Biomarkers, January–February 2005; 10(1): 58–71

Taylor & Francis
Taylor & Francis Group

Scale of classification based on biochemical markers in mussels: application to pollution monitoring in Mediterranean coasts and temporal trends

J. F. NARBONNE, N. AARAB, C. CLÉRANDEAU, M. DAUBÈZE, J. NARBONNE, O. CHAMPEAU, & P. GARRIGUES

Laboratoire de Physico et Toxicochimie des Systèmes Naturels, UPRESA CNRS 5472, Université Bordeaux, Talence, France

Abstract

A battery of biochemical parameters was used to evaluate the response of mussels to a contaminated coastal environment. A multimarker approach was developed, establishing a scale for the classification of the water quality in European coastal sites (BIOMAR European programme). This study allows the evaluation of the temporal trends of this scale when applied to selected sites of European Mediterranean coast (BEEP Biological Effects of Environmental Pollution in Marine Coastal Ecosystems: European programme). Acetylcholinesterase activity (AChE) is highly sensitive to organophosphorus and carbamate insecticides and, to some extent, also to heavy metals. Catalase activity (CAT) and lipid oxidation (evaluated as malonedialdehyde) are markers of oxidative stress, glutathione S-transferase (GST) activity is related to conjugation of organic compounds and benzo(a)pyrene hydroxylase activity (BPH) is a marker of effect of certain planar organic compounds (e.g. polycyclic aromatic hydrocarbons, PAHs). These parameters were measured either in gills (AChE, GST) or digestive gland (BPH, GST, CAT, MDA). For each biomarker, a discriminatory factor was calculated (maximum variation range/confidence interval) and a response index was allocated. For each site, a Multimarker Pollution Index (MPI) was calculated as the sum of the response index of each of the five more discriminating biomarkers. As the result of our calculation method, the quality of the coastal environment at each site can be classified according to a five levels scale. Samples collected for five cruises in May 2001, 2002, 2003, and September 2001 and 2002 showed MPI evolutions. The results show that water quality can be classified from class 1 (clean areas in some sites of France, Italy and Spain) to class 4 (high pollution in main harbours). Results of the use of the biomarker scale in WP3 (Work Package Concernant Biomonitoring Programmes in Mediterranean Sea) during the BEEP programme make a strong contribution to the establishment of standardized strategies and methods for internationally agreed protocols for biomarker-based monitoring programmes. In comparison with scale pollution methodology used in the BIOMAR programme, the main contribution of BEEP was (1) to select from discriminatory analysis the biomarkers to be included in calculation of scale pollution; (2) to improve the use of the biomarker index in order to identify the main contaminants by analysis of individual contributions to the MPI; and (3) to apply methodology for temporal trends at sampled sites.

Keywords: *Molecular biomarkers,* Mytilus galloprovincialis, *benzo(a)pyrene hydroxylase, glutathione S-transferase, catalase, malonedialdehyde, acetyl cholinesterase, Mediterranean Sea*

(Received 28 September 2004; accepted 27 January 2005)

Correspondence: J. F. Narbonne, Toxicologie Biochimique, LPTC CNRS 5472, Avenue des Facultés, F-33405 Talence Cedex, France. E-mail: jf.narbonne@lptc.u-bordeaux1.fr.

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500500071339

Introduction

Over the past decade, early molecular and cellular biomarkers have been extensively used in pollution monitoring in aquatic environments (McCarthy and Shugart 1990, Huggett et al. 1992). Biomarkers were selected among early molecular events occurring in the toxicological mechanisms of major contaminants (PAHs, PCBs, heavy metals, pesticides, etc.) and are potentially useful tools for detecting either exposure to, or effects of, chemicals. Benzo(a)pyrene hydroxylase (BPH) activity was used in a number of field studies in mussels and some correlations were found with PAH pollution (Suteau et al. 1988, Garrigues et al. 1990, Narbonne et al. 1991, Michel et al. 1994). Lipid peroxidation (malonedialdehyde, MDA formation), glutathione S-transferase (GST) and catalase (CAT) activities were found to be modulated by metal or organic contaminants both under field and laboratory exposure (Pellerin-Massicotte 1994, Prahash and Rao 1995, Regoli and Principato 1995). Recently, cholinesterase activities have appeared as biomarkers of exposure for some pesticides and other pollutants on wildlife (Boquene et al. 1990, Najimi et al. 1997). These data can be useful to scientists to evaluate the specificity of the responses to natural or anthropogenic changes, but it is very difficult for the environmental manager to interpret increasing or decreasing changes in biomarker data. Except for some examples in limited areas and time (Bayne et al. 1988, McCarthy et al. 1990), the translation of biochemical data into environmental information is limited due to the difficulty to interpret the temporal and spatial extent of biomarker variation. The practical approach carried out by the official agencies in charge of environmental survey is to establish indexes of environmental quality taking into account chemical or biological criteria in order to classify the sites being monitored in a scale from 'clean' to 'highly polluted' (usually four for microbiological criteria, or five levels for chemical criteria). Marine mussels are commonly used as sentinel organisms for the detection of environmental pollution in coastal waters due to their capacity to accumulate several organic and inorganic contaminants (Goldberg et al. 1975, Livingstone 1991). During the BIOMAR European programme (1994–98), the pollution index scale was developed and first applied to the European coast. Work Package 3 (WP3 Concernant Biomonitoring Programmes in Mediterranean Sea) was part of BEEP programme (supported by the European Commission) and aimed to validate the multimarker approach in pollution monitoring of European coastal environments. The aims of this study are to evaluate the temporal trends of this scale applied to selected sites of the European Mediterranean coast. Working sites (identified as clean, intermediate and polluted) were selected on the Italian, French and Spanish coasts based on historical knowledge (chemical and biological data) by participant scientific groups.

