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Multixenobiotic resistance protein expression in *Mytilus edulis*, *M. galloprovincialis* and *Crassostrea gigas* from the French coasts

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ABSTRACT: Multixenobiotic resistance (MXR) is a membrane-transport mechanism that allows organisms to exclude many compounds from their cells and tissues. It is thus a first line of defence against a variety of toxic compounds. Since mussels and oysters possess MXR proteins, an analysis of the expression level of these membrane-transporters has been conducted in relation to their body burden of some major environmental contaminants. Mussels *Mytilus edulis* and *M. galloprovincialis* and the oyster *Crassostrea gigas* were sampled from a total of 43 sites along the French coasts. High expression levels were found in animals from the major French estuaries (Seine, Loire and Gironde), at a few sites in Brittany and in nearly all sites from the Mediterranean mainland coasts. Multivariate analysis of the data for both species of blue mussel did show significant differences between groups of samples. Results indicated that expression of MXR protein was strongly associated with contaminant concentrations in mussels, and that polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) were directly correlated with MXR protein concentration. However, multivariate analysis of oysters, which were collected in less-contaminated sites, at least for organic pollutants, did not show any significant differences between MXR protein expression and contaminants. Although the results do not infer a causal linkage between mussel MXR protein, PAHs and PCBs, since many other chemical contaminants are also present at some sites, they do show clearly that MXR protein expression can be used as an indicator of pollutant exposure in blue mussels. The findings also highlight the need to use alternative analytical methods for the interpretation of complex environmental data, and that non-parametric multivariate statistical methods are appropriate for this task.

KEY WORDS: MXR · P-glycoprotein · Biomonitoring · *Mytilus edulis* · *Mytilus galloprovincialis* · *Crassostrea gigas*

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INTRODUCTION

Mussels and oysters have gained recognition as 'indicator organisms', serving as biomonitors to detect environmental perturbations that may affect living organisms (Philips 1978, Cantillo 1998). The rationale for a bivalve sentinel system is based on several factors (Farrington et al. 1983): they are cosmopolitan, sedentary, filter feeders, relatively pollutant resistant and commercially valuable organisms. Bivalve species are concentrating and integrating chemical exposure as they filter water for food. Moreover, their biological responses and mechanisms of adaptation can be used to provide insight into the potentially detrimental effects of their surrounding environment. Consequently, much ecotoxicological research and many monitoring studies have focused on mussels and oysters (Gosling 1992, Moore et al. 2004).

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Several studies have shown that mussels and oysters possess a multixenobiotic resistance (MXR) mechanism (Minier et al. 1993, Lüdeking & Köhler 2002) similar to the multidrug resistance (MDR) phenomenon first discovered in mammalian cells and later identified in many other organisms including bacteria, plants, fungi, invertebrates and vertebrates (Lage 2003). Characteristics of this defence system have been established in studies on mammalian cell lines isolated for resistance to a single cytotoxic drug, which subsequently were shown to be cross-resistant to numerous structurally and functionally unrelated drugs (Biedler & Riehm 1970, Akiyama et al. 1985). The cytotoxic compounds are predominantly alkaloids and antibiotics of plant and fungal origin (Gottesman & Pastan 1993). The pharmacological basis for the resistance appears to be decreased drug accumulation and retention, mediated by the ATP-consuming action of an integral membrane P-glycoprotein (Pgp) (Bodo et al. 2003). This transport protein, a member of a wide family of traffic ATPases termed 'ABC transporters', is always expressed in normal human tissues. Its localisation in kidney, liver, pancreas, intestine, brain and testis suggests that this protein plays a role either in preventing the absorption or, otherwise, in the transepithelial and transendothelial transport of toxic xenobiotics or endogenous metabolites (Cordon-Cardo et al. 1990, Schinkel & Jonker 2003).

Mussels and oysters possess several ABC-related genes (Lüdeking & Köhler 2002, Minier et al. 2002) and express 1 or 2 proteins immunologically related to mammalian Pgp (Minier et al. 1993). Bivalve cells are able to bind and transport anticancer compounds against which mammalian cells are resistant (Galgani et al. 1995, Minier & Moore 1996). It has been proposed that these molluscan Pgp could be part of a defence system against toxic compounds including anthropogenic wastes (Smital et al. 2004). Several studies performed on mussels support this hypothesis: (1) transport of rhodamine, a fluorescent compound used to obtain preliminary indication of the occurrence of multi-drug transport activity, can be modulated in a competitive manner by environmental contaminants (reviewed in Bard 2000); (2) increased Pgp expression level and rhodamine exclusion activity can be experimentally induced by treating aquatic organisms with drugs or environmental contaminants including polycyclic aromatic hydrocarbons (PAHs) (Smital et al. 2003); (3) field studies indicate that mussel MXR protein expression or activity is related to tissue pollutant concentrations (Minier et al. 1993, Kurelec et al. 1996, Smital et al. 2003).

Since the MXR expression may be related to pollutant exposure, assessment of the tissue protein level is a potentially powerful environmental monitoring tool. In this study we used the C219 monoclonal antibody for assessing MXR expression levels in mussels and oysters collected at 43 sites along the French coasts. Relationships between MXR expression and body burdens of some major chemicals of concern found in bivalves were evaluated.

MATERIALS AND METHODS

Cells and animals. Bivalves were collected at 43 sites along the French coasts (Fig. 1) in October and November 1993. Depending on the sites and occurrence of the species of interest, mussels *Mytilus edulis* (15 sites, size 4 to 5 cm) and *M. galloprovincialis* (10 sites, size 4 to 6 cm) or oysters *Crassostrea gigas* (18 sites, size 5 to 7 cm) were sampled. Bivalves used for biological analysis were immediately frozen and kept at –30°C until further processing (within 3 mo), while samples used to assess contaminant body burdens were first cleaned of epibiota and then depurated for 24 h in decanted seawater from the collection site in order to eliminate faeces and pseudofaeces. Soft tissues were removed from the shell using a stainless-steel scalpel, homogenized in a Virtis grinder and freeze-dried. The alga *Prorocentrum micans* Ehrenberg, cultured in the laboratory for many generations in toxic-free medium, i.e. in filtered (4 and 0.2 µm) and sterilized (20 min at 120°C) Provasoli enriched seawater (Provasoli et al. 1957), was used as a P-glycoprotein (Pgp) negative control (Minier et al. 1993).

