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Relationship Between Kinetics of Benzo[a]pyrene Bioaccumulation and DNA Binding in the Mussel *Mytilus galloprovincialis*

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Presence of persistent contaminants in the marine environment is of great concern as some of them have been identified as genotoxic or carcinogenic to several biological systems. In the aim to monitor pollution levels and impacts on marine flora and fauna, large biomonitoring programs have been developed leading to important multidisciplinary *in situ* studies. A large number of enzymatic biomarkers of pollutant exposure have been developed and integrated in field studies. Specific biomarkers of genotoxicity have also been assessed as 'early warning' of pollution effects (Burgeot et al. 1996). Due to their bio-ecological particularities and caging possibility, mussels are widely used in biomonitoring. They are the key sentinel organisms of the American Mussel Watch Program and French biomonitoring network, RNO (Réseau National d'Observation).

Among the genotoxicity biomarkers, DNA adducts have been widely studied for the last decade as their formation is thought to be a crucial event in chemical carcinogenesis initiation. In marine environment biomonitoring, several studies have been conducted in benthic fishes using the ^{32}P-postlabeling technique. In *situ* dose-response relationships have been demonstrated between total Polycyclic Aromatic Hydrocarbon (PAHs) concentration in sediment and hepatic DNA adduct levels (French et al. 1996). Because DNA adduct levels have been shown to be correlated with the prevalence of hepatic neoplasms and related lesions (Stein et al. 1994) they can be used as early markers of chemical carcinogenesis and hence for cancer risk assessment. These observations demonstrate the potential use of DNA adducts as a biomarker of both PAH exposure and effects.

With the future aim to study the different pathways involved in PAH DNA adduct formation, a mesocosm model to assess pollutant exposure has been developed for the mussel, *Mytilus galloprovincialis*. As model PAH, Benzo[a]pyrene (B[a]P) contamination of mussels has already been noted via sediment and water column and DNA adduct formation has been demonstrated for the latter exposure scenario (Venier and Canova, 1996). Because contamination in bivalves occurs via their filter-feeding activity, uptake via food supply was chosen for this experiment. During a 13 day exposure period, kinetics of B[a]P bioaccumulation in mussel tissues (gills, digestive gland,

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mantle) have been studied to assess the bioavailability of the pollutant for the exposed organisms. B[a]P DNA binding kinetics were also studied in both gills and digestive gland to check the ability of the dose applied to induce DNA damage. For the original way of exposure described, a concentration of environmental concern is reached in whole mussel, sufficient enough to target DNA. The results obtained validated the use of this model for Ruther works on DNA adduct formation in mussels.