Materials and methods

Sample collection and preparation

Mussels (*Mytilus galloprovincialis*) were collected from stations on the European coast by either grab or skin divers (in water depth up to 40 m). Coastal sites from the Mediterranean (France, Italy and Spain) were expected to present a wide variety of contamination patterns, from preserved areas (Aragnon, Porto Fino, Cala Monjoy), intermediate areas (Lavera, Cortiou, Voltri, Fangal, Los Alfaques) to highly polluted harbours on the coast (Fos, Genoa, Barcelona). Five sampling cruises took place in

the same area (May, September 2001, May, September 2002, May 2003). Gills and digestive glands were dissected and immediately stored in liquid nitrogen before analysis.

Biochemical measurements

Biochemical determinations were carried out as described (Labrot et al. 1996). In gills, AChE and GST activities were measured in the post-mitochondrial fraction (S9) by using acethylthiocholine (Ellman et al. 1961) and 1-chloro-2-4-dinitrobenzene (Habig et al. 1974), respectively. In digestive gland, BPH activity was measured in the microsomal fraction (Michel et al. 1994). GST, CAT activities (Clairborne 1985) and MDA were determined in post-mitochondrial fraction (S9). The concentration of endogenous MDA was also measured after reaction with thiobarbituric acid (Buege and Aust 1978). All these data were expressed in relation to protein concentration estimated according to the method of Lowry et al. (1951).

Statistical procedure and indices calculation

An ANOVA analysis was used to determine the statistical significance of the individual biochemical variables among sites. Tukey's test was used to determine the significance for individual variables between sites to determine the integrated response of mussel to the environmental conditions at each sampling site. All the individual biomarkers were considered jointly within a multivariate context using a canonical discriminant analysis procedure (Statistica software 6.0 StatSoft, Inc., 2002 Edn). A variable selection procedure (Adams et al. 1999) was also used to identify and select those variables that contributed most to the discrimination among the integrated biomarker response for each site.

In lieu of a common expression of biomarker results (increased or decreased activity), we developed a simple scoring approach to provide a relative comparison among sites that exhibited multiple biomarker responses. The multimarker pollution index (MPI) for each site was calculated as follows:

$$\mathrm{MPIi} = \sum_{\mathrm{j=i}} \mathrm{BPI}$$

where i is the site, j is the biomarker, BPI is the biomarker pollution index from the table of conversion (Table I) for individual mean (X_i), related to discriminatory factor (DF) of the measure:

$$\mathrm{DF} = (X_{\max} - X_{\min} + \mathrm{CI})/\mathrm{CI}$$

Table I. Index of response for each biomarker according to their rank in a scale based on discriminatory factors.

	Discriminatory factors				
Number of levels	1	2	3	4	5
Index of response	4	10			
	3	6	12		
	2	4	7	12	
	1	2	4	8	14

where X_{max} is the mean maximum, X_{min} is the mean minimum and CI is the confidence interval given by Tukey's test.

Finally a pollution scale was established including five levels (from lightly to highly contaminated). The global biomarker index of each site and for each cruise was converted to a pollution level and associated to a colour (red, orange, yellow, green and blue for classes from 5 to 1). These colours were reported on the map of the collected sites in order to visualize easily the temporal changes in effects of pollution.

The MPI classification scale from 1 to 5 (Narbonne et al. 1999) was first applied in European BIOMAR Programme.

Results

Discriminant analysis

As result of discriminant analysis, the individual biomarker variables are shown in Table II. In order to select the biomarkers with the most influence in distinguishing among site responses, a discriminatory power was calculated by ranking analysis (Table III).

Discriminatory power was calculated using:

$$DP_i = \sum R_1 V_1 + \frac{\sum R_2 V_2}{2} + \frac{\sum R_n V_i}{n}$$

where i is the biomarker, n is the number of cruises, r is the root of discrimination and RV_i is the rank number of the discriminatory variable for biomaker i and root r.

The order of importance in term of discriminatory power was GSTg > CATdg > AChEg > BPHdg > GSTdg > MDAdg. Therefore, the first five biomarkers were selected for MPI calculation.

Index calculation

The results of biomarker measurements to built scale are presented in Table IV; the discriminatory factors are presented in Table V; the MPI and BPI are presented in Table VI; and the contribution of each biomarker by station are presented in Figure 1.

The Multimarker Pollution Index (MPI) of each station, for each site for each cruise is converted in five pollution levels (from highly to lightly contaminated) and associate with a colour from blue to red (or a number from 1 to 5) related to the pollution level. These colours are reported in Figure 2, respectively, for French, Italian and Spanish sites in order to visualize easily potential 'hot spots' and the temporal changes in effects of pollution. Supposed 'hot spots' and gradients of contamination were selected by local research groups in Italy, France and Spain. The relationship between biomarker response and environmental characteristics was investigated using the MPI and the profiles of biomarker contributions to the MPI. Moreover, temporal trends may be estimated by MPI seasonal variations related to successive sampling cruises.

In French sites, data from BEEP 1 cruise showed (Figure 2A) that the higher MPI was measured in Cortiou (supposed hot spot), but there was no significant differences between sites in the Gulf of Fos (a supposed gradient). Results of temporal trends showed that the MPI remained high in Cortiou (except in May 2002). The mean MPI

Table II. Individual biomarker variables (canonical roots) measured in mussels (*Mytilus galloprovincialis*) sampled from the Mediterranean Sea during the BEEP project used for the discriminatory analysis procedure.

