1992), thus accounting for the marked bioaccumulation of these compounds by molluscs, and the process being explainable on the basis of a simple lipid/water equilibrium model (Burns and Smith, 1981). The fundamental reason for this phenomenon is presumably that whereas uptake is mainly passive and determined by physicochemical principles, metabolism (i.e. enzyme activities) is intrinsic and largely determined (limited) by endogenous considerations, i.e. energy costs and endogenous functions of the biotransformation enzymes. Other factors that undoubtedly contribute to the long residence times of organic xenobiotics in molluscs, and other marine invertebrates, are a slow release of metabolites into the seawater, and the covalent binding of xenobiotics to macromolecules (Livingstone, 1991a, 1992).

Induction studies

A fundamental feature of most biotransformtion enzymes in mammals and other vertebrates is that the enzymes are induced by exposure of the organism to the xenobiotic. However, differences in gene regulation and induction are seen between different animal groups, e.g. cytochrome *P*-450 IIB1 is inducible in mammals but not apparently in fish (Stegeman, 1989). The amount of information available for molluscs is limited, but generally indicates that the enzymes, particularly the MFO system, are less responsive, and the increases in enzyme activities are less than in vertebrates. Responses of biotransformation enzymes to xenobiotic exposure have recently been reviewed (Livingstone, 1991b). Increases in MFO system components and activities, including BPH, occur with experimental exposure to organic xenobiotics (3-methylcholanthrene (3MC), BaP, PCBs, hydrocarbon mixtures (diesel oil

and crude oil), β-naphthoflavone), but the results are variable, including absence of responses. Increases in microsomal cytochrome *P*-450 specific content of digestive gland have been reasonably consistently observed with exposure of *M. edulis* and *M. galloprovincialis* to PAH and other hydrocarbons. Changes in cytochrome *P*-450 isozyme composition have been indicated for *M. edulis*. With one exception (Galli et al., 1988), no responses have been seen with exposure to phenobarbital or related compounds. Similar variability has been seen in field comparisons of molluscs from clean and polluted sites, e.g. no differences were evident in *M. edulis* from around Cape Cod, U.S.A. and the Shetland Islands, U.K., but increases in digestive gland cytochrome *P*-450 content were seen in Langesundfjord, Norway. Increases with pollution have been observed for the 418-peak of digestive gland microsomes of *M. edulis*, *C. edule* and the periwinkle, *Littorina littorea*, which in some cases have been associated with low or undetectable levels of cytochrome *P*-450. It is postulated that the haemoprotein responsible for the 418-peak may be some sort of microsomal breakdown product, possibly derived from cytochrome *P*-450. Elevated NADPH-cyctochrome *c* (*P*-450) reductase activity and levels of cytochrome *P*-450 and the 418-peak were seen in digestive gland microsomes of *Mytilus* sp. from polluted sites on the Catalan coast, Spain (Porte et al., 1991). In a study of *M. galloprovincialis* from sites around the Mediterranean Sea, whole body microsomal BPH activity (total metabolites) was correlated with sediment levels of PAH (Garrigues et al., 1990).

The flavoprotein monooxygenase system has been shown not to be inducible, at least in *M. galloprovincialis* (Britvić and Kurelec, 1986). Epoxide hydratase activity of whole body microsomes of *M. galloprovincialis* was increased following exposure to 3MC-type inducers (BaP, 3,4,3',4'-tetrachlorobiphenyl (TCBP), diesel oil emulsion), and to the phenobarbital-type inducer 2,4,5,2',4',5'-hexachlorobiphenyl (Suteau et al., 1988a, b). In contrast, decreases in activity only have been observed in field mussels with increasing body burdens of PAHs and PCBs (Suteau et al., 1988b). Indirect evidence for increases in epoxide hydratase activity in *M. galloprovincialis* was seen in the increased ability of digestive gland postmitochondrial fractions to detoxify styrene oxide in a yeast genotoxicity test following pre-exposure of the mussels to phenobarbital (Galli et al., 1988).

Glutathione *S*-transferase activity (enzyme substrate: CDNB) was increased in whole body of *S. corneum* after exposure of the bivalve to dieldrin or lindane (Boryslawskj et al., 1988), and in digestive gland of the freshwater mussel, *Anodonta cygnea* after exposure to diesel oil or polluted river water (Kurelec and Pivčević, 1989). With styrene oxide as the substrate for the enzyme, glutathione *S*-transferase activity in whole body of *M. galloprovincialis* increased in animals exposed to TCBP (Suteau et al., 1988a), but not in those exposed to diesel oil emulsion or in mussels from polluted field sites (Suteau et al., 1988b). Sulphotransferase activity in *T. philippinarum* increased following exposure to PCP; increases in activity also

occurred with exposure to resorcinol, *o*-cresol, *p*-chlorophenol and *p*-nitrophenol but not phenol (Kobayashi, 1985). Changes have also been seen in antioxidant enzymes with exposure to xenobiotics (see section on oxyradical metabolism p.443–446).

Heavy Metal Metabolism

On entering cells, heavy metals are primarily complexed by thiol-containing molecules such as amino acids, glutathione and, in particular, the metal-binding detoxication proteins, metallothioneins. In addition, part of the metal is compartmentalized in the lysosomal vacuolar system, or trapped in different types of specialized inorganic granules.