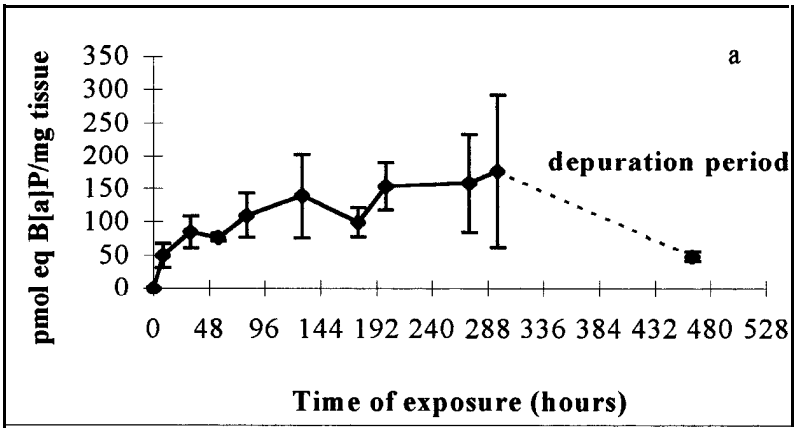
MATERIALS AND METHODS

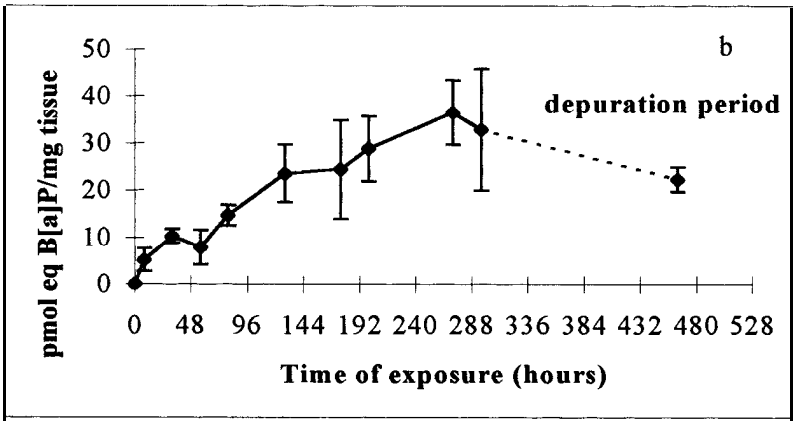
Specimens of mussels *Mytilus galloprovincialis* were bought (Viviers d'Aquitaine, La Teste) in Arcachon Bay (France). Originating from Rya de la Rosa (Gallice, Spain), they were kept in wet docks before being commercialized. In the laboratory, 150 animals were selected to build up a group of similar sized individuals and kept in 35 ‰ seawater filled glass aquariums. Water was pumped from the Bay of Arcachon. Its temperature was stabilized at room temperature (18°C) using an air conditioning system. Water was renewed every morning. The volume provided per individual (volume of water/ number of animals = 1L) was fixed during the time of the experiment: animals removed for testing were replaced in the aquarium. Oxygen was provided by the use of a continuous air-bubbler system. Animals were fed with Marine Liquifry, a commercial solution for marine invertebrates prepared by INTERPET. The suitability of particle size for the mussel was checked by laser granulometry. Each animal received a daily amount of 28 mg equivalent d.wt of Marine Liquifry. Feed was delivered over 8 hr into the aquariums by using a peristaltic pump. Every morning, feed was freshly contaminated with a mixture of B[a]P/ [H^3]B[a]P (1364: 1) to reach a concentration of 1.786 µg B[a]P/ mg Marine Liquifry. Hence, each individual was nominally exposed to a daily dose of 50 mg B[a]P/ kg d.wt. A 4 day acclimatization period was observed before the beginning of the experiment.

Experiment was conducted over 13 days and sampling occurred at 0, 8, 32, 56, 80, 128, 176, 200, 272 and 296 hr after the acclimatization period. It has been followed by a 7 day depuration period characterized by the same abiotic parameters, as previously described, but in total absence of B[a]P in feed. A sample was taken at the end of the depuration period to assess the ability of mussels to recover from B[a]P exposure. At each sampling time, 12 individuals were removed: 4 animals were used for the study of B[a]P concentration in mussel tissues (gills, digestive gland, mantle) and 4 other ones for the study of B[a]P DNA binding in both gills and digestive gland. The rest were used for the time-course study of the mean individual dry weight as an index of the physiological state of the organisms.

At each sampling time, the individual dry weight of 4 whole mussels was gravimetrically determined by weighing the samples before and after a 48 hour period at 60°C in a drying oven.

Determination of [^{3}H]B[a]P concentration in each individual tissue was carried out by Liquid Scintillation Counting (LSC). Liver, gills and mantle of four individual