	Variable	Root 1	Root 2	Root 3
May 2001	AChE g	−0.515	−0.785	0.554
BEEP	BPH dg	−0.724	−0.365	0.830
	CAT dg	−0.402	−0.924	−1.793
	MDA dg	−0.277	0.126	0.176
	GST dg	−0.027	2.171	0.427
	GST g	1.948	−0.296	−0.115
September 2001	AChE g	−1.804	−0.927	0.875
BEEP	BPH dg	0.212	0.556	0.076
	CAT dg	0.447	1.181	−0.588
	MDA dg	0.251	0.062	0.224
	GST dg	−0.568	0.121	−0.905
	GST g	1.334	−0.883	−0.361
May 2002	AChE g	−0.119	−0.937	−2.022
BEEP	BPH dg	−0.622	0.111	−0.143
	CAT dg	−1.549	0.002	0.740
	MDA dg	1.239	1.091	−0.138
	GST dg	−0.061	1.089	1.609
	GST g	1.180	−1.460	0.219
September 2002	AChE g	−1.241	−0.178	0.093
BEEP	BPH dg	−0.050	1.094	0.335
	CAT dg	−0.781	−1.474	0.520
	MDA dg	−0.245	0.195	0.173
	GST dg	0.214	0.017	−2.088
	GST g	1.885	0.032	1.148
May 2003	AChE g	−0.032	0.626	0.345
BEEP	BPH dg	−1.084	−0.105	0.043
	CAT dg	−0.329	0.100	−0.463
	MDA dg	0.238	−0.535	−0.707
	GST dg	−0.020	0.242	0.141
	GST g	−0.214	0.808	−0.495

All biomarker abbreviations are explained in the abstract.

from four cruises was 38. The MPI average from five cruises appeared relatively low in Fos and Lavera (24) with a slight seasonal variation (green or blue). Aragnon exhibited intermediates MPI (mean = 28). Biomarker contribution profiles suggested

Table III. Discriminatory power calculated by ranking analysis of variables for each biomarker of response in mussels (*Mytilus galloprovincialis*) in Table I.

	Root 1						Root 2						Root 3						Discriminatory Power
	B1	B2	B3	B4	B5	Σ	B1	B2	B3	B4	B5	Σ	B1	B2	B3	B4	B5	Σ	
AChE g	5	1	5	2	5	18	3	2	5	5	5	20	5	2	1	5	5	18	34
BPH dg	2	5	5	5	1	18	5	5	5	2	5	22	2	5	5	5	5	22	36
CAT dg	5	5	1	3	5	19	2	1	5	1	5	14	1	5	3	5	5	19	32
MDA dg	5	5	2	5	5	22	5	5	3	5	5	23	5	5	5	5	1	21	41
GST dg	5	5	5	5	5	25	1	5	2	5	5	18	5	1	2	1	5	14	39
GST g	1	2	5	1	5	14	5	3	1	5	1	15	5	5	5	2	5	22	29

Table IV. Results of biomarker measurements in mussels (*Mytilus galloprovincialis*) for each station at each of the three sites during BEEP cruises in the Mediterranean Sea between 2001 and 2003.