Analysis of P-glycoprotein. *Prorocentrum micans* cells and roughly 400 mg of each bivalve gill sample were sonicated twice for 10 s at 20 000 Hz and 70 W in Tris-buffered saline (TBS: Tris/HCl 20 mM, 137 mM NaCl, pH 7.6) with 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02 TUI ml^{-1} aprotinine. Sodium dodecyl sulphate (SDS) was then added at a final concentration of 0.1%, and the homogenates were centrifuged at $4000 \times g$ for 10 min at 4°C. Supernatant protein content was determined using the BCA protein assay reagent (Pierce) with bovine serum albumin (BSA) as a standard.

For Western blot analysis, gel electrophoresis of protein extracts (20 µg per well) was performed in 9% polyacrilamide gels containing 0.1% (w/v) SDS. Proteins were transferred to nitro-cellulose membranes at 500 mA for 50 min. For dot blot analysis, 10 µl of protein extracts (2 to 10 µg) were spotted onto nitro-cellulose sheets (MSI, hybridisation transfer membrane). C219 monoclonal antibody (CIS Biointernational Nantes, France) was used as primary antibody (1 µg ml^{-1}). This monoclonal antibody, raised against hamster MDR carrier is specific for a highly conserved sequence among MXR proteins (Georges et al. 1990).

MXR proteins were revealed using the enhanced chemiluminescence assay (Whitehead et al. 1979). Blots were incubated in luminol provided in the ECL™ Western blotting kit (Amersham) following manufacturer's instructions, and light emission was detected after 1 min exposure to autoradiography film. Staining responses were then quantified using a densitometer.

Ten mussel protein extracts (from 10 individuals) for each sample site were analysed. To provide semi-quantitative results, each blot was loaded with 1 µg of negative (algae protein extracts) and positive samples (pooled mussel extracts from Sites 6 and 40).

Chemical analysis. Analytical procedure for organic compounds included a solid–liquid (Soxhlet, hexane: acetone 80:20) extraction step on freeze-dried samples followed by adsorption chromatography on open alumina–silica columns to remove lipids and other unwanted organic material. Total PAHs were analyzed by high performance liquid chromatography with spectrofluorometric detection (Michel 1983). Organochlorine compounds, including polychlorinated biphenyls (PCBs) and dichloro-diphenyl-chloroethanes (dichloro-diphenyltrichloro-ethane, DDT, dichloro-diphenyl-dichloro-ethane, DDD, and dichloro-diphenyl-ethane, DDE) were analyzed by gas chromatography with an electron capture detector (Luçon & Michel 1986).

Mercury was determined by flameless atomic absorption spectrophotometry (Thibaud 1983a). The other metals (cadmium, copper, zinc and lead) were determined by atomic absorption spectrophotometry equipped with electrothermal atomisation (Thibaud 1983b).

Results of the chemical analysis are part of the data from the French mussel watch, which analyzes 43 sites along the coasts 4 times a year since 1979. Previous results corresponding to the period 1979 to 1989 and full description of the techniques used have been previously published (Claisse 1989).

Statistical analysis. MXR protein expression and contaminant data for *Crassostrea gigas*, *Mytilus edulis* and *M. galloprovincialis* were analysed using non-parametric multivariate analysis software, PRIMER ver. 6 (PRIMER-E Ltd, Plymouth, UK; Clarke 1999). Cluster analysis and non-metric multi-dimensional scaling (MDS) analysis, derived from Euclidean distance similarity matrices was used to visualise dissimilarities between sample groups. All data were log-transformed and normalised prior to analysis. The results were further tested for significance using analysis of similarity (PRIMER ver. 6 ANOSIM), which is analogous to a univariate ANOVA and reflects on differences between treatment groups in contrast to differences among replicates within samples (the R

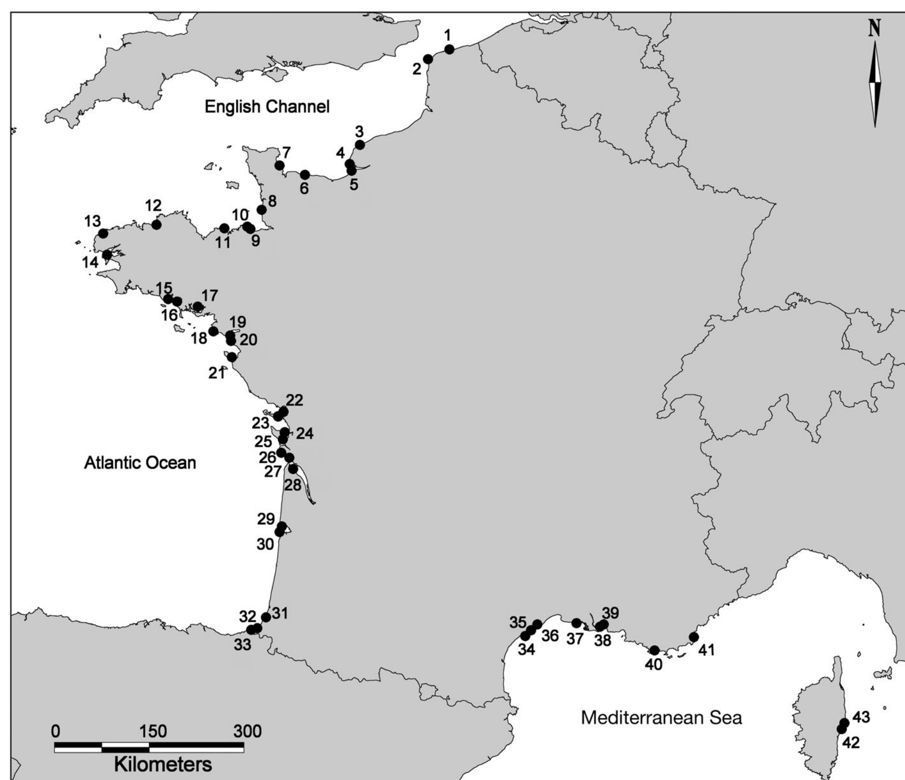

Fig. 1. Locations of sampling sites for mussels and oysters along the French coasts

statistic). Under the null hypothosis, H_0 ('no difference between samples'), R = 0, and this was tested by a non-parametric permutations approach; there should be little or no effect on the average R value if the labels identifying which replicates belong to which samples are randomly rearranged (Clarke 1999).

Pairwise scatterplots (draftsman plots) were used to examine the relationships between the variables.

Finally, the PRIMER ver. 6 BIO-ENV routine linking multivariate patterns was used to identify 'influential parameters'—small subsets of MXR and chemical parameters capturing the full MDS pattern.

