PAH-metabolizing enzymes in whole mussels as biochemical tests for chemical pollution monitoring

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ABSTRACT: Enzyme activities related to PAH metabolism, benzo(a)pyrene hydroxylase (B(a)PH), epoxide hydrolase (EH) and glutathione S-transferase (GST), in subcellular preparations from whole mussels *Mytilus edulis*, were assayed during the GEEP Workshop as possible biochemical tests for pollution monitoring. No response was observed along a field pollution gradient in Langesundfjord, owing to the variability within each biochemical measurement and to the limited number of samples/animals assayed per site. In mesocosm exposures to a diesel oil and copper mixture, inductions of B(a)PH and EH activities were related to some extent to the PAH gradient. These specific biochemical variables were more sensitive than a general biochemical variable related to the physiological state of the animal, the cytosolic glutathione content.

INTRODUCTION

Cytochrome P-450 dependent mono-oxygenase or mixed function oxidase (MFO) reactions are involved in many species in the metabolism of xenobiotics, including polynuclear aromatic hydrocarbons (PAH). The occurrence of oxidative hydrocarbon metabolism in some marine invertebrate groups (Lee 1981, Stegeman 1981) and particularly in bivalve molluscs has been established only recently (Livingstone & Farrar 1984, Stegeman 1985, Suteau & Narbonne 1987). This hydrocarbon metabolism involves both activation to toxic derivatives by MFO and epoxide hydrolase activities and the detoxification of these metabolites by conjugation; it may also imply a possible connection between environmental PAH and neoplasms in marine bivalves (Mix 1983). Increases in the activity of the MFO system and in the level of its protein components (i.e. induction) reflect exposure of the organism to organic xenobiotics (Livingstone et al. 1986) and such responses have been proposed as a means of identifying the biological impact of organic pollution in the field. Several such studies have been carried out with fish (e.g. Kurelec et al. 1977) but information is more limited for molluscs. Bivalves readily accumulate hydrocarbons such as PAH and polychlorinated biphenyls (PCB) from the environment, and have been widely used as 'bioaccumulators' in environmental monitoring programmes (Goldberg et al. 1978). Induction of some components of the molluscan MFO system, including benzo(a)pyrene hydroxylase (B(a)PH) (Anderson 1978), cytochromes P-450 and b5 (Gilewicz et al. 1984, Livingstone et al. 1985) and NADPH neotetrazolium reductase (Moore 1980), shows promise as a biological counterpart to xenobiotic accumulation.

Previous studies in our laboratory (Suteau 1986) showed the natural variability of PAH-metabolizing enzymes from *Mytilus galloprovincialis*. Moreover, experiments with pure inducers showed that benzo(a)pyrene (B(a)P) and 3,4,3',4'-tetrachlorobiphenyl (both coplanar 3-methylcholanthrene type inducers) increased both B(a)PH and epoxide hydrolase (EH) activities, while 2,4,5,2',4',5'-hexachlorobiphenyl (a non-coplanar phenobarbitone-type inducer) increased the conjugation of epoxide metabolites as shown by EH and glutathione S-transferase (GST) activities. Drug metabolizing enzyme activities appeared to be specific and sensitive indicators of the presence of hydrocarbons, since significant responses occurred at water concentrations as low as 0.4 µmol l⁻¹. These activities were therefore examined in *Mytilus edulis*, for the laboratory and field pollution gradients studied at the
GEEP Workshop. In addition, since glutathione is involved in the protection of organisms against general pollutant stress (Meister & Anderson 1983), and declines with decreasing animal condition, we measured total cytosolic glutathione content in mussels as a general physiological index.

MATERIAL AND METHODS

*Mytilus edulis* was sampled from the experimental exposures to diesel oil and Cu (as described by Bakke et al. 1988) and from field sites in Langesundfjord (as described by Follum & Moe 1988). Table 1 summarises PAH water concentrations and PAH, PCB and Cu residue concentrations in these samples (data taken from Appendix 1).

<table>
<thead>
<tr>
<th>Water</th>
<th>Tissue PAH</th>
<th>Tissue Cu</th>
<th>Tissue PCB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>12240</td>
<td>14.2</td>
<td>77</td>
</tr>
<tr>
<td>2</td>
<td>5870</td>
<td>16.6</td>
<td>180</td>
</tr>
<tr>
<td>3</td>
<td>11430</td>
<td>16.5</td>
<td>225</td>
</tr>
<tr>
<td>4</td>
<td>15450</td>
<td>17.0</td>
<td>275</td>
</tr>
<tr>
<td>Basin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>60</td>
<td>1080</td>
<td>7.3</td>
</tr>
<tr>
<td>L</td>
<td>100</td>
<td>6210</td>
<td>16.3</td>
</tr>
<tr>
<td>M</td>
<td>130</td>
<td>22810</td>
<td>26.8</td>
</tr>
<tr>
<td>H</td>
<td>520</td>
<td>8200</td>
<td>59.0</td>
</tr>
</tbody>
</table>