Site	Station	Campain	AChE activity in g (nmol/mn/mg protein) (mean ± MCI)	GST activity in g (nmol/mn/mg protein) (mean ± MCI)	GST activity in dg (nmol/mn/mg protein) (mean ± MCI)	CAT activity in dg (µmol/mn/mg protein) (mean ± MCI)	BPH activity in dg (pmol/mn/mg protein) (mean ± MCI)	MDA level in dg (nmol/mg protein) (mean ± SE)
France								
	111 Aragnon	May 2001	52.45 ± 13.0	405.13 ± 140.5	267.48 ± 30.4	67.20 ± 16.8	96.85 ± 18.3	0.904 ± 0.21
	211 Aragnon	September 2001	39.11 ± 5.3	320.00 ± 51.4	141.11 ± 26.6	80.42 ± 21.9	28.51 ± 14.9	0.737 ± 0.39
	311 Aragnon	May 2002	50.30 ± 7.8	364.76 ± 64.3	157.67 ± 26.4	134.07 ± 15.5	14.67 ± 8.0	0.127 ± 0.04
	411 Aragnon	September 2002	88.53 ± 14.4	450.24 ± 68.0	92.35 ± 11.3	59.70 ± 9.9	38.49 ± 186	0.288 ± 0.05
	511 Aragnon	May 2003	20.00 ± 2.0	391.25 ± 91.1	305.98 ± 47.8	125.37 ± 23.2	nd	2.143 ± 0.77
	112 Lavera	May 2001	42.51 ± 13.0	309.83 ± 140.5	209.37 ± 30.4	50.86 ± 16.8	70.02 ± 18.3	0.993 ± 0.31
	212 Lavera	September 2001	31.38 ± 5.3	356.36 ± 51.4	114.69 ± 26.6	93.69 ± 21.9	23.62 ± 14.9	0.362 ± 0.24
	312 Lavera	May 2002	40.44 ± 7.8	221.09 ± 64.3	104.84 ± 26.4	92.99 ± 15.5	14.09 ± 8.0	0.211 ± 0.03
	412 Lavera	September 2002	51.61 ± 14.4	420.22 ± 68.0	69.93 ± 11.3	44.49 ± 9.9	11.72 ± 18.6	0.344 ± 0.08
	512 Lavera	May 2003	14.31 ± 2.0	463.63 ± 91.1	256.28 ± 47.8	119.43 ± 23.2	7.88 ± 6.1	1.300 ± 0.50
	113 Fos harbour	May 2001	41.64 ± 13.0	258.49 ± 140.5	210.02 ± 30.4	68.53 ± 16.8	35.10 ± 18.3	0.801 ± 0.23
	213 Fos harbour	September 2001	41.86 ± 5.3	406.73 ± 51.4	113.25 ± 26.6	54.35 ± 21.9	30.47 ± 14.9	0.557 ± 0.30
	313 Fos harbour	May 2002	39.84 ± 7.8	330.74 ± 64.3	127.87 ± 26.4	76.65 ± 15.5	7.69 ± 8.0	0.203 ± 0.07
	413 Fos harbour	September 2002	57.71 ± 14.4	455.77 ± 68.0	69.26 ± 11.3	51.99 ± 9.9	35.50 ± 18.6	0.275 ± 0.12
	513 Fos harbour	May 2003	10.69 ± 2.0	320.42 ± 91.1	262.63 ± 47.8	115.01 ± 23.2	12.47 ± 6.1	0.950 ± 0.54
	114 Cortiou	May 2001	32.47 ± 13.0	433.81 ± 140.5	262.64 ± 30.4	87.00 ± 16.8	7.45 ± 18.3	0.917 ± 0.28
	214 Cortiou	September 2001	30.27 ± 5.3	361.83 ± 51.4	173.60 ± 26.6	140.62 ± 21.9	63.26 ± 14.9	0.495 ± 0.15
	314 Cortiou	May 2002	42.54 ± 7.8	397.91 ± 64.3	165.07 ± 26.4	100.86 ± 15.5	15.14 ± 8.0	0.156 ± 0.04
	414 Cortiou	September 2002	22.94 ± 14.4	645.59 ± 68.0	107.39 ± 11.3	70.20 ± 9.9	11.31 ± 18.6	0.284 ± 0.13
	514 Cortiou	May 2003	nd	nd	nd	nd	nd	nd
Italy								
	121 Porto Fino	May 2001	58.87 ± 13.0	733.02 ± 140.5	194.74 ± 30.4	75.30 ± 16.8	65.94 ± 18.3	0.946 ± 0.28
	221 Porto Fino	September 2001	33.61 ± 5.3	260.22 ± 51.4	132.64 ± 26.6	97.13 ± 21.9	15.38 ± 14.9	0.764 ± 0.36
	321 Porto Fino	May 2002	45.18 ± 7.8	634.09 ± 64.3	162.62 ± 26.4	75.16 ± 15.5	15.60 ± 8.0	0.192 ± 0.09
	421 Porto Fino	September 2002	57.38 ± 14.4	531.98 ± 68.0	92.06 ± 11.3	75.38 ± 9.9	27.31 ± 18.6	0.390 ± 0.19
	521 Porto Fino	May 2003	20.73 ± 2.0	805.11 ± 91.1	319.27 ± 47.8	119.38 ± 23.2	16.35 ± 6.1	0.715 ± 0.24

Table IV (*Continued*)