RESULTS

Contaminant body burdens

Sites where the oysters *Crassostrea gigas* were collected are characterised by high concentrations of metals that accumulated in the mollusc tissues (Table 1). Although differences in bioconcentration factors are well described between mussels and oysters (O'Connor 2002), the results highlighted the fact that the southwest coastline is highly contaminated, especially with cadmium, copper and zinc. The Gironde Estuary

Table 1. *Mytilus edulis*, *M. galloprovincialis* and *Crassostrea gigas*. Concentration of metals and organic pollutants along the French coasts. Total: DDD + DDE + DDT. Metals and PAH are in µg g^{-1} dry wt; other compounds are in ng g^{-1} dry wt

Site	Cd	Cu	Hg	Pb	Zn	DDD	DDE	DDT	Total	α-HCH	γ-HCH	PAH	PCB
Mytilus edulis													
1	0.42	7.28	0.07	0.98	66.75	3.70	6.05	12.10	21.85	1.57	4.40	3.07	653.50
2	0.66	7.58	0.10	1.90	75.25	4.10	15.15	9.35	28.60	0.80	2.98	2.26	598.50
3	3.20	8.75	0.52	2.75	78.00	4.55	7.60	21.50	33.65	1.10	3.07	3.85	1286.25
4	4.35	8.10	0.16	2.55	121.75	13.95	19.70	17.05	50.70	0.67	4.08	9.40	3612.25
5	3.85	8.33	0.13	2.90	94.75	12.70	18.15	6.90	37.75	0.67	4.58	6.77	3091.00
6	1.25	7.13	0.13	1.70	61.00	4.55	6.10	9.05	19.70	0.85	2.50	3.86	804.00
7	0.60	5.30	0.05	1.10	46.75	2.85	8.15	20.15	31.15	1.40	9.20	1.05	308.50
8	0.40	6.70	0.09	1.45	57.50	1.30	5.25	3.25	9.80	0.45	2.43	1.87	241.25
9	0.35	6.33	0.07	1.10	47.00	1.45	4.40	0.75	6.60	1.07	4.63	1.34	154.00
11	0.30	5.90	0.08	0.80	44.00	2.15	3.35	2.80	8.30	1.57	2.38	1.04	187.75
12	0.76	5.45	0.13	2.60	224.75	1.70	1.00	1.80	4.50	1.05	2.53	2.88	351.00
15	0.89	8.15	0.12	1.75	214.00	7.25	12.50	18.70	38.45	1.17	7.78	11.86	512.25
18	0.57	5.70	0.11	2.43	84.00	3.45	8.25	4.65	16.35	0.60	12.37	5.60	409.75
19	0.87	6.98	0.07	2.58	77.25	9.90	15.55	1.60	27.05	0.90	4.80	1.87	863.75
20	1.06	7.65	0.11	3.30	104.25	6.75	10.35	3.25	20.35	0.50	4.33	2.00	508.50
Mytilus galloprovincialis													
34	0.74	9.00	0.12	6.60	220.67	22.65	33.15	11.95	67.75	0.43	3.18	3.68	335.00
35	0.95	7.58	0.05	1.50	148.75	17.00	94.65	17.85	129.50	1.27	5.27	3.73	393.50
36	0.74	7.35	0.06	1.70	140.50	17.20	28.55	12.10	57.85	0.73	2.55	2.91	425.75
37	1.09	6.30	0.11	2.30	199.00	23.75	27.45	25.95	77.15	1.15	2.88	2.52	478.25
38	0.39	5.48	0.05	1.55	117.00	25.85	23.00	19.30	68.15	0.93	4.63	4.50	438.50
39	0.52	6.70	0.21	3.10	119.00	18.10	20.35	8.40	46.85	0.43	2.58	5.33	486.75
40	0.71	7.73	0.53	8.33	171.00	7.75	9.05	4.40	21.20	0.40	1.70	6.65	521.25
41	1.07	6.85	0.20	6.05	314.25	2.70	6.60	3.85	13.15	0.85	2.70	3.86	225.25
42	0.61	5.53	0.10	0.40	91.25	1.80	3.20	2.50	7.50	0.77	2.67	1.50	80.50
43	0.53	5.15	0.13	0.75	146.50	3.45	4.95	3.35	11.75	0.80	2.97	1.83	133.25
Crassostrea gigas													
10	2.03	142.73	0.20	1.63	2053.33	2.15	5.50	1.95	9.60	0.70	2.98	2.66	168.75
13	0.98	40.68	0.08	0.83	922.50	4.60	6.50	4.70	15.80	0.80	2.00	1.85	388.75
14	1.09	59.45	0.15	5.93	1261.00	5.05	6.35	7.15	18.55	9.40	16.00	2.11	308.25
16	1.35	51.65	0.20	1.20	1952.00	2.75	5.20	10.05	18.00	1.47	8.48	1.25	237.50
17	1.34	128.60	0.15	1.15	2598.75	5.60	9.95	6.05	21.60	1.07	6.23	1.61	303.25
21	1.82	145.68	0.26	1.60	2282.75	6.30	8.25	1.50	16.05	1.15	3.95	0.92	203.00
22	3.33	470.28	0.26	1.63	3803.00	11.75	17.45	1.65	30.85	1.98	6.40	1.41	207.25
23	2.72	191.18	0.27	1.53	2989.00	20.55	18.60	4.60	43.75	1.68	6.33	1.52	252.25
24	4.79	398.83	0.30	1.73	3422.25	8.65	13.20	2.75	24.60	20.80	8.58	1.80	210.25
25	5.68	288.23	0.35	1.45	3860.75	8.45	21.00	3.00	32.45	10.95	7.25	1.52	256.25
26	11.27	314.80	0.19	1.80	2577.75	16.75	24.10	8.95	49.80	16.20	7.43	1.54	462.25
27	30.17	1064.20	0.22	2.20	6062.00	18.90	68.80	11.45	99.15	14.70	6.73	2.19	500.00
28	55.97	985.20	0.22	2.58	3972.25	20.60	39.90	2.05	62.55	2.20	3.43	2.44	583.25
29	1.90	92.98	0.17	1.40	2768.25	15.25	9.55	10.45	35.25	2.15	4.05	1.64	192.25
30	1.13	135.58	0.22	1.25	2717.75	16.80	14.05	9.75	40.60	1.00	3.68	3.24	143.75
31	2.86	335.45	0.23	2.18	3999.25	6.55	18.10	6.05	30.70	1.63	6.98	4.28	434.25
32	1.36	269.45	0.18	2.43	4026.50	25.65	43.95	21.90	91.50	1.73	5.10	51.93	800.75
33	1.97	559.10	0.17	3.83	4881.25	10.40	37.85	14.25	62.50	3.73	4.23	17.02	971.25

(Sites 26 to 28) contributes to the greatest environmental problem brought to light by the mussel watch program (Boutier & Chiffoleau 1986). Cadmium body burdens were in the range 10 to 60 μg g^{-1} dry wt of tissues.