Metallothioneins

Metallothioneins are a class of low molecular weight, soluble (generally cytosolic), thiol-rich (high cysteine content) proteins with a high heavy metal content (Viarengo, 1989). They contain Zn^{2+} and Cu^{2+}, but can also bind xenobiotic metals such as Hg^{2+}, Cd^{2+}, Au^{2+} and Ag^{2+}. Amongst other identified or putative physiological roles, e.g. Cu/Zn homeostasis and oxyradical scavenging, metallothioneins mainly function to maintain low levels of free heavy metal cations in cells, initially through the displacement of Zn in existing metallothionein and the binding of the xenobiotic metal, and then through induction by the synthesis of increased amounts of metallothionein.

Metallothioneins have a wide tissue distribution in mussels, e.g. gills, mantle and digestive gland in *M. galloprovincialis* (Viarengo et al., 1981). Copper-binding (Viarengo et al., 1984), Cd-binding (George et al., 1979) and Hg-binding (Roesijadi and Hall, 1981) proteins (molecular weight 10,000 to 15,000 daltons, or more) have been isolated and characterized from *M. edulis* or *M. galloprovincialis*. Copper displaced Zn (but not Cd) from Cd, Zn-thioneins in digestive gland of *M. galloprovincialis* (Viarengo, 1989). Elevation of metallothionein levels have been seen in various tissues of mussels exposed to Cu (Viarengo et al., 1981), Cd (Noel-Lambot, 1976) and Hg (Roesijadi, 1982), and in other molluscs (Viarengo, 1989). Enhanced Hg-tolerance in *M. edulis* was related to the induction of Hg-binding proteins (Roesijadi et al., 1982a). Mercury-tolerance was also enhanced by pre-exposure to other heavy metals capable of inducing metallothioneins (Roesijadi and Fellingham, 1987). Metallothionein levels were elevated in the larvae of Hg-exposed *M. edulis* (Roesijadi et al., 1982b).

Lysosomes

Metal accumulation in lysosomes in mussels is well-documented, although information on the molecular processes involved is limited (Moore et al., 1989; Viarengo, 1989). Lysosomes are present in considerable numbers in digestive gland and kidney (see also p.449–452). Tertiary lysosomes accumulate undegradable end-products of lipid peroxidation (oxidized lipid and protein polymers), so-called lipofuschin. In kidneys, lipofuschin granules have been shown to bind metals in two ways, viz. (a) metals weakly bound by acidic groups in the outer region of the granules, and thus able to dissociate and be in equilibrium with cations in the cytoplasm; and (b), metals sterically 'trapped' in a nontoxic form in the centre of the developing granules (George, 1983). Active excretion of these residual bodies by exocytosis leads to metal elimination. A second method of metal elimination has been indicated for Cu in the digestive gland, involving the accumulation of Cu-rich thionein-like proteins in lysosomes, followed again by elimination of residual bodies (Viarengo, 1989; Viarengo et al., 1989). Neither of these two biochemical elimination pathways appear to play a major role in Cd removal in *Mytilus* sp., with the result that the biological half-life for Cu is nine days compared to seven months for Cd (Viarengo, 1989).

Inorganic granules and vesicles

Two major types of metal-containing granules, involved in heavy metal detoxification, are found, viz. Cu-sulphur-containing granules and calcium-containing granules. An association of Cu with sulphur has been seen in membrane-limited vesicles of oyster granular amoebocytes (Viarengo, 1989). Heavy metal cations are trapped in calcium-insoluble concretions as ortho- and pyrophosphates, e.g. in the kidneys of scallops (Fowler, 1987). Kinetic relationships are likely to exist between these concentrations of metals, and those of lysosomes and metallothioneins.

Toxic Effects

Oxyradical metabolism

Oxyradical generation, oxidative damage and antioxidant defenses in mussels and other molluscs have been reviewed (Viarengo, 1989; Livingstone et al., 1990b). The normal fate of molecular oxygen is tetravalent reduction to water, coupled to oxidative phophorylation and the production of energy. However, small amounts are continually partially reduced by endogenous and xenobiotic-stimulated processes to reactive oxygen species, so-called oxyradicals. These oxyradicals include the superoxide

anion radical (O_2^-; univalent reduction), hydrogen peroxide (H_2O_2; bivalent reduction) and the highly reactive hydroxyl radical (•OH; trivalent reduction). Oxyradicals are implicated in oxidative tissue damage and free radical pathology. Deleterious molecular effects include enzyme inactivation, lipid peroxidation and DNA damage, the latter being implicated in carcinogenesis. Combating oxyradicals are antioxidant defences which include various free radical scavengers and specific antioxidant enzymes. Only when these defences are overcome, either through their depletion, or a marked increase in oxyradical production, are pro-oxidant processes thought to result in significant molecular damage.

NADH-dependent and/or NADPH-dependent generation of O_2^-, H_2O_2 and •OH have been observed variously in digestive gland microsomes of *M. edulis* (Winston et al., 1990), *R. cuneata* and the mussel, *Geukensia demissa* (Wenning and Di Giulio, 1988a). Redox cycling metals such as Fe (and probably Cu) are necessary for •OH generation to catalyze the Haber–Weiss reaction (Winston et al., 1990), viz.

$$O_2^- + Fe^{3+} = O_2 + Fe^{2+}$$
$$H_2O_2 + Fe^{2+} = \bullet OH + OH^- + Fe^{3+}$$

Net (Haber–Weiss reaction): $O_2^- + H_2O_2 = O_2 + \bullet OH + OH^-$

The Haber–Weiss reaction which produces •OH from O_2^- and H_2O_2 is thermodynamically favourable but kinetically very slow, and therefore in biological systems the production of •OH is thought to be dependent on, and site specific to, the presence of a suitable chelated metal catalyst (probably Fe or Cu), of which nothing is known in molluscs.