Site	Station	Campain	AChE activity in g (nmol/mn/mg protein) (mean ± MCI)	GST activity in g (nmol/mn/mg protein) (mean ± MCI)	GST activity in dg (nmol/mn/mg protein) (mean ± MCI)	CAT activity in dg (μmol/mn/mg protein) (mean ± MCI)	BPH activity in dg (pmol/mn/mg protein) (mean ± MCI)	MDA level in dg (nmol/mg protein) (mean ± SE)
	122 Voltri	May 2001	52.08 ± 13.0	584.81 ± 140.5	192.77 ± 30.4	80.35 ± 16.8	68.51 ± 18.3	1.057 ± 0.25
	222 Vollri	September 2001	28.50 ± 5.3	211.047 ± 51.4	95.07 ± 26.6	59.90 ± 21.9	13.83 ± 14.9	0.630 ± 0.30
	322 Voltri	May 2002	46.73 ± 7.8	372.42 ± 64.3	122.96 ± 26.4	64.48 ± 15.5	12.03 ± 8.0	0.218 ± 0.11
	422 Votri	September 2002	47.40 ± 14.4	452.30 ± 68.0	64.10 ± 11.3	46.33 ± 9.9	32.47 ± 18.6	0.287 ± 0.09
	522 Voltri	May 2003	19.68 ± 2.0	751.66 ± 91.1	369.11 ± 47.8	142.71 ± 23.2	34.96 ± 6.1	0.415 ± 0.22
	123 Genoa in	May 2001	45.71 ± 13.0	472.94 ± 140.5	181.74 ± 30.4	74.58 ± 16.8	28.46 ± 18.3	1.016 ± 0.47
	223 Genoa in	September 2001	24.68 ± 5.3	300.72 ± 51.4	125.93 ± 26.6	42.04 ± 21.9	26.83 ± 14.9	0.248 ± 0.09
	323 Genoa in	May 2002	33.79 ± 7.8	515.83 ± 64.3	170.63 ± 26.4	66.13 ± 15.5	25.27 ± 8.0	0.405 ± 0.06
	423 Genoa in	September 2002	40.68 ± 14.4	415.52 ± 68.0	58.69 ± 11.3	32.94 ± 9.9	25.87 ± 18.6	0.252 ± 0.07
	523 Genoa in	May 2003	15.46 ± 2.0	408.97 ± 91.1	227.26 ± 47.8	135.77 ± 23.2	34.96 ± 6.1	0.256 ± 0.10
	124 Genoa out	May 2001	57.50 ± 13.0	648.75 ± 140.5	188.45 ± 30.4	72.21 ± 16.8	44.74 ± 18.3	1.029 ± 0.29
	224 Genoa out	September 2001	30.44 ± 5.3	237.15 ± 51.4	91.93 ± 26.6	58.00 ± 21.9	88.83 ± 14.9	0.582 ± 0.22
	324 Genoa out	May 2002	45.91 ± 7.8	426.07 ± 64.3	157.80 ± 26.4	68.68 ± 15.5	17.06 ± 8.0	0.279 ± 0.11
	424 Genoa out	September 2002	43.85 ± 14.4	399.81 ± 68.0	51.61 ± 11.3	33.47 ± 9.9	75.45 ± 18.6	0.329 ± 0.14
	524 Genoa out	May 2003	11.72 ± 2.0	424.53 ± 91.1	271.59 ± 47.8	129.97 ± 23.2	14.01 ± 6.1	0.543 ± 0.22
Spain								
	131 Cala Monjoy	May 2001	47.83 ± 13.0	589.60 ± 140.5	229.54 ± 30.4	85.77 ± 16.8	52.24 ± 18.3	0.679 ± 0.13
	231 Cala Monjoy	September 2001	27.79 ± 5.3	265.99 ± 51.4	171.16 ± 26.6	135.94 ± 21.9	18.49 ± 14.9	0.481 ± 0.22
	331 Cala Monjoy	May 2002	nd	nd	nd	nd	nd	nd
	431 Cala Monjoy	September 2002	60.80 ± 14.4	466.38 ± 68.0	87.25 ± 11.3	84.95 ± 9.9	28.68 ± 18.6	0.271 ± 0.03
	531 Cala Monjoy	May 2003	12.24 ± 2.0	270.66 ± 91.1	271.09 ± 47.8	112.18 ± 23.2	27.33 ± 6.1	1.023 ± 0.47
	132 Fangal	May 2001	55.62 ± 13.0	382.51 ± 140.5	167.31 ± 30.4	44.53 ± 16.8	46.08 ± 18.3	0.677 ± 0.06
	232 Fangal	September 2001	14.89 ± 5.3	189.94 ± 51.4	123.24 ± 26.6	116.82 ± 21.9	36.62 ± 14.9	1.373 ± 0.86
	332 Fangal	May 2002	nd	nd	nd	nd	nd	nd
	432 Fangal	September 2002	42.65 ± 14.4	241.55 ± 68.0	62.04 ± 11.3	28.63 ± 9.9	34.52 ± 18.6	0.242 ± 0.08
	532 Fangal	May 2003	14.50 ± 2.0	275.59 ± 91.1	216.19 ± 47.8	107.51 ± 23.2	10.12 ± 6.1	0.699 ± 0.35
	133 Los Alfaques	May 2001	55.79 ± 13.0	351.42 ± 140.5	128.94 ± 30.4	42.79 ± 16.8	38.73 ± 18.3	0.812 ± 0.38
	233 Los Alfaques	September 2001	27.27 ± 5.3	598.15 ± 51.4	120.11 ± 26.6	61.74 ± 21.9	9.96 ± 14.9	0.294 ± 0.23
	333 Los Alfaques	May 2002	nd	nd	nd	nd	nd	nd

Table IV (*Continued*)

Site	Station	Campain	AChE activity in g (nmol/mn/mg protein) (mean ± MCI)	GST activity in g (nmol/mn/mg protein) (mean ± MCI)	GST activity in dg (nmol/mn/mg protein) (mean ± MCI)	CAT activity in dg (μmol/mn/mg protein) (mean ± MCI)	BPH activity in dg (pmol/mn/mg protein) (mean ± MCI)	MDA level in dg (nmol/mg protein) (mean ± SE)
	433 Los Alfaques	September 2002	25.31 ± 14.4	360.81 ± 68.0	70.14 ± 11.3	34.189 ± 9.9	13.85 ± 18.6	0.123 ± 0.05
	533 Los Alfaques	May 2003	16.57 ± 2.0	481.56 ± 91.1	186.48 ± 47.8	110.82 ± 23.2	5.07 ± 6.1	0.471 ± 0.11
	134 Barcelona	May 2001	43.90 ± 13.0	1054.39 ± 140.5	236.40 ± 30.4	40.34 ± 16.8	46.35 ± 18.3	1.516 ± 0.68
	234 Barcelona	September 2001	8.15 ± 5.3	466.66 ± 51.4	119.36 ± 26.6	38.03 ± 21.9	18.48 ± 14.9	0.278 ± 0.08
	334 Barcelona	May 2002	nd	nd	nd	nd	nd	nd
	434 Barcelona	September 2002	30.45 ± 14.4	458.27 ± 68.0	77.11 ± 11.3	31.32 ± 9.9	17.78 ± 18.6	0.266 ± 0.08
	534 Barcelona	May 2003	5.99 ± 2.0	427.61 ± 91.1	275.83 ± 47.8	175.99 ± 23.2	11.46 ± 6.1	0.821 ± 0.31

n.d., Not determined; g, gills; dg, digestive gland; MCI, mean confidence interval; SE, Standard error.

Table V. Discriminatory levels for biomarkers of response obtained for each BEEP cruise in the Mediterranean Sea between 2001 and 2003.