High PCB and PAH concentrations were measured in *Mytilus edulis* in the Seine Estuary (Sites 4 and 5). The measured PAH concentrations were above 6000 ng g^{-1} dry wt of mussel tissues, and the PCB body burdens exceeded 3000 ng g^{-1} dry wt of mussel tissues (Table 1). The Seine Estuary is thus one of the most highly polluted sites in the world in regard to such compounds (Minier et al. 2006).

In the Mediterranean Sea, the mussels *Mytilus galloprovincialis* were mainly characterised by their relatively high levels of DDD, DDE and DDT accumulated in the tissues. The sum of the 3 compounds was 50 to 130 ng g^{-1} dry wt of mussel tissues for Sites 34 to 39. In contrast, Corsican sites (Sites 42 to 43) had relatively low body burdens for all measured contaminants.

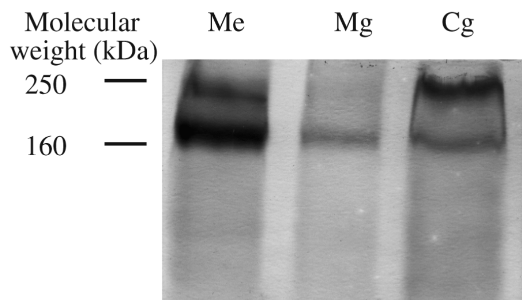

Fig. 2. Western blot of *Mytilus edulis* (Me), *M. galloprovincialis* (Mg) and *Crassostrea gigas* (Cg) protein extracts using the C219 monoclonal antibody

MXR expression levels

Analysis of the Western blots obtained with the C219 monoclonal antibody showed that mussels *Mytilus edulis* and *M. galloprovincialis* and oysters *Crassostrea gigas* express MXR proteins of approximately 170 and 220 kDa (Fig. 2). Despite some staining responses sometimes found for low molecular weights and likely to correspond to degraded products, the C219 staining was specific as no cross reaction was observed with the whole-cell *Prorocentrum micans* extracts. The dot blot method was thus used to obtain staining responses related to the protein quantities spotted on nitrocellulose sheets. A significant linear correlation ($r = 0.92$, $p < 0.001$, $n = 8$) between staining intensity and protein quantities was observed in the range of 0.2 to 20 μg of proteins.

Fig. 3 shows the MXR-protein expression levels in bivalves across all 43 sites. Significant differences were found for each bivalve species in relation to the sampling sites ($p < 0.05$). Although comparisons might not be valid between species, analysis of the results showed highest expression levels in the Seine Bay and a little further north, at 2 sites in Brittany, at the Loire and Gironde estuaries and in the Mediterranean Sea (with the noteworthy exception of the Corsican sites—Sites 42 and 43).

Statistical analysis

Multivariate analysis of oyster data did not show any significant differences between MXR protein expression and contaminant burden. However, multivariate

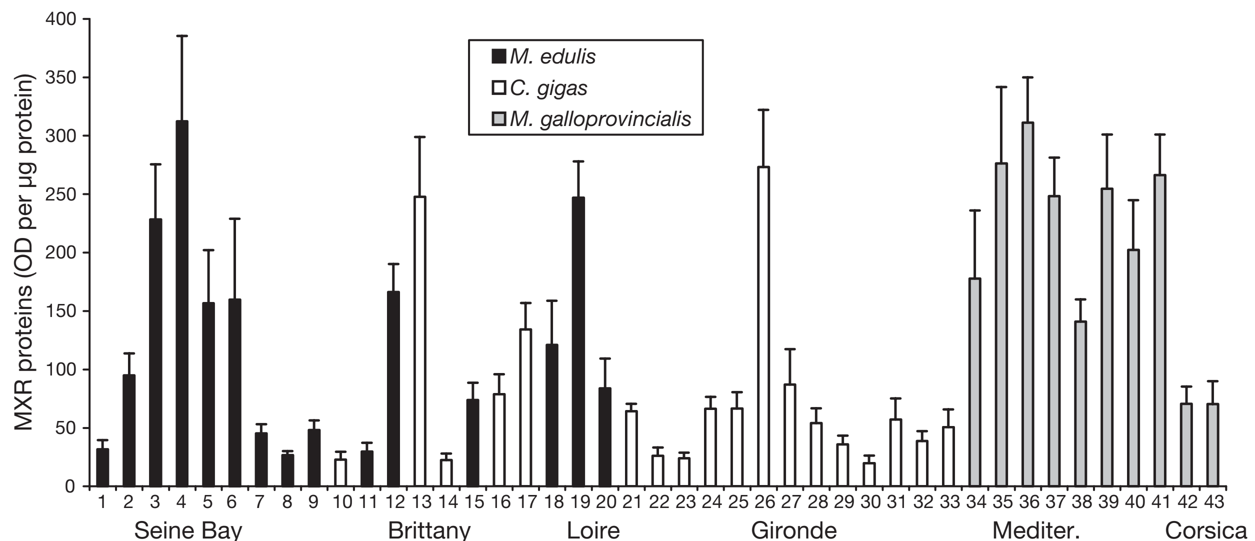

Fig. 3. *Mytilus edulis*, *M. galloprovincialis* and *Crassostrea gigas*. MXR-protein expression levels in mussel and oyster gills along the French coasts. Bars indicate 95% confidence intervals. OD: optical density