Tissues from 2 mussels (1 male and 1 female) were pooled to give each sample for biochemical measurements. Sex was determined by examining a smear of the mantle tissue under a light microscope for the presence of sperm or oocytes. The following procedures were carried out at 4°C. Tissues were rinsed, weighed and homogenized in ice cold, 0.05 M K2HPO4 buffer, pH 7.5, containing 1 mM EDTA and 15% glycerol. Subcellular fractions were obtained by differential centrifugation of homogenates according to a protocol for teleost organs described by Narbonne et al. (1987). Microsomal fractions were resuspended in the same buffer to give a protein concentration of 2 to 4 mg ml⁻¹. Microsomal and cytosolic (105 000 × g supernatant) preparations were stored at -70°C until use. Four samples from each treatment/site were assayed, with a minimum of 2 replicates per sample.

Total protein concentrations were determined by the method of Lowry et al. (1951). B(a)PH activity was assayed radiometrically (Van Cantfort et al. 1977). Microsomal samples (100 μl) were incubated at 25°C in a reaction mixture containing 3 mM MgCl2 and 50 mM Tris, pH 7.4. No NADPH was added, since previous observations showed an inhibition of B(a)PH activity related to addition of NADPH to the incubation mixture. The reaction was initiated by addition of the substrate ([3H]-B(a)P: 11.1 MBq mmol⁻¹) and was stopped after 30 min by addition of 0.5 N NaOH in 86% ethanol. Following removal of non-polar substrate by hexane, aliquots of the aqueous phase were counted by liquid scintillation. Glutathione S-transferase (GST) activity was assayed radiometrically by the method of Oesch et al. (1971a, b). Microsomal samples were incubated at 31°C in a reaction mixture containing 0.025% Tween 80, 49 mM 7-[³H]-styrene oxide (1.02 MBq mmol⁻¹, Amersham) and 0.125 M Tris, pH 9. After removal of non-polar substrate by petroleum ether, styrene glycol was selectively extracted by ethyl acetate and counted by liquid scintillation. Glutathione S-transferase (GST) activity was assayed by a modification of the radiometric method of Marniemi & Parkki (1975). Cytosolic fractions were incubated at 31°C in a reaction mixture containing 3.8 mM GSH, 49 mM 7-[³H]-styrene oxide (1.02 MBq mmol⁻¹, Amersham) and 0.1 M Tris, pH 8.0. After removal of the non-polar substrate by petroleum ether, conjugated styrene oxide was counted by liquid scintillation.

Total cytosolic glutathione content was estimated by the method of Akerboom & Sies (1981). Reduced glutathione (GSH) and glutathione disulphide (GSSG) from cytosolic fractions were determined using a kinetic assay in which catalytic amounts of GSH, GSSG and glutathione reductase brought about the continuous reduction of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) by NADPH at room temperature. The rate of 5-thio-2-nitrobenzoate (TNB) formation was compared to that obtained with an equimolar mixture of GSSG and GSH. Results were expressed in GSH equivalents.

RESULTS

All activities and concentrations assayed were detectable in whole mussel (*Mytilus edulis*) preparations. Results are expressed per milligram of total microsomal or cytosolic proteins and are given in Figs. 1 to 4.

For the mesocosm experiment, involving 4 dosing levels (C: control, L: low, M: medium, H: high-dose), analysis of variance (ANOVA) revealed a significant global variation (F = 6.45, p < 0.01) for B(a)PH activity between the basins. Based on t-tests, mussels from...
Basin M showed significantly higher B(a)PH activities ($p < 0.02$) than all other groups. No significant differences were observed between C, L and H. ANOVA of EH activity also showed a significant global variation ($F = 3.61, p < 0.05$). C mussels had the lowest EH activity, and differed significantly from M ($p < 0.05$) and H ($p < 0.02$). No significant variations between treatments were observed for GST activity. Total cytosolic glutathione content varied globally ($F = 6.77, p < 0.01$), with H differing significantly from C and L ($p < 0.02$). No significant difference was observed between C, L and M.

In the field study, there were no significant variations between the Langesundfjord sites (1, 2, 3 and 4) in B(a)PH and EH activities, or in total cytosolic glutathione content. Only GST activity showed some variation ($F = 5.88, p < 0.05$) owing to the higher activity in mussels from Site 2 compared to all other sites ($p < 0.02$). This did not match the contaminant gradient seen in mussel tissues (Table 1).

Table 2. Mytilus edulis. Least significant differences (LSD) in mean response, necessary to distinguish any 2 mesocosm or field conditions, for the biochemical variables B(a)PH and EH activity (pmol and nmol min$^{-1}$ mg microsomal protein$^{-1}$ respectively), GST activity (nmol min$^{-1}$ mg cytosolic protein$^{-1}$) and glutathione content (GSH, nmol mg cytosolic protein$^{-1}$). LSD = t.SD.$(2/n)$, where $t$ is the 1% point of $t$ on $4(n-1)$ df, SD is the residual standard deviation and $n$ the number of replicates (= 4).