Cruise	Biomarkers	Response factor (RF)	Response range (RR)	Confidence interval (CI)	Discriminatory factor (DF)	Discriminatory levels (DL)
May 2001	AChE g	3.77	63.93	26.00	3.46	2
BEEP1	GST g	8.01	1181.97	281.00	5.21	4
	GST dg	3.44	234.24	60.80	4.85	3
	CAT dg	5.26	111.11	33.60	4.31	3
	BPH dg	293.50	122.78	36.60	4.35	3
Sept. 2001	AChE g	12.33	44.29	10.60	5.18	4
BEEP 2	GST g	4.47	570.74	102.80	6.55	4
	GST dg	17.85	189.39	53.20	4.56	3
	CAT dg	9.16	170.37	43.80	4.89	3
	BPH dg	50.60	135.90	29.80	5.56	4
May 2002	AChE g	1.49	16.50	7.83	2.05	2
BEEP 3	GST g	2.87	413.00	64.31	4.21	4
	GST dg	1.63	65.79	26.43	2.24	2
	CAT dg	2.08	69.59	15.15	3.30	3
	BPH dg	3.29	17.58	7.97	2.10	2
Sept. 2002	AChE g	3.86	65.59	14.45	3.27	3
BEEP 4	GST g	2.67	404.04	67.98	3.97	4
	GST dg	2.08	55.78	11.3	3.47	4
	CAT dg	2.97	56.32	9.91	3.84	4
	BPH dg	6.67	64.14	18.59	2.73	3
May 2003	AChE g	3.46	14.74	1.98	4.72	4
BEEP 5	GST g	2.97	534.4	91.08	3.93	4
	GST dg	1.36	182.63	47.76	2.91	3
	CAT dg	1.64	68.48	23.25	2.47	2
	BPH dg	6.90	29.89	6.12	3.44	3

pollution by organic compounds in Aragnon, pollution by PAHs in Fos (especially in May 2001) and mixed pollution (heavy metals and organic compounds) in Cortiou.

Italian sites (Figure 2B) may be ranged by using the MPI average from five cruises as follows: Porto Fino (31), Genoa in (30), Voltri (29) and Genoa out (28). However, wide seasonal variations were shown in Genoa harbour (from orange to green) and in Voltri (from orange to blue). This last site appeared to be strongly impacted in May 2003. Biomarker contribution profiles suggested PAHs and heavy metals pollution in Genoa harbour and Voltri, especially in May 2001 and 2003. Porto Fino appeared impacted by organic pollution.

Spanish sites (Figure 2C) ranged from higher to lower MPI averages from four cruises: Barcelona (34), Cala Monjoy (32), and Los Alfaques and Fangal (22). The main temporal trend was observed in Barcelona harbour (from green to orange). Biomarker contribution profiles suggest pollution by heavy metals and organic compounds in Barcelona harbour and mixed pollution in Cala Monjoy (perhaps agricultural waste collected by the Lobregat River).

Genoa and Barcelona harbours were suspected to be hot spots from biomarker data collected during previous BIOMAR programme. Temporal trends were very high in these sites, suggesting 'false-negative' results related to a decrease in enzyme activities due to the toxic effects in biota due to high pollution levels.