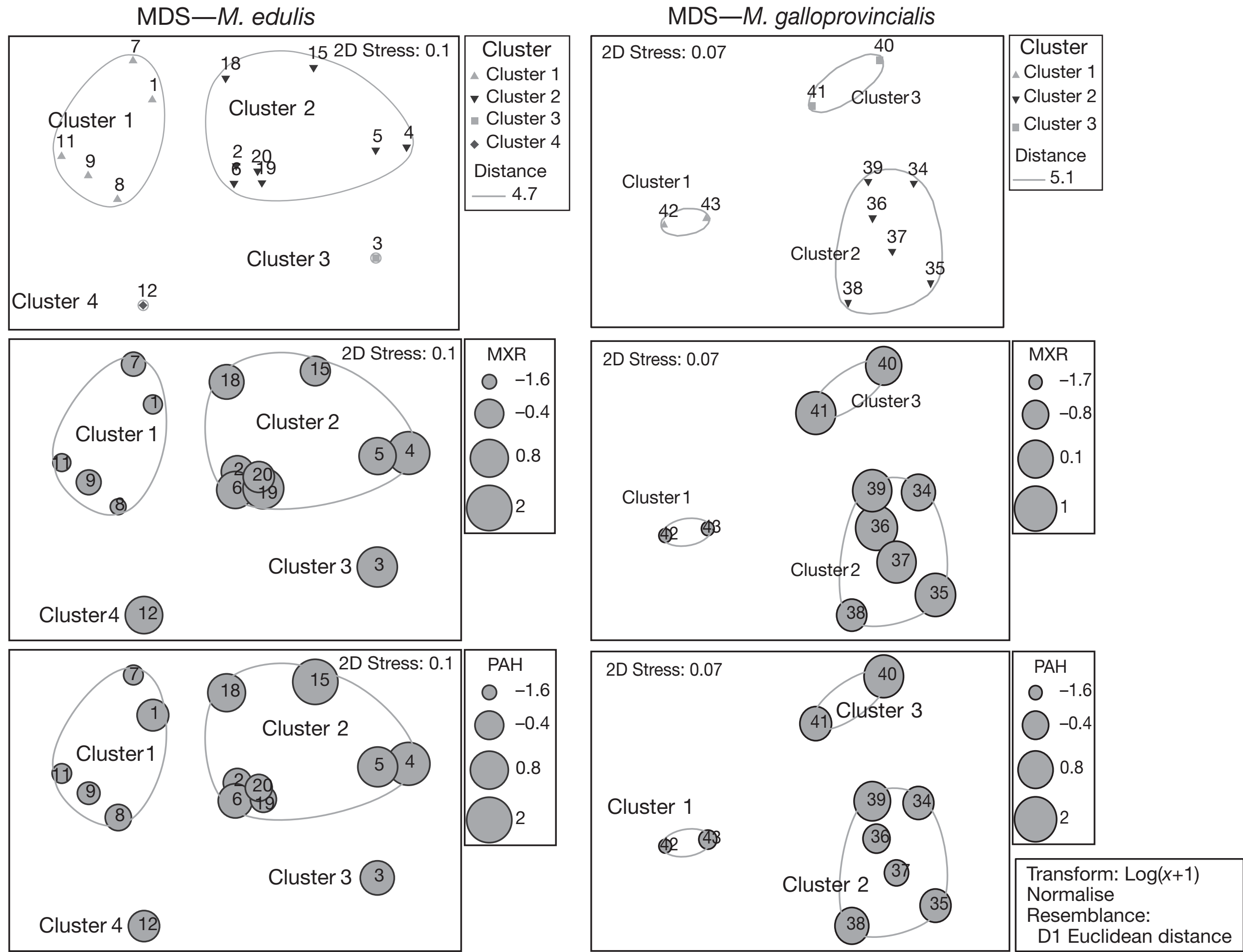

Fig. 4. *Mytilus edulis* and *M. galloprovincialis.* Results of multivariate analysis of MXR protein and all measured contaminants for both species of blue mussel. Non-metric multidimensional scaling analysis with superimposed cluster analysis indicates 4 distinct groups for *M. edulis* (MDS stress 0.1) and 3 for *M. galloprovincialis* (MDS stress 0.07). Bubble plots are shown for MXR protein expression and PAHs only as representative ubiquitous indicators of pollution

analysis using MDS, cluster analysis and ANOSIM of blue mussel data did show significant differences between groups of samples when the MXR protein and all of the pollutant data were analysed together for *Mytilus edulis* and *M. galloprovincialis* respectively (Fig. 4, Table 2). These findings indicate that that increased MXR protein is significantly associated with increased pollution in both species of mussel.

Since previous data have indicated that induction of MXR protein is linked with PAHs and PCBs, the data sets were reanalysed using only MXR protein, PAH and PCB concentrations. The results of these analyses are shown in Fig. 5; there is a general similarity to the clusters obtained using all of the pollutant data (MDS stress values are reduced in comparison with the full data sets). Sites 4 and 5 at the mouth of the Seine (*Mytilus edulis* from Le Havre and Villerville) are a distinct group in Cluster 4 (Fig. 5); both sites are characterised by the highest tissue concentrations of cadmium, PCBs and PAHs.

Table 2. *Mytilus edulis* and *M. galloprovincialis.* ANOSIM 1-way analysis of similarity (R statistic) for blue mussel data (only statistically significant comparisons are shown)

Variables	Global test	Clusters 1 & 2	Clusters 1 & 4	Clusters 2 & 4
Mytilus edulis				
All parameters	R = 0.764	R = 0.683		
	p ≤ 0.001	p ≤ 0.002		
MXR protein,	R = 0.919	R = 0.876	R = 1.000	R = 0.948
PAH, PCB	p ≤ 0.001	p ≤ 0.001	p ≤ 0.048	p ≤ 0.028
Mytilus galloprovincialis				
All parameters	R = 0.824	R = 1.000		
	p ≤ 0.002	p ≤ 0.036		
MXR protein,	R= 1.000	R = 1.000		
PAH, PCB	p ≤ 0.022	p ≤ 0.022		
Combined species				
MXR protein	R= 0.870	R = 0.858	R = 0.996	R = 0.976
PAH, PCB	p ≤ 0.001	p ≤ 0.001	p ≤ 0.022	p ≤ 0.010