Microsomal NAD(P)H-dependent oxyradical production is stimulated by the redox cycling compounds menadione (2-methyl-1,4-naphthoquinone) (Livingstone et al., 1989b), nitrofurantoin (*N*-(5-nitro-2-furfurylidene)-1-aminohydantoin) (Garcia Martinez et al., 1989) and paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) (Wenning and Di Giulio, 1988b). More recently, 4-nitroquinoline *N*-oxide has been observed to stimulate NAD(P)H-oxyradical production by microsomes and cytosol of digestive gland of *M. edulis* (Garcia Martinez et al., 1992). Likely loci for the oxyradical production are cytochrome *P*-450 reductase and NADH-dependent microsomal flavoprotein reductases (autoxidation, redox cycling), and cytochrome *P*-450 (autoxidation, oxidase activity). Menadione-stimulated NADPH-dependent oxyradical production was indicated to be limited by the presence of a microsomal DT-diaphorase (EC 1.6.99.2) catalyzing the two-electron reduction of menadione to the hydroquinone, so preventing one-electron reduction to the semiquinone and redox cycling. Oxyradical

production was also supported by the cytosolic fraction of *M. edulis* digestive gland (Winston et al., 1990).

Antioxidant enzymes have been detected in a number of bivalves, viz. superoxide dismutase (SOD; EC 1.15.1.1), catalase (EC 1.11.1.6), NAD(P)H-dependent DT-diaphorases and selenium-dependent (EC 1.11.1.9) and selenium-independent glutathione peroxidases (GPX) variously in, for example, *M. edulis* (Goldfarb et al., 1989; Winston et al., 1990; Livingstone et al., 1992) and the giant clam, *Tridacna maxima* (Shick and Dykens, 1985). A wide tissue distribution is indicated, with activities related to the potential for oxyradical production, e.g. high in digestive gland and gills. CuZnSOD (cytosolic form) and MnSOD (mitochondrial form) were found in *G. demissa*, *R. cuneata* (Wenning and Di Giulio, 1988a), the clams, *Calyptogena magnifica*, *M. mercenaria* (Blum and Fridovich, 1984), and in *M. edulis* (Livingstone et al., 1992). Small molecular weight free radical scavengers, such as glutathione, vitamins C (ascorbic acid), A (retinol) and E (α-tocopherol), and carotenoids, including β-carotene, have been detected variously in *M. edulis* (Ribera et al., 1991; Viarengo et al., 1991a), *M. galloprovincialis* (Ribera et al., 1989), *G. demissa* and *R. cuneata* (Wenning and Di Giulio, 1988a). Levels of antioxidant defenses vary seasonally in digestive gland of *Mytilus* spp. (Viarengo et al., 1991b).

Changes in antioxidant defences have been observed with exposure to xenobiotics. Catalase and SOD activities were transiently elevated in digestive gland of *G. demissa* exposed to paraquat: the increases were maximal after 6–12h, but had declined to control levels by 24h (Wenning et al., 1988). Minimal increases were seen or indicated in catalase, SOD, GPX and DT-diaphorase activities in digestive gland of *M. edulis* exposed to BaP and menadione (Livingstone et al., 1990b). Catalase and SOD, but not GPX, activities were elevated in digestive gland of *M. edulis* from polluted sites on the Catalan coast, Spain (Porte et al., 1991). GPX activities in digestive gland of *A. cygnea* were unaffected by exposure of mussels to polluted river water or diesel oil (Kurelec and Pivčević, 1989).

Glutathione occasionally increased in digestive gland of *G. demissa* exposed to paraquat (Wenning et al., 1988), whereas no change or decreases were seen in whole body *M. edulis* exposed to diesel oil/copper mixture, or to PAH and PCBs in the field (Suteau et al., 1988b). Glutathione in the digestive gland and gills of *M. edulis* decreased with exposure to Cu^{2+}, but increased slightly with exposure to Cd^{2+} or Zn^{2+} (Viarengo et al., 1988). Changes in the status of glutathione and vitamins A and E were observed variously in digestive gland, gills and remaining tissues of *M. edulis* exposed to menadione, BaP or carbon tetrachloride (Ribera et al., 1991). Total carotenoids increased in *M. galloprovincialis* exposed to a mineral oil/hypoxia condition (Karnaukhov et al., 1977).

Lipid peroxidation has been observed in vitro and in vivo. Enzyme-mediated lipid peroxidation was demonstrated in mantle microsomes of *M. edulis* (Musgrave et al.,

1987). Lipid peroxidation (malonaldehyde equivalents) increased in digestive gland of *G. demissa* exposed to paraquat (Wenning et al., 1988), in gill and digestive gland of *M. edulis* exposed to Cu^{2+} (Viarengo et al., 1988), and in various tissues of *M. edulis* exposed to menadione or BaP (Livingstone et al., 1990b; Ribera et al., 1991). Other toxic aldehydes such as 4-hydroxyalkenals were also detected in *M. edulis* (Viarengo et al., 1988). Lipofuschin formation increased in digestive gland of *M. edulis* following exposure to organic pollution in the field (Moore, 1988). Increased levels of lipid peroxide have been correlated with reduced antioxidant defenses in digestive gland of *Mytilus* spp. in relation to age (animal size) (Viarengo et al., 1991a) and seasonal variability (Viarengo et al., 1991b). Copper is considered to be the most likely metal peroxidative agent in the field, with seasonal variations in membrane lipid composition, and antioxidant defenses also being important (Viarengo, 1989).