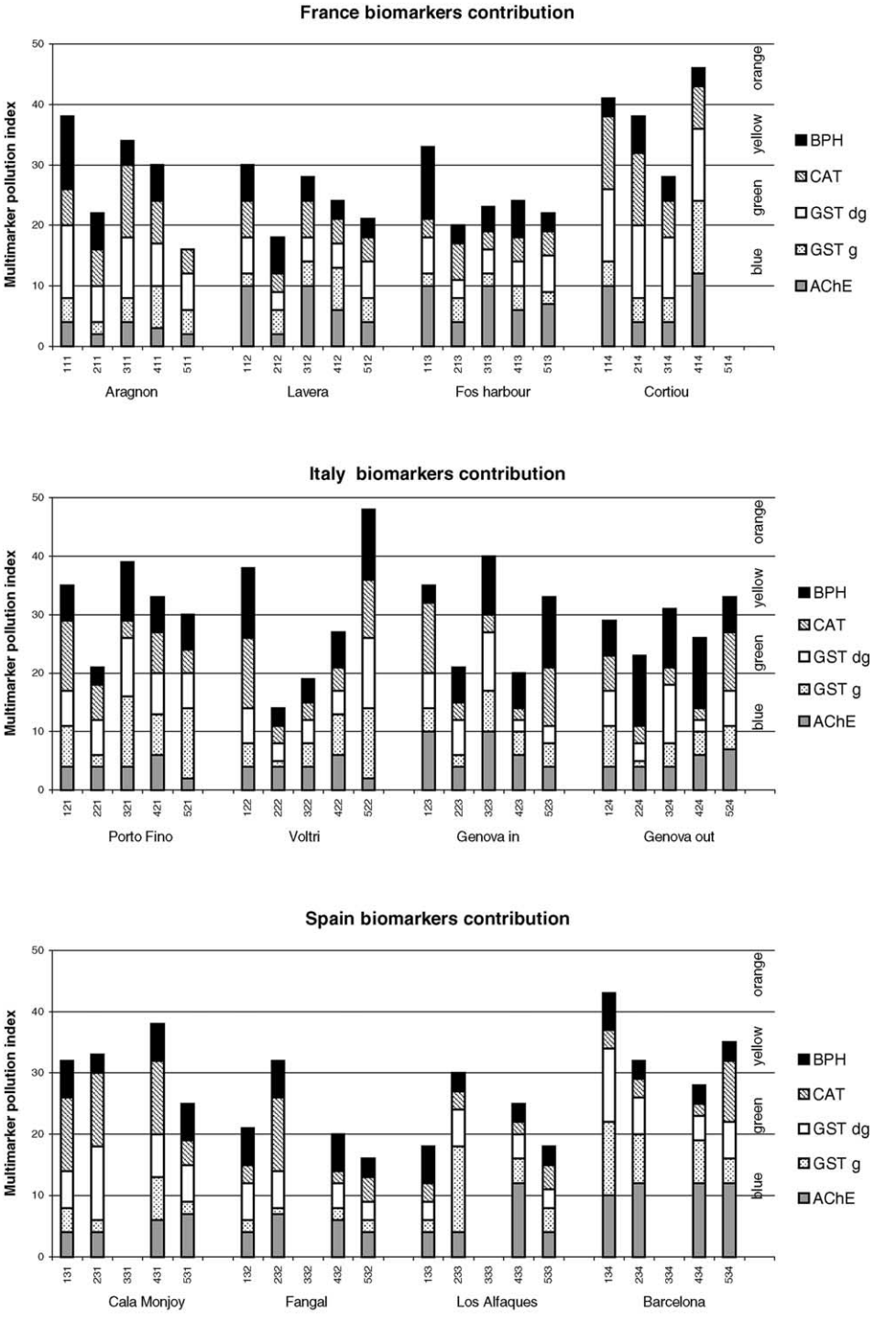

Figure 1. Contribution of each biomarker for each site studied during BEEP cruises 2001–03.

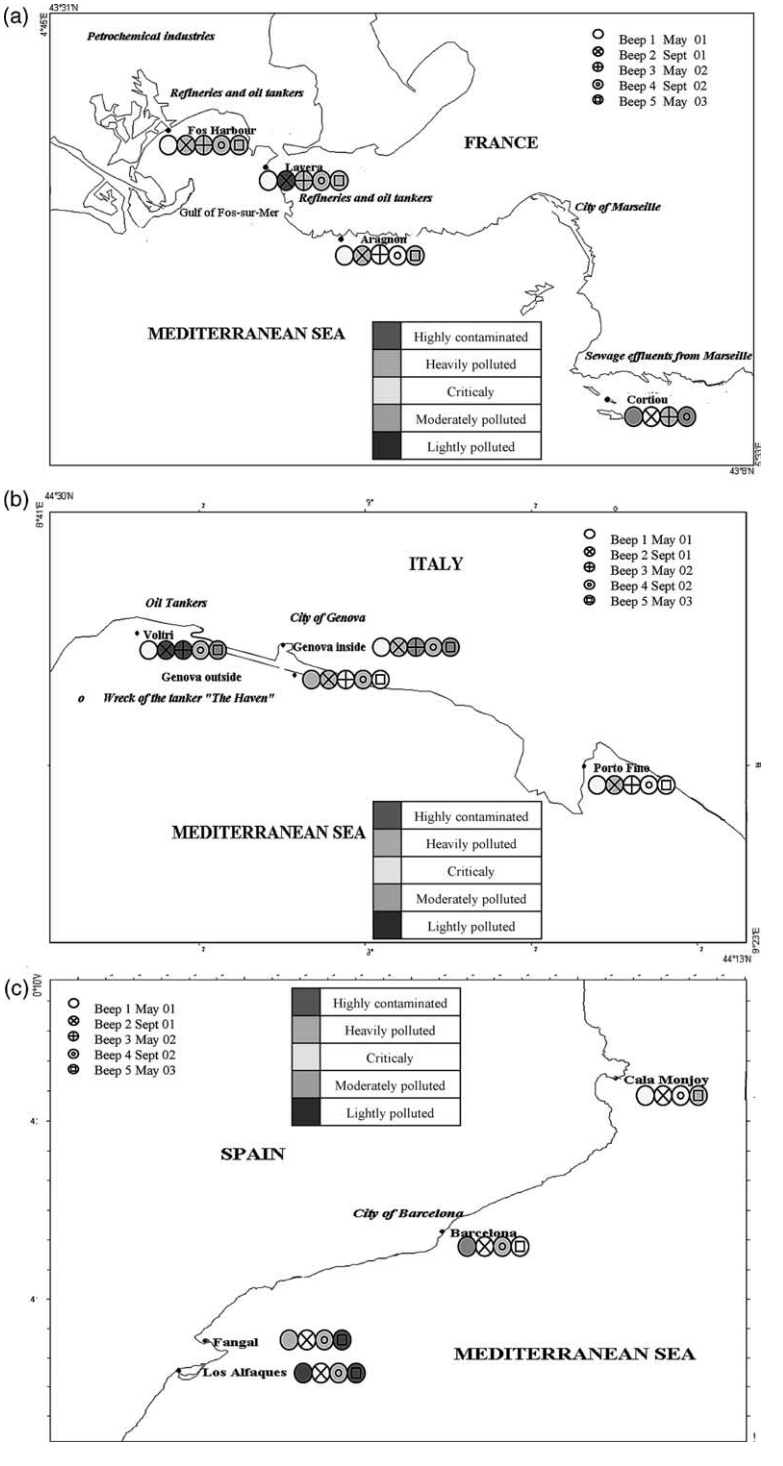

Figure 2. Temporal trends in multimarker pollution indexes (MPI) in working sites selected off France (A), Italy (B) and Spain (C) throughout the five BEEP cruises.