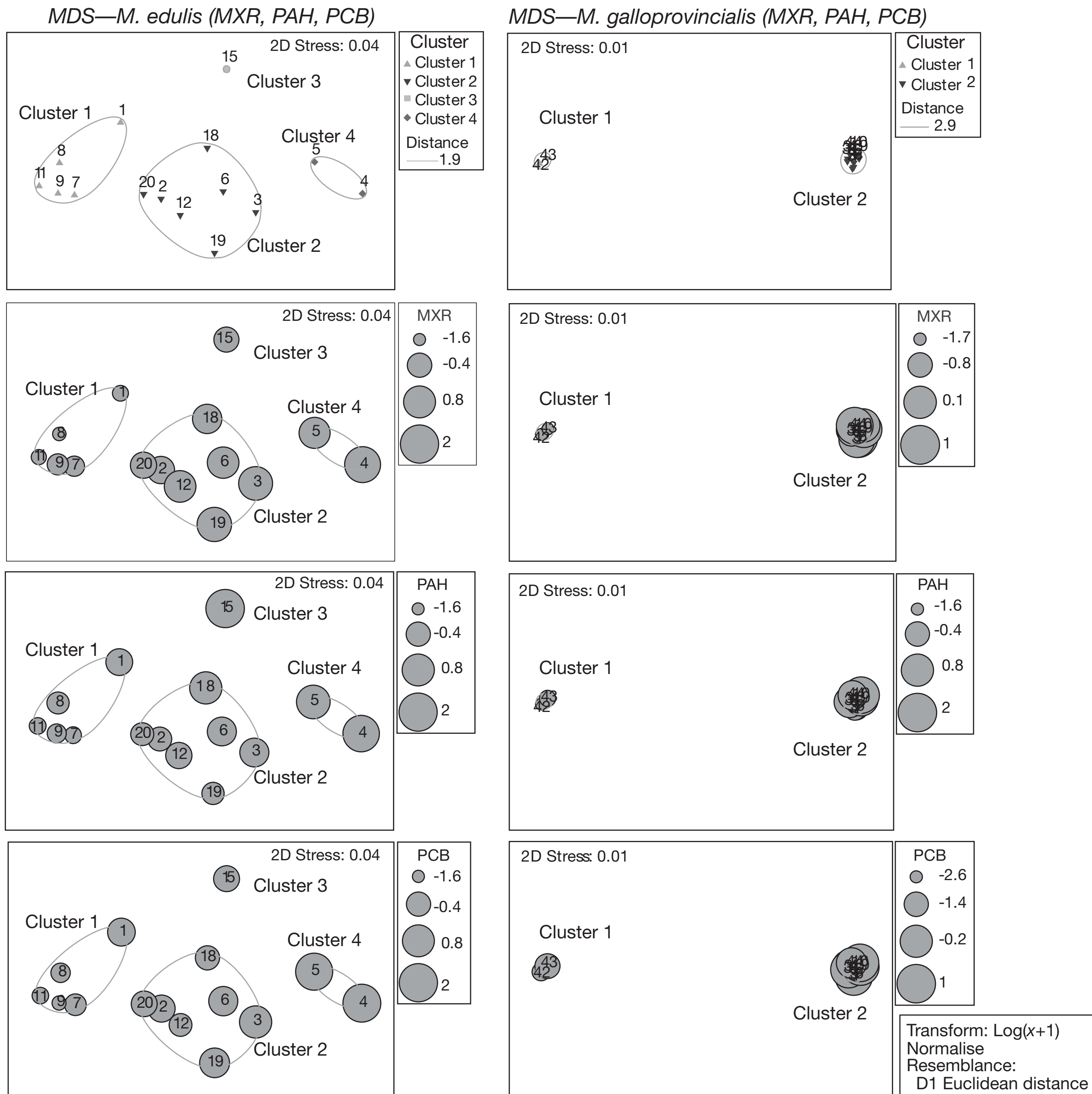

Fig. 5. *Mytilus edulis* and *M. galloprovincialis.* MDS and superimposed cluster analysis for MXR protein, PAHs and PCBs only. Cluster analysis indicates 4 distinct groups for *M. edulis* and 2 groups for *M. galloprovincialis*. Bubble plots are shown for MXR protein expression, PAHs and PCBs

ANOSIM indicated significant differences between the relatively less-contaminated sites in Cluster 1 for both species compared with the more contaminated clusters (Table 2, Fig. 5). Spearman rank correlations derived from the BIOENV routine indicated that the 3 parameters essentially captured the full MDS profile for both species of blue mussel (Table 3).

MDS and cluster analysis of combined species data for MXR protein, PAHs and PCBs from both species of blue mussels essentially showed a simple combination of the separate analyses (Figs. 6 & 7). ANOSIM showed that there were significant differences between Cluster 1 and Clusters 2 and 4, as well as between Clusters 2 and 4 (Table 2, Fig. 7). The only noticeable difference in the MDS plot was a shift for Sites 2 and 20 from Cluster 2 in the *Mytilus edulis* analysis to Cluster 1 in the combined analysis; and again, Sites 4 and 5 at the mouth of the Seine formed a distinct cluster. These analyses indicate that it was probably a reasonable assumption to treat both closely related species together.

Table 3. *Mytilus edulis* and *M. galloprovincialis.* Spearman rank correlations (PRIMER ver. 6 BIOENV) for separate and combined blue mussel data. Variables: (1) MXR protein; (2) PAHs; (3) PCBs

Number of variables	Correlation	Selections
Mytilus edulis		
3	1.000	1, 2, 3
2	0.913	1, 2
2	0.857	2, 3
2	0.825	1, 3
Mytilus galloprovincialis		
3	1.000	1, 2, 3
2	0.961	1, 2
2	0.948	2, 3
2	0.816	1, 3
Combined species		
3	1.000	1, 2, 3
2	0.871	1, 2
2	0.869	1, 3
2	0.838	2, 3

DISCUSSION

Immunochemical analyses have proven very useful and accurate in assessing multixenobiotic resistance protein expression levels in mammals (Fredericks et al. 1991), and such experiments have been proposed as a tool for assessing the cell degree of resistance (Chan et al. 1990) and for predicting the response of tumour cells to chemotherapy (Leonard et al. 2003, Efferth & Volm 2005). In this study, MXR expression was assessed in both mussels and oysters along the French coasts. High P-glycoprotein concentrations were found in samples collected in the Seine Bay and the Loire and Gironde estuaries, which are likely to be areas impacted by numerous xenobiotics carried by the rivers. High expression levels were also found in nearly all sites from the Mediterranean mainland coasts, and multivariate analysis of pooled blue mussel data did show that expression of MXR protein was strongly associated with contaminant concentrations in mussels. With the exception of results from the Mediterranean Sea, a similar pattern of biological response has previously been observed with other biomarkers such as P-450-mediated ethoxyresorufin O-deethylase activity (Burgeot et al. 1994), acetylcholine-esterase activity (Bocquené et al. 1993) and the micronucleus assay (Burgeot et al. 1995).