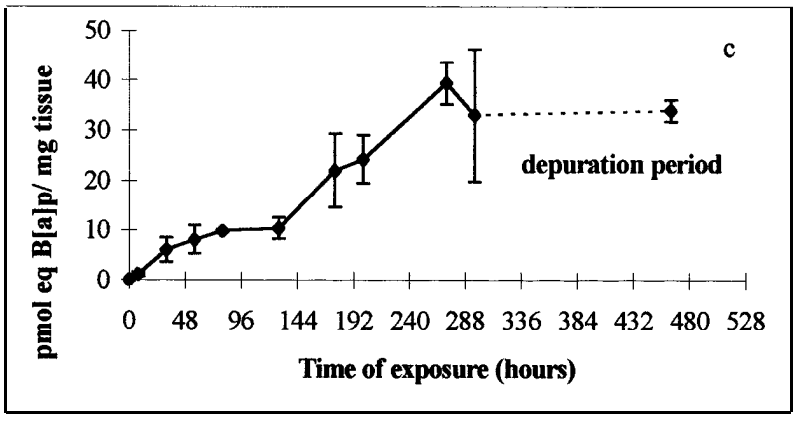


Figure 1. B[a]P bioaccumulation kinetics in (a) digestive gland (b) gills and (c) mantle of exposed mussel.

mussels were dissected out at each sampling time. After a brief rinse in acetone to remove adsorbed B[a]P, they were kept at -80 °C before analysis. After weighing, thawed tissue remained overnight in Lumasolve (LUMAC LSC, 5 mL/ g fresh tissue) under moderate agitation at 50°C. Aqualuma plus (LUMAC, LSC), was then added to a volume equivalent to 1 mg tissue (6 mL/ mL) for radioactivity counting. Radioactivity of the samples was recorded using a Searle Delta 300 Counter. Taking into account the dilution factor of [^{3}H]B[a]P into B[a]P, results were expressed in pmol equivalent B[a]P per mg fresh tissue.

For DNA binding, the liver and the gills of 4 individual mussels were dissected out and briefly rinsed in acetone to remove adsorbed B[a]P. Samples were kept at - 80°C prior analysis. DNA of each individual tissue was isolated as described by Venier and Canova (1996). DNA titration was carried out by UV spectrophotometry at 260 and 280 nm. Radioactivity was recorded as previously described for tissue samples. Aqualuma Plus was added for each sample to a volume equivalent to 500 µg DNA. Results were expressed in pmol equivalent B[a]P/ mg DNA.

For the time-course study of both mean individual dry weight and DNA binding, raw data were analyzed by carrying out a one-way ANOVA ($p = 0.05$) taking B[a]P exposure time as a factor (Statsoft, Inc. 1993). Variance homogeneity was previously checked by conducting the Hartley test.

RESULTS AND DISCUSSION

No significant difference was observed in the mean individual dry weight of the exposed organisms as a function of exposure time. Animals did not lose weight during the time of the experiment, suggesting a relatively constant physiological condition of the test organisms over a short experimental period. This stability can be interpreted as a result of the filter feeding activity.

In either digestive gland, gills and mantle, the concentration of B[a]P increases with exposure time (Fig. 1). B[a]P bioaccumulation kinetics follow a linear mathematical model in both gills ($y = 0.1344\ x$, $R = 0.826$, $p < 0.001$) and mantle ($y = 0.12310\ x$, $R = 0.915$, $p < 0.001$), whereas in the digestive gland the concentration increases following a logarithmic model ($y = -14.819+67.306$ *log10(x)+eps, $R = 0.609$, $p = 0.001$). The comparison of the values obtained before and after the depuration period shows a decrease of B[a]P concentration in both digestive gland and gills, by respectively 73 and 32%. However, the conduction of Student t tests on these values shows no significant decrease. Lack of statistical significance is explained by the high standard deviations of means obtained, reflecting inter-individual variability in B[a]P bioaccumulation. In the mantle, B[a]P concentration remains constant after depuration. Digestive gland, gills and mantle represent respectively, 14%, 24% and 20% of the mean individual wet weight (determined during the course of the experiment). Taking into account the weight distribution of each tissue type and the concentration of B[a]P recorded in each tissue, the estimated concentration in whole mussels reaches 58.5 mg B[a]P/ kg d.wt mussel (9.964 mg B[a]P/kg w. wt) at the end of