Genotoxicity

The primary role of biotransformation enzymes is to detoxify organic xenobiotics by converting them to polar excretable products. Paradoxically, however, some of the biotransformations result in products that are more mutagenic or carcinogenic than the parent compound. Although information in molluscs is limited, such processes are indicated and genetic effects with exposure to xenobiotics have been observed.

Sister chromatid exchange (SCE) frequencies in developing eggs of *M. galloprovincialis* were higher in mussels from a polluted compared to a clean, field site (Brunetti et al., 1986; see also p.361–363 Chapter 7). Increased incidence of chromosomal aberrations were found in embryos of *M. edulis* (aneuploidy) (Dixon, 1982) and gill cells of *M. galloprovincialis* (aberrant metaphase) (Al-Sabti and Kurelec, 1985) in animals from polluted field sites. The percentage of aberrant metaphases in *M. galloprovincialis* was increased in a dose-dependent manner by exposure to BaP (Al-Sabti and Kurelec, 1985). In contrast, neoplastic diseases are found in bivalves but no convincing association with environmental pollution could be demonstrated (Mix, 1986; see also p.453–454).

Mutagenic chemicals have been found in the tissues of *M. edulis* and other molluscs, and a relationship is seen with pollution, season and the digestive gland (Moore et al., 1989). The identity of the mutagenic chemicals was not established, and therefore, it is not known to what extent they were bioaccumulated or subsequently produced through biotransformation. However, mutagenic activity of material extracted from the tissues with concentrated nitric acid was increased by incubation with mussel microsomes (Parry et al., 1981).

The digestive glands of *M. edulis, M. mercenaria* and *C. virginica* produced the proximate carcinogen BaP-7,8-dihydrodiol in in vitro incubations (Anderson, 1985; Stegeman, 1985), but showed only minimal or no mutagenic activation of BaP or 3MC

in Ames bacterial (*Salmonella typhimurium*) tests (Anderson and Döös, 1983; Britvić and Kurelec, 1986; Marsh et al., 1992), consistent with the predominance of quinones in the in vitro metabolism of BaP by cytochrome *P*-450. Similarly, using ^{32}P-postlabelling analysis of DNA adducts, incubation of digestive gland homogenate of *M. galloprovincialis* with BaP either showed no adduct, or a very weak adduct spot (1 adduct per 1–4 x 10^9 nucleotides) (Kurelec et al., 1988). In contrast to PAH, mutagenic activation was seen with aromatic amines such as AA, AF, AAF, *N*-hydroxy-2AAF, aminobiphenyl and 4-amino-trans-stilbene, and the properties of the activating ability were consistent with microsomal flavoprotein monooxygenase being the principal enzyme responsible (Anderson and Döös, 1983; Kurelec et al., 1986; Britvić and Kurelec, 1986; Marsh et al., 1992). Digestive gland homogenates of *M. galloprovincialis* incubated with AF showed one major and one minor DNA-adduct in the range of 1 to 4 x 10^8 nucleotides (Kurelec et al., 1988). Inhibitor studies established that *N,O*-acetyltransferase, sulfotransferase and paraoxon sensitive cytosolic enzyme (deacetylase) are also involved in the mutagenic activation (*S. typhimurium*) of *N*-hydroxy-2AAF, AF and AAF in digestive gland cytosol of *M. galloprovincialis* (Kurelec and Krča, 1987). The nitroaromatics 4-nitroquinoline *N*-oxide (Garcia Martinez et al., 1992) and 1-nitropyrene (Marsh et al., 1992) were activated to bacterial mutagens by subcellular fractions of digestive gland of *M. edulis*.

Mechanisms of xenobiotic activation have also been investigated using SCE and alkaline unwinding as endpoints of genetic damage. The promutagen cyclophosphamide (cytochrome *P*-450-activated), increased frequencies of SCE in both adult and larval *M. edulis* (Dixon et al., 1985). Pre-exposure to phenobarbital resulted in an increase in the levels of SCE produced by cyclophosphamide, possibly indicating induction of enzymes such as cytochrome *P*-450 or epoxide hydratase. In contrast, 9,000g supernatant of digestive gland of *M. galloprovincialis* did not activate cyclophosphamide in a yeast genotoxicity test (Galli et al., 1988). The frequency of SCE was not affected in eggs of *M. galloprovincialis* (Brunetti et al., 1986) and larvae of *M. edulis* (Dixon and Prosser, 1986) by exposure to, respectively, nitriloacetic acid and *bis*(tributyl)tin oxide.

The effects of BaP and 4-nitroquinoline *N*-oxide on damage to DNA in haemolymph of *M. galloprovincialis* were examined using the alkaline elution technique which measures alkali-labile sites and single strand breaks in the DNA (Bihari et al., 1990). Following injection of the xenobiotics into the pallial cavity, dose-dependent DNA damage was observed for both compounds 1.5h after exposure. However, the effects decreased after two days for BaP and five days for 4-nitroquinoline *N*-oxide, indicating the presence of significant DNA repair mechanisms in mussel haemolymph. The formation of peroxides and/or radicals was postulated as the mechanism of genotoxic action of BaP.

Damage to DNA (oxidation) can also be effected by enhanced oxyradical generation. Thus, redox cycling, formation of quinones from PAHs (BaP), and elevation of cytochrome *P*-450 content and cytochrome *P*-450 reductase activity represent possible mechanisms for linking pollution exposure to DNA and other damage in the digestive gland of molluscs (Livingstone et al., 1990b).