The physiological function of Pgp is not known in detail but might include detoxification and excretion of xenobiotics (Bodo et al. 2003). Differential MXR protein levels in bivalves might be related to the presence of toxic compounds inducing its expression as seen in mammals. Indeed, in knockout mice, which lack various P-glycoprotein isoforms, tissue distribution and elimination of drugs are affected (Schinkel et al. 1994). A 2- to 20-fold increase in accumulation and subsequent increase in toxicity have been reported in various organs when compared with wild type mice, demonstrating that these proteins are essential in preventing the accumulation of xenobiotics in multiple organs. Similarly, mussel MXR proteins are induced by exposure to toxic compounds (Minier & Moore 1996,

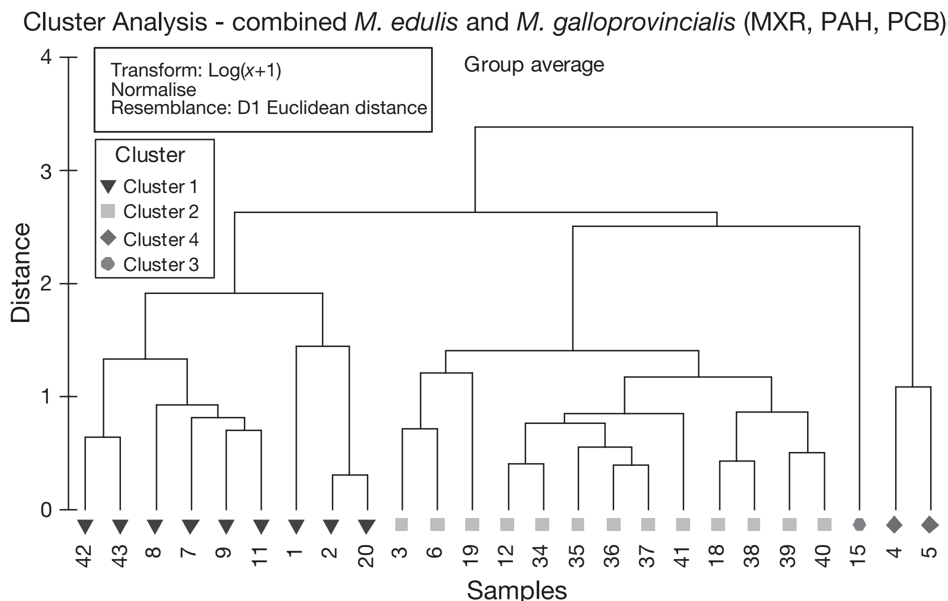

Fig. 6. *Mytilus edulis* and *M. galloprovincialis.* A dendrogram derived from cluster analysis for combined mussel data for MXR protein, PAHs and PCBs. Sites 1–9, 11, 12, 15 and 18–20 are *M. edulis* and Sites 34–43 are *M. galloprovincialis*

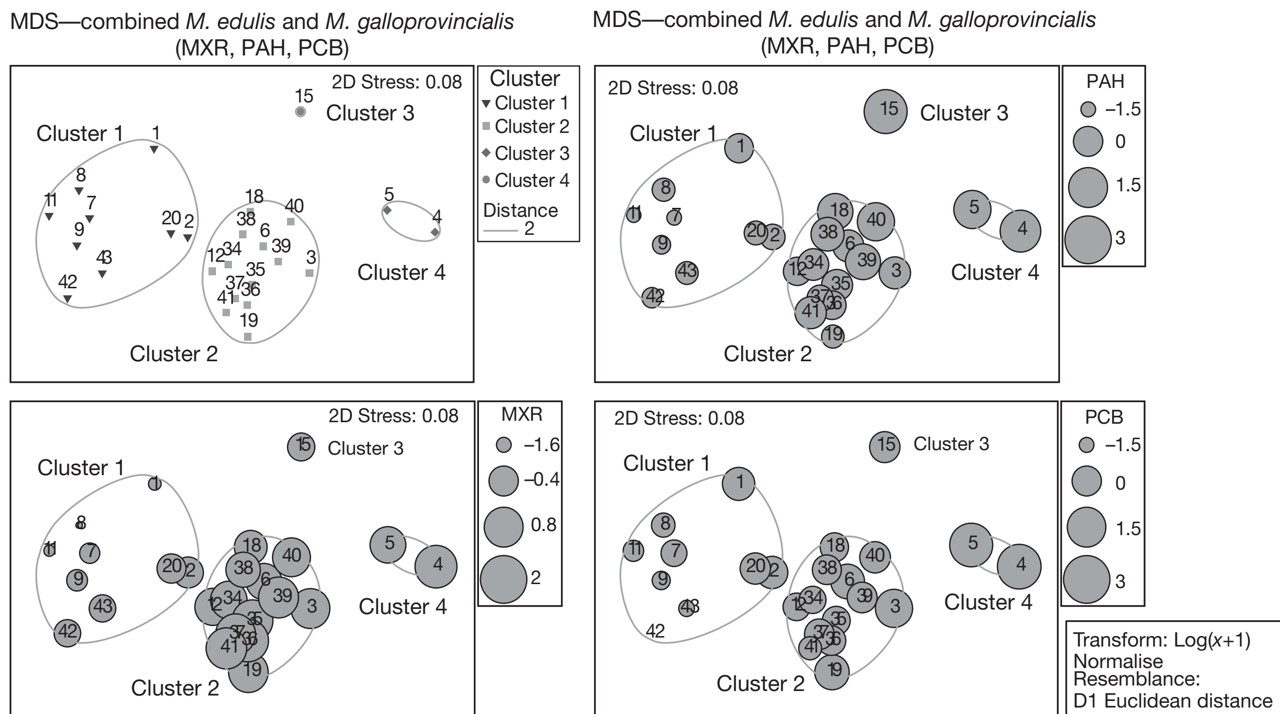

Fig. 7. *Mytilus edulis* and *M. galloprovincialis.* MDS (stress 0.08) and superimposed cluster analysis for MXR protein, PAHs and PCBs only in combined data for both species of blue mussel. Cluster analysis indicates 4 distinct groups. Bubble plots are shown for MXR protein expression, PAHs and PCBs

Smital et al. 2003). In this study, the results of the multivariate analysis show clearly that MXR protein is strongly correlated with PAHs and PCBs. This does not imply a causal linkage, since it is probable that many other chemical contaminants, not included in the chemical determinations, are also present at some sites. However, exposure to PAHs has been implicated in induction of MXR expression in mussels (Kurelec 1995, Smital et al. 2003) and other organisms (Hamdoun et al. 2002). To our knowledge, PCBs have not yet been used as inducers *in vitro*. But studies on mussels have already shown that these compounds can interact with the mussel MXR system, thus altering membrane transport. In *Mytilus galloprovincialis*, PCBs compete with rhodamine (a known Pgp substrate), leading to an increase in either dye accumulation or impaired export (Galgani et al. 1995). In *M. californianus*, the same effects on rhodamine transport were also correlated with MXR protein titer (Eufemia & Epel 1998).