<table>
<thead>
<tr>
<th>Condition</th>
<th>B(a)PH</th>
<th>EH</th>
<th>GST</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesocosm</td>
<td>16.40</td>
<td>2.38</td>
<td>3.05</td>
<td>3.05</td>
</tr>
<tr>
<td>Field</td>
<td>7.69</td>
<td>2.25</td>
<td>3.18</td>
<td>4.28</td>
</tr>
</tbody>
</table>

Table 2 shows least significant differences' (e.g. Steel & Torrie 1960) between 2 field or mesocosm conditions, required to demonstrate significance at $p < 0.01$. EH activity excepted, variabilities were higher for the field data.

**DISCUSSION**

Hydrocarbon accumulation by Mytilus edulis is influenced by many factors, such as the hydrocarbon concentration in water, the period of exposure and the physiological state of the mussel. In contrast to the long-term exposure (115 d) of the other groups, mussels from Basin H of the mesocosm experiment were exposed only for a period of 25 d before biochemical responses were measured, and their PAH body burden (measured after 18 d) was lower than that expected from the water concentration and accumulation rate of
Mussels readily accumulated the dosed copper for all treatments and the high tissue concentration of copper for group H seems likely to have induced toxic effects and decreased oxygen consumption and filtration activity (Manley 1983). In this group, the dose was lethal (by 7 wk) and we observed a drop in the membrane bound enzyme activities (B(a)PH and EH) and a decreased total cytosolic glutathione content, see Figs. 1, 2 and 4. Group H mussels were clearly in a poor physiological state and the following discussion on sub-lethal biochemical responses is therefore based only on results from the other 3 basins.

A hydrocarbon gradient was clearly evident in mussel tissues from both the mesocosm and field studies, with the highest levels being somewhat greater for the mesocosm experiment. In previous work (Suteau 1986) we observed a significant increase in MFO activities with water concentrations of PCB or B(a)P above 100 ppb. Comparable significant effects were not apparent in the present field data, perhaps due to lower levels of organic contamination and, more likely, to both the high variability of enzyme activities and the relatively small number of mussels sampled (2 in each of 4 replicate pools per site).

Field and laboratory exposure conditions clearly differ in many ways. Laboratory experiments over periods of months can be difficult to carry out without the mussels being affected. The physiological condition of mussels may deteriorate as a result of many factors (Livingstone & Bayne 1974, Widdows et al. 1982), and retardation of gametogenesis (Widdows et al. 1982) can occur in apparently well-maintained animals. We observed that mesocosm mussels were in a spawning phase whereas most of the field mussels had already spawned. It is therefore not surprising that B(a)PH and GST activities, and total cytosolic glutathione contents, were lower in mussels from the mesocosm than in the field. In the mesocosm, significant responses were observed for B(a)PH and EH, and this is consistent with observed induction in other studies (see Introduction).

Activities of B(a)PH and EH in the mesocosm were significantly related, by linear regression, to the pollution gradient (total PAH concentration in water), as indicated by the correlation coefficients \( r = 0.68 \) and \( r = 0.66 \) respectively \((p < 0.05)\). By combining the ‘least significant differences’ of Table 2 with the linear regressions it is possible to estimate a minimum water concentration for detectable decline can be computed. It is ca 600 ng \( l^{-1} \), suggesting that the specific biochemical variables associated with hydrocarbon biotransformation, such as B(a)PH and EH activities, are more sensitive than general variables such as cytosolic GSH content, relating to the mussel’s physiological state. However, this analysis must be treated with caution since no account is taken of the variability in water concentration measurements in separate samples from the same basin.

Table 2 allows comparison of the variability of enzyme activities between field and mesocosm studies, and suggests that the variance is smaller in the long-term experimental conditions (though this effect is partially confounded with the likely occurrence of increasing variability with increasing mean). In previous laboratory work (Suteau 1986), the time of exposure was short (2 d) and the variability of enzyme activities was similar to that observed for the present field samples, so it is possible that a long period in laboratory conditions reduces response variabilities and increases the sensitivity of the biochemical measures. The variabilities and the level of EH activity did not change markedly between field and laboratory conditions suggesting that this enzyme is not influenced by physiological status. These data are consistent with previous results indicating that EH was not significantly modified within the seasonal cycle (Suteau et al. 1985).

In summary, the use of whole preparations Mytilus edulis for enzyme assays may reflect the global mussel response to chemical stress; moreover, chemical analysis and body burden evaluations are generally carried out in whole mussels. Biochemical variables such as whole mussel B(a)PH and EH activities, related to PAH biotransformation, offer the potential to develop tests for organic pollution monitoring, and appear to be more sensitive than variables such as tissue GSH concentrations which are related to mussel general physiological condition. EH activity may be particularly suitable, as it does not seem to be affected by natural factors and is inducible by the 2 classical types of inducer.

**LITERATURE CITED**