Little is known of metal-mediated genotoxicity (Viarengo, 1989), although a role in enhanced oxyradical generation is possible. Copper accumulated in appreciable amounts in the nuclei of exposed *M. galloprovincialis*. Although mRNA syntheis was increased in nuclei isolated from digestive gland and subsequently exposed to Cu, the activities of nuclear polymerases I and II (involved in, respectively, rRNA and mRNA synthesis) were decreased. In contrast, nuclear polymerase activities were unaffected in whole mussels exposed to Cu or Hg.

Other effects

Many other molecular and subcellular toxic interactions are possible. Identified ones include those on intermediary metabolism, lysosomes, mitochondria, endoplasmic reticulum, and calcium homeostasis. Effects on the adenosine phosphate system are variable, with decreases, increases and no change in adenylate energy charge (AEC) being recorded: similar variability has been seen for the different enzymes of intermediary metabolism (Livingstone, 1985; Viarengo, 1989). Lysosomal membranes are readily destablized by Cu, Cd and various organic xenobiotics such as PAHs (Moore et al., 1989; Viarengo, 1989). Decreases in cytochrome *P*-450 content and BPH activity occurred in *M. edulis* exposed to Cd (Livingstone, 1988). Calcium levels were elevated in tissues of *Mytilus* spp. exposed to Cu, Cu/PAHs mixture, or from polluted field sites, possibly affecting its key role as a secondary messenger: a mechanistic basis for this, involving oxidative damage and impared Ca^{2+} pumping (Ca, Mg-ATPases), has been proposed (Viarengo, 1989).

CELLULAR ASPECTS

It is possible to detect structural and functional alterations at the cellular level resulting from exposure to environmental contaminants at an early stage of the stress response. The tissues and cell types investigated generally reflect potentially sensitive indicators of an adaptive response in the mussel with research concentrated on the cells associated with the processes of feeding and digestion, reproduction and defence. Studies on contaminant induced cellular alterations can be broadly divided into three categories: (a) descriptive histopathology, where pathological changes in cell and

tissue morphology are recorded; (b) quantitative histology, where subtle changes in cell (or organelle) size and numerical ratios are recorded, and (c) cytochemistry, where changes relating morphology to cellular biochemistry are recorded. The first two categories are described together under headings for feeding and digestion, reproduction, and internal defence. Cytochemistry which investigates subcellular organelles and can generally be applied to various cell and tissue types is dealt with separately in the final subsection.

Feeding and Digestion

Laboratory studies have been made on the acute effects of a wide range of potential pollutants on the gills of mussels (see Fig. 9.2 and Chapter 2 for details on gill structure). An inflammatory reaction consisting of enlargement of the postlateral cells, dilation of the blood spaces and invasion by granular haemocytes resulted from exposure to a number of heavy metals, diesel oil and *N*-nitroso compounds (Rasmussen, 1982; Sunila, 1988; Auffret, 1988; Hietanen et al., 1988). In addition exposure to high concentrations of Zn caused swelling and degeneration of mucus secretory cells and necrosis of haemocytes within the gill (Hietanen et al., 1988). Lead and *N*-nitroso compounds caused loss of lateral cilia and sloughing of lateral cells from the chitinous rod, while treatment with Ag resulted in vacuolation of endothelial cells (Rasmussen, 1982; Sunila, 1988). Uncoupling of the interfilamentar junctions of the gill resulted from exposure to Cu and Cd (Sunila and Lindstrom, 1985); in addition Cu caused fusion of the gill filaments (Sunila, 1986a). Field studies, taking samples from polluted sites on the southern coast of Finland, and at dredge spoil dumpsites in New Haven, U.S.A., demonstrated similar effects in gill tissues (Arimoto and Feng, 1983; Sunila, 1987); however, no chemical analysis, to determine the levels or types of contaminant present at the sites or in the mussel tissues, was carried out.

Few studies have investigated contaminant effects on the cells of the stomach or digestive gland ducts. In contrast, however, there is a considerable body of work on the digestive diverticula of mussels (see Chapter 2 for details on structure of digestive organs). Mussels exposed to Cu and a mixture of diesel oil and Cu showed cytoplasmic erosion and invasion by clusters of brown cells of the epithelial cells lining the stomach wall; vacuolization, loss of cilia and cytoplasmic erosion of the ciliated columnar cells in the digestive gland ducts were also noted (Calabrese et al., 1984; Auffret, 1988). Mussels dosed with Ag accumulated it as black deposits in the basement membranes and connective tissue around the stomach, intestine and digestive diverticula (Calabrese et al., 1984; George et al., 1986).