No correlation was observed for *Crassostrea gigas* between MXR content and contaminant concentrations. In the oyster *C. virginica*, Keppler & Ringwood (2001) also failed to find any correlation between MXR proteins and pollutants in another field study. Differences in organism physiology or in site characteristics might explain these discrepancies. In this study, sites where oysters were collected were characterised by their high levels of metal contamination (especially cadmium, copper and zinc) as indicated by the mollusc body burdens. This specific pattern of contaminants may contribute to the absence of correlation, although cadmium has been shown to induce Pgp expression in other mollusc species (Eufemia & Epel 2000, Legeay et al. 2005).

The results of the multivariate analysis show clearly that MXR protein expression can be used as an indicator of pollutant exposure in blue mussels, but not oysters (at least in this study); and that up-regulated MXR protein expression is strongly linked with elevated contaminant burdens of PAHs and PCBs (Fig. 8). The findings demonstrate that multi-dimensional data can be effectively represented in a 2-dimensional way using multi-dimensional scaling. Empirical evidence and simulation studies of MDS stress values indicate that stress increases not only with reducing dimensionality but also with increasing quantity of data (Clarke & Warwick 2001). A general rule is that stress values less than 0.1 correspond to a good ordination with no real prospect of a misleading interpretation, and values <0.05 give an excellent representation with no prospect of misrepresentation (a perfect representation would probably be one with stress values < 0.01). Since all of the MDS-derived stress values were between 0.01 and 0.1, the interpretations are probably reasonable.

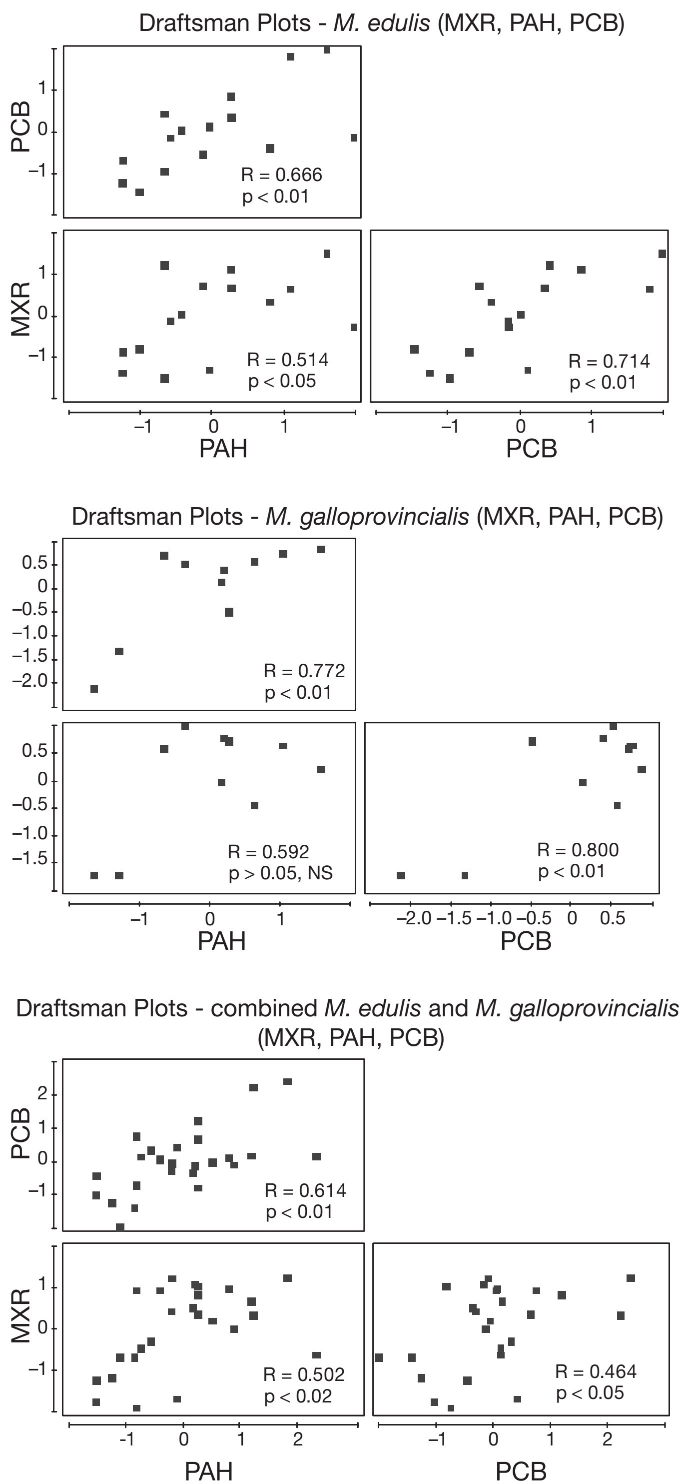

Fig. 8. *Mytilus edulis* and *M. galloprovincialis.* Draftsman plots for both species of blue mussel and combined data for MXR expression, PAHs and PCBs. Standard product moment correlation coefficients between every pair of variables are shown on each plot. It is clear from these pairwise scatterplots that MXR expression and concentration of PAHs and PCBs are correlated (all significant at $p < 0.05$, except for MXR vs PAH for *M. galloprovincialis*)

The selection of PAHs and PCBs as toxicologically meaningful variables along with MXR protein for MDS had the effect of reducing the stress in all cases.

The cluster and MDS analysis identifies mussels with either high or low MXR protein content. It also consistently shows that the samples from the mouth of the Seine (Sites 4 and 5) have different characteristics from the other groups (Figs. 4, 5 & 7). Since these animals (Sites 4 and 5) were characterised by the highest tissue concentrations of cadmium, PCBs and PAHs, a possible explanation may lie in the existence of a maximum MXR protein expression level that is reached when contaminant concentrations are high or, otherwise, through antagonistic interactions between contaminants. As the MXR transport mechanism is capable of saturation (Toomey & Epel 1993), doses exceeding the maximum induction level or the presence of compounds that interfere with normal MXR expression may endanger the health of the animals.

Overall, the findings highlight the need to use alternative analytical methods for the interpretation of complex environmental data, and that non-parametric multivariate statistical methods are appropriate for this task (Wedderburn et al. 1998, Astley et al. 1999, Galloway et al. 2002, Allen & Moore 2004).

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