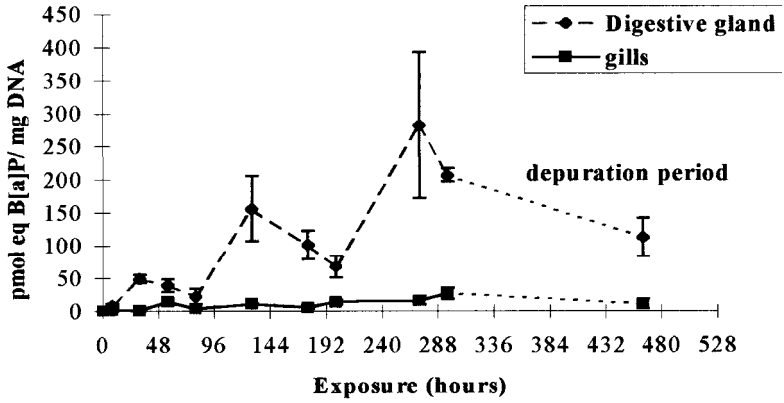


Figure 2. B[a]P DNA binding kinetics in mussel digestive gland and gills.

the 13 day of exposure. This concentration is similar to that recorded at the highly polluted site of Nice-La Reserve for total PAHs (54.4 mg/ kg d.wt mussel) by the RNO (Beliaeff et al. 1998).

These results suggest that for the conditions of exposure described, B[a]P is bioavailable for the organisms. The linear increase of B[a]P concentration with exposure time shows that in both gills and mantle, steady state is not reached at the end of the 13 days of exposure. In the digestive gland, the logarithmic increase of B[a]P concentration illustrates a different pattern of bioaccumulation. In this tissue, the logarithmic kinetics involve a fast absorption phase during the first 32 hours. For this period, the uptake rate in the digestive gland is of 2.87 pmol B[a]P hr^{-1} for only 0.34 and 0.18 pmol hr^{-1} for gills and mantle respectively. In digestive gland, a plateau is reached after a 100 hr period whereas saturation is not evident after 280 hr exposure in gills and mantle. Difference observed between tissues can be interpreted as a classical difference between contact tissue and compartments of distribution via the circulating system. In mussel, feed particles are filtrated through gills and dispatched up to the digestive gland haemolymph to be metabolized, explaining the high bioaccumulation in this tissue. In the other hand, enzymes involved in the biotransformation of both endogenous and exogenous substrates are mainly localized in the digestive gland in mussel (Livingstone and Pipe 1993). Benzo[a]pyrene is known to be metabolized to more water soluble products that can be excreted via conjugates formation. The balance between these two opposite processes explains the non linear increase of B[a]P concentration in the digestive gland.

The decrease of B[a]P concentration afier the 7 day depuration period illustrates the ability of digestive gland to recover from B[a]P exposure via its biotransformation and excretion. The weak release of stored metabolites observed during depuration in the mantle may be linked with the low level of biotransformation process in this tissue. It seems to be due also to its rich lipid

content, responsible of its role as a storage compartment in the mussel. As previously reported by Suteau et al. (1988), the digestive gland appears as the main tissue of benzo[a]pyrene bioaccumulation.

Results obtained for the digestive gland and the gill DNA are presented in Fig. 2. For the digestive gland and the gills, [^{3}H]B[a]P associated radioactivity was recorded in DNA. DNA binding time-course was analyzed using a one way ANOVA. Data were log transformed to obtain homogenized variances. Binding of B[a]P metabolites increases with time in a jagged pathway in both the digestive gland ($F = 31.881$, $\sqrt{} = 9/18$, $p < 0.001$) and the gills ($F = 20.995$, $\sqrt{} = 9/19$, $p < 0.001$) DNA. Binding in the digestive gland is up to 10 times higher than in the gills. Conduction of Student t tests, show that the amount of bound metabolites decrease in both tissues during depuration. The calculation of a binding index (pmol bound/ mg DNA)/ (Dose, µmol/ kg) as previously proposed by James et al. (1995) gives values for digestive gland and gills of, respectively, 1.04 and 0.13.