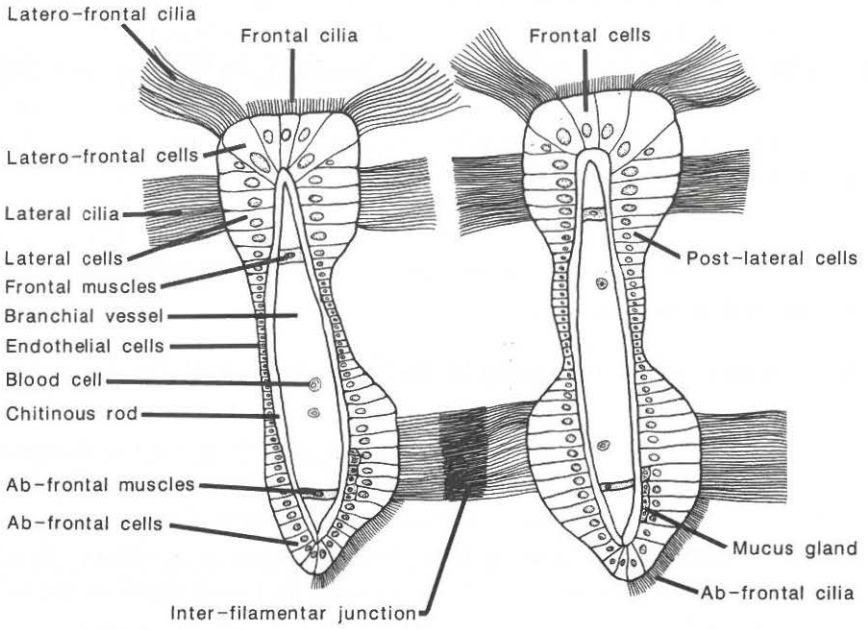

Fig. 9.2. Diagram of transverse section across adjacent gill filaments of *Mytilus edulis* showing histological structure under normal conditions.

The response of the digestive diverticula of mussels to contaminant exposure has been well-documented from both laboratory and field studies. Changes vary from gross pathology involving atrophy of cells to more subtle alterations in phasic activity of the tubules or variations in the lysosomal-vacuolar system. Mussels challenged with a wide range of contaminants develop some degree of atrophy of the digestive tubules (see Fig. 9.3); in extreme cases, such as injection with *N*-nitroso compounds, this involves severe necrosis of the digestive epithelium and replacement with collagenous scars (Rasmussen, 1982; Rasmussen et al., 1983a, b, 1985a). The degree of tubule thinning can be quantified using stereological techniques (Lowe et al., 1981). Other more sophisticated measurements, such as the ratio of lumen surface area to digestive cell volume can also be estimated using stereology (Lowe and Clarke, 1989). The breakdown of the digestive epithelium appears to be a generalized stress response, resulting not only from exposure to a wide range of contaminants (Pipe and Moore, 1986; Sunila, 1986b; Moore et al., 1987; Lowe, 1988; Lowe and Clarke, 1989), but also physiological extremes such as increased salinity and starvation (Thompson et al., 1974; Pipe and Moore, 1985a). Associated with the epithelial breakdown is an increase

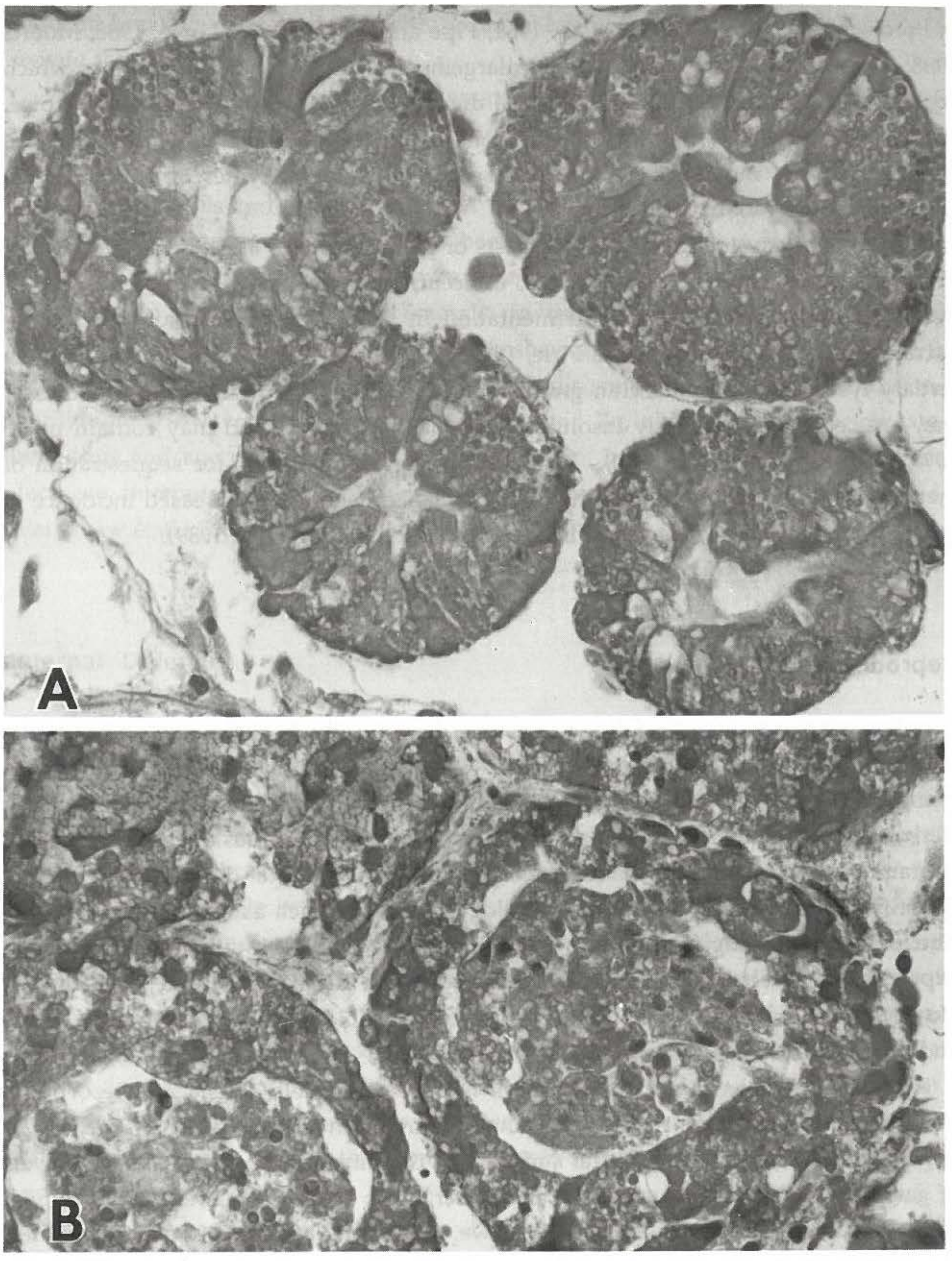

Fig. 9.3. Micrographs of transverse sections across the digestive gland of *Mytilus edulis* showing the structure of the digestive diverticulae under (A) normal and (B) oil-exposed conditions; notice thinning of the tubules and accumulation of material within the lumen. (Unpublished micrographs courtesy of D.M. Lowe).

in lipid within the cells (Wolfe et al., 1981; Pipe and Moore, 1986; Lowe, 1988; Moore, 1988; Lowe and Clarke, 1989), and an enlargement of the secondary lysosomes which are the organelles responsible for internal digestion of food (Lowe et al., 1981; Lowe, 1988; Moore, 1988). Experimental evidence suggests that the breakdown of the digestive cells is mediated through release of hydrolytic enzymes of pre-lysosomal origin (Pipe and Moore, 1986).

The excretory system in mussels consists of paired kidneys and pericardial glands. These organs are also the principal sites of accumulation of heavy metals. Metals are detoxified by intracellular compartmentation in tertiary lysosomes and eventually excreted in the urine (George and Pirie, 1979; George et al., 1982; George, 1983). These tertiary lysosomes or lipofuschin granules are found in large numbers in the kidney; they are composed of highly insoluble lipoprotein pigments and may contain up to 10% metal (George et al., 1982). While the kidney forms a site for sequestration of metals, it is not itself immune to the toxic effects, and shows an increased incidence of renal cysts in mussels subjected to long term Cu exposure (Sunila, 1989).

Reproduction

Gametogenesis in mussels follows a seasonal cycle which varies according to the location of the population (see Chapter 4). The reproductive state of mussels can be quantified in terms of the cellular composition of the mantle tissue using stereological techniques (Lowe et al., 1982; Pipe, 1985); this enables the subtle effects of contaminant exposure, such as slowing down of gamete development, to be quantified in addition to the gross pathological changes such as oocyte degeneration. The use of stereological analysis has demonstrated that low-level hydrocarbon exposure can reduce the levels of stored reserves in the mantle tissue of *M. edulis*. However, the indications are that this effect is reversible following depuration (Lowe and Pipe, 1986, 1987).

A frequently observed phenomenon in mussels is gamete atresia and resorption; this may be brought about by a wide range of environmental conditions (Pipe, 1987a, b). Exposure to a number of metal and organic contaminants induces enhanced levels of gamete breakdown (Maung Myint and Tyler, 1982; Rasmussen et al., 1983a; Sunila, 1984, 1986b; Lowe and Pipe, 1986, 1987; Lowe, 1988). Molluscan lysosomal membranes are known to be sensitive to environmental conditions (Moore et al., 1987); the susceptibility of the gametes to breakdown may therefore be due, in part, to their considerable lysosomal compartment (Pipe and Moore, 1985b; Pipe, 1987a; Pipe and Da Silveira, 1989). An inflammatory response often accompanies the contaminant

induced breakdown of gametes, with granular haemocytes infiltrating both male and female follicles (Sunila, 1984).

The suppression of gamete development has been reported following exposure of mussels to Cu, Cd and Zn (Maung Myint and Tyler, 1982; Calabrese et al., 1984; Kluytmans et al., 1988). This may well result from a lack of nutrients caused by damage to the gills and digestive tract. A number of contaminants, including metallic and *N*-nitroso compounds, have also been shown to stimulate spawning activity (Rasmussen, 1982; Sunila, 1986b; Kluytmans et al., 1988).

The mode of action of heavy metals, in terms of respiration rate, has been investigated (Akberali et al., 1984, 1985; Earnshaw et al., 1986). The results show that addition of Cu to unfertilized eggs from *M. edulis* results in an initial stimulation of respiration. However, pre-incubation with Cu and Zn inhibits the respiration rate of both eggs and sperm (Akberali et al., 1984, 1985). In addition, both metals cause a decrease in sperm motility, with Zn also causing some mitochondrial damage (Earnshaw et al., 1986).

Internal Defence

The internal defence of mussels relies upon circulating haemocytes which play a key role during inflammation, wound repair and phagocytosis of pathogens. The circulating haemocytes of *M. edulis* have been classified into at least two morphologically distinct types: granular and agranular cells (Moore and Lowe, 1977; Cheng, 1981; Rasmussen et al., 1985b; Pipe, 1990a). The granular haemocytes have been shown recently to be heterogeneous in terms of the lectin binding characteristics of their granules, indicating the possibility that a number of cell types or different developmental stages are present (Pipe, 1990a). The mechanisms available for destroying invading pathogens include release of hydrolytic enzymes (Pipe, 1990b) and the generation of reactive oxygen metabolites (Pipe, 1992). Further aspects of the internal defence system of *M. edulis* have been reviewed recently (Renwrantz, 1990).