These results suggest that B[a]P incorporated into the food supply has been absorbed, bioaccumulated and biotransformed in mussel. Its biotransformation in digestive gland and gills has led to the production of electrophilic metabolites that covalently bind to DNA. As recorded in Table 1, mussel tissues present a different balance between phase I and phase II biotransformation activities. Phase I activities and particularly those related to the MFO system are greater in the digestive gland. Benzo[a]pyrene Hydroxylase (BPH) activity only has been

Table 1. Phase I and Phase II enzymatic activities in mussel tissues. [1]DG: digestive gland, G: gills, M: mantle, [2]M: microsomes, C: cytosol, S: S9 supernatant, [3]fs: field study, ms: mesocosm study, 4 Glutathion-S-Transferase.

Enzy. Activities	Tissue	Values recorded	References
NADPH	DG[1]/ M[2]	7.6	Stegeman (1985), fs[3]
cyt c reductase	G/ M	3.2	Stegeman (1985), fs
nmol/ min/ mg prot	M/ M	2.6	Stegeman (1985), fs
	DG/ M	21.8 ± 19	Suteau and Narbonne (1988), fs
BPH	DG/ M	0.9 ± 0.6	Nasci et al. (1989), fs
UF/ min/ mg prot			
B[a]P metabolism	DG/ M	0.035	Stegeman (1985), fs
nmol/ min/ mg prot	G/ M	0.013	Stegeman (1985), fs
	M/ M	ND	Stegeman (1985), fs
GST[4]	DG/ C	55.9- 82.3	Solé et al. (1996), fs
nmol/ min/ mg prot	DG/ M	8.5- 25	Solé et al. (1996), fs
	DG/ C	65 ± 15	Unpublished results, ms

recorded in this tissue until now. As a result, B[a]P biotransformation is much more efficient in the digestive gland than in the other tissues. The digestive gland in this experiment, is the tissue presenting the highest B[a]P concentration and known phase I activities (Table 1), it is hence not surprising to record in this tissue the highest DNA binding values. It is also important to underline that phase I activities have been reported to be induced by aromatic compounds, demonstrating the existence of adaptive mechanisms in polluted environment. The discontinuous increase of DNA binding with time, notably in the digestive gland (jagged pattern) may reflect the existence of an opposite process. A decrease observed after depuration support this hypothesis. In vertebrates, DNA repair mechanisms have already been demonstrated. In mollusks, no similar mechanism has been clearly identified.

If we consider that all the B[a]P taken up via feeding per individual was available, 6.9 % of the total dose applied is recovered in whole mussel, B[a]P transfer from tissue to DNA is low, less than 0.1% (Table 2.).

Table 2. Tissue distribution and DNA transfer of recovered B[a]P [1] in relation to the concentration of the corresponding tissue, ND: undetermined.

	Digestive gland	Gills	Mantle	Rest
Tissue distribution (%)	57	20	16	7
% of DNA binding[1]	0.1	0.08	ND	ND

These results show that the mesocosm system developed is suitable for the study of DNA adduct formation in mussel. B[a]P in feed is bioavailable for the organisms: for the dose applied, its accumulation and biotransformation in mussel result in DNA binding of B[a]P metabolites. Because B[a]P bioaccumulation and DNA binding vary from one tissue to another due to differences in uptake and biotransformation abilities, DNA adduct formation is expected to occur to different extents among various tissues. Data obtained during depuration demonstrate the ability of mussel to recover from B[a]P exposure in term of internal tissue dose and DNA damage.

DNA adduct formation is the resulting integration of both absorption, metabolism and detoxification processes. In mussel, persistence of genotoxin-DNA adducts has been recently demonstrated in *Mytilus edulis* (Harvey and Parry, 1998) pointed out the limited capacity of the DNA repair mechanisms in mussel. Because mussels are chronically exposed to pollutants in the marine environment, DNA adduct measurement appears so of considerable promise as a cumulative index of current and past exposure to genotoxin compounds. Further work based on this mesocosm will be carried out to improve the knowledge on stable and depurinating adduct formation in this sentinel organism.

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