There have been relatively few studies concerned with the direct effects of contaminants on the cells of the immune system in mussels. Oil emulsions and Cu and Cd have been shown to cause fluctuations in haemocyte numbers and, in addition, it has been demonstrated that oil affects their phagocytic ability (Livingstone, 1984; Sunila, 1984; McCormick-Ray, 1987). Silver has been shown to accumulate within the vacuoles of connective tissue macrophages (which are also responsible for phagocytosis), where it was associated with sulphur (George et al., 1986). The haemocyte response to contaminant exposure has been noted in numerous field and experimental studies; a general response to a wide range of contaminants is

haemocytic infiltration of tissues (Rasmussen, 1982, Sunila, 1984, 1988; Auffret, 1988; Hietanen et al., 1988). Another non-neoplastic response to a wide range of environmental pollutants is the formation of granulocytomas (for definition see p.543 Chapter 12). This condition was first described by Lowe and Moore (1979) in the digestive gland and mantle tissues of *M. edulis*. Rasmussen et al. (1985a) were able to induce a similar pathological effect following chronic chemical exposure; more recently it has been described in mussel tissues exposed to chemical contamination under field and experimental conditions (Auffret, 1988). A virus-associated granulocytoma has also been described in *M. edulis* (Rasmussen, 1986).

Proliferative blood cell disorders have been described in *M. edulis* from different locations worldwide (Mix, 1986). Early work (Lowe and Moore, 1978), implicated environmental contamination, in particular PAHs, as potential causative agents of the haematopoietic neoplasms. Subsequent studies have demonstrated that mussels inhabiting highly contaminated environments did not display neoplastic disorders, while others have shown neoplasms from nonpolluted sites (Mix, 1988). Recent studies (Elston et al., 1988) have indicated that the haemic neoplasm can be transmitted allogenically and suggest a retrovirus as the causative agent (see p.557 Chapter 12). This does not preclude a possible role for environmental pollutants which may initiate the expression of an integrated viral genome.

Cytochemistry

A quantitative approach to the functional disturbances of subcellular organization resulting from contaminant exposure in mussels has concentrated on the use of cytochemical techniques. The lysosomal system and the endoplasmic reticulum are the organelles which have received most attention. Increases in permeability of lysosomal membranes, resulting from exposure to a wide range of contaminants, has been extensively reported, (Moore, 1991). The technique used to measure lysosomal permeability or stability is based on substrate penetrability (hydrolase latency), and bears a quantitative relationship to the magnitude of the stress imposed. Destabilization of the lysosomal membrane appears to lead to enhanced protein catabolism and cellular atrophy (Moore, 1985; Moore and Viarengo, 1987). In addition to quantifying effects in terms of membrane damage, the accumulation of compounds within lysosomes can also be measured. Changes in the lysosomal content of lipofuschin, neutral lipid and metallothionein have all been quantified following contaminant exposure (Viarengo et al., 1987; Moore, 1988). Accumulation of neutral lipid in enlarged lysosomes appears to be as a consequence of exposure to lipophilic xenobiotics such as PAHs (Moore et al., 1988; Moore, 1991).

The smooth endoplasmic reticulum forms the site of metabolism of many lipophilic organic xenobiotics. NADPH-ferrihaemoprotein reductase activity is associated with cytochrome *P*-450 reductase of the MFO system, and is measured cytochemically using tetrazolium salts (Altman, 1972; van Noorden and Butcher, 1986; Moore, 1988). Field and experimental studies have demonstrated stimulation of cytochemically determined NADPH-ferrihaemoprotein reductase following exposure to a range of organic xenobiotics (Moore et al., 1987; Moore, 1988).

POLLUTION MONITORING

The need for biological effects measurements in pollution monitoring has long been appreciated. The extensive use of mussels for such purposes has resulted in the development of various molecular and cellular indices of stress for application in environmental impact assessment (Moore et al., 1987; Livingstone et al., 1989c). The details of many such responses have already been described in the previous sections, and the main points of general interest are highlighted below.

Induction of metallothioneins (Viarengo, 1989) and the MFO system (Livingstone, 1991b) have been used as specific indicators of impact by, respectively, metals and organic pollutants. More success has been achieved with the former than the latter, and in the case of the MFO system a multiparameter approach has been suggested, e.g. measurement of cytochrome *P*-450, 418-peak, BPH and other parameters. In contrast to the variability, or lack of change, of biochemical measures of the MFO system, cytochemically measured NADPH-ferrihaemoprotein (NADPH-neotetrazolium) reductase activity has been used extensively as an indicator of impact by organic pollution. Other less specific, or general, molecular stress indices that have been considered include AEC, antioxidant enzymes, free radical scavengers, and end-points of biological damage such as lipid peroxidation and genotoxicity.

At the molecular/subcellular level, lysosomal latency has been routinely used as a general indicator of stress. Cellular measurements have included stereological quantification of gametogenic and digestive conditions.

Future directions in the development of molecular and cellular indices of biological effect—so-called biomarkers—for molluscs, are likely to include modern molecular biological and immunological techniques. The former is likely to focus on the expression of genes (measured at either the mRNA or protein levels) concerned with producing such molecules as biotransformation enzymes (e.g. cytochrome *P*-450 (Spry et al., 1989)) and multidrug resistance proteins (Kurelec and Pivčević, 1991). The extent to which a molecular biomarker for impact by organic pollution will be developed will depend upon the inducibility of the system concerned and the depth of

understanding of its functioning in molluscs (Livingstone, 1991b). For example, the current extensive use of EROD activity, and other measurements of cytochrome *P*-450 IA1 expression, as a specific biomarker for impact by organic pollution in fish, is based on a thorough characterization of the enzyme and its functioning in this animal (Stegeman, 1989).

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