Discussion

Biomarkers are used increasingly in monitoring programmes for assessing environmental contamination. Many studies have, however, focused on single test organisms or limited arrays of biomarker responses to assess the health condition of complex ecosystems (Adams and Ryon 1994). These studies have demonstrated successful and cost-effective environmental assessments. Several biomarkers were also used in a pollution-monitoring programme for the Mediterranean Sea (Med Pol, UNEP 1997) with the aim of evaluating the toxic effects of pollutants on the marine organisms. The same test organisms were used across the study area and were chosen based on appropriate features and wide geographical distribution (the main selected organisms were mussels). Furthermore, a suite of relatively simple, sensitive and low-cost biomarkers (biomarkers of effect and exposure) was employed by the programme.

Lafontaine et al. (2000) measured five biomarkers (MT, EROD, DNA strand breaks, LPO, VG) in the soft tissues of zebra mussels (*Dreissena polymorpha*) in order to assess the spatial variation of exposure to contaminants along the St Lawrence River, Canada. The highest responses were measured in specimens from contaminated sites, indicating that the measurement of biomarker responses was feasible for monitoring programmes

Moore et al. (1999) reported on a large-scale biomarker-based biomonitoring programme for the Black Sea. The lysosomal integrity was strongly associated with suspended solid input and perceived pollution gradients. The study clearly demonstrated the biomarker's ability to pinpoint problem sites with complex contamination profiles allowing a more focused and effective use of the limited analytical resources.

A rapid assessment of the marine pollution (RAMP) programme developed by scientists from Plymouth University, UK (Wells et al. 2001), has been in operation in Brazil for 3–4 years. Local scientists received training in the easy-to-use, inexpensive and robust procedures.

Through programmes such as GIPME (Global Investigation of Pollution of the Marine Environment) funded by UNEP, techniques to assess the effects of contaminant on marine organisms using biomarkers have been developed. In addition, it is suggested that mussels are considered for use as test species.

With the use of more than one biomarker in monitoring, powerful multivariate statistics can be used to investigate the data and look for grouping or trends. Chevre et al. (2003), for example, evaluated effects at the cellular and molecular levels in the clam *Mya arenaria* with discrimination methods. Rough set analysis was used to classify sites and identify important biomarkers for defining the groups. The improvement of the methodology presented in this paper and carried out during the BEEP programme provides a procedure to select an appropriate biomarker of exposure able to discriminate hot spots and multi-exposure effects (discriminatory analysis).

Burgeot et al. (1996) used several biomarkers to assess biological and genotoxic effects of marine pollutants in the north-western Mediterranean Sea. A simple method summarizing biomarker responses was developed, thereby aiding interpretation. They used star plots to display results for a range of biomarkers and the integrated response was computed as the star plot area. The integrated response was then used to investigate spatial and temporal variation in contaminant exposure. The approach was applied to the Baltic Sea and English Channel sites during the BIOMAR programme,

and the integrated biomarker responses compared well with PAH and PCB levels measured in mussel and fish tissues (Beliaeff and Burgeot 2002).

In the same BIOMAR programme, the present authors have developed the MPI approach in order to give decision-makers enough information expressed in a simplified form (pollution scale; Narbonne et al. 1999). The pollution scale is now expressed as a coloured scale easily used for mapping pollution. This made it possible for 4000 km of the north coast of the Mediterranean coastline to be surveyed (BIOMAR and BEEP programmes). The MPI procedure has been used by other countries, especially from North Africa (Morocco and Tunisia).

However, it is generally reported that there is a difficulty in interpreting biomarker responses and the chemical analysis of main contaminants. The integrated suite of biomarkers of exposure, i.e. BPH, CAT and GST activities in digestive gland, AChE and GST in gills, expressed as an individual contribution to MPI, was used to identify the class of contaminant impacting on the aquatic environment.

It is very difficult to find any specific examples in the literature of failures of biomarker programmes. Undoubtedly failures have occurred, but these remain unreported. This is perhaps a reflection of the lack of general acceptance of biomarkers into routine monitoring programmes (Handy et al. 2003). Although the MPI procedure appeared well adapted to detect pollution gradients, trends measured in hot spots (Cortiou, Barcelona, Genoa), indicating a transitory decrease in the pollution index, suggest a false-negative response in highly polluted sites. In this case, low enzymatic activity was not related to a low pollution level but to acute toxicity inducing a decreased cellular metabolism. Selection of non-specific biomarkers indicating an acute toxic effect might be tested in order that is integrated into the MPI calculation.

Acknowledgements

The European Union (BEEP Project, Contract EVK3-CT2000-00025) is acknowledged for financial support, the IFREMER for shipping facilities, and G. Bocquéné and T. Burgeot for AChE and BPH biomarker measurements.

References

Adams SM, Bevelhimer MS, Greeley MS, Levine DA, Teh SJ. 1999. Ecological risk assessment in a large river-reservoir: 6. bioindicators of fish population health. Environmental Toxicology and Chemistry 18:628–640.

Adams SM, Ryon MG. 1994. A comparison of health assessment approaches for evaluating the effects of contaminant-related stress on fish populations. Journal of Aquatic Ecosystem Health 3:15–25.

Bayne BL, Clarke KR, Gray JS. 1988. Biological effects of pollutants: Results of a practical workshop. Marine Ecology Progress Series, 46, Oslo.

Beliaeff B, Burgeot T. 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environmental Toxicology Chemistry 21:1316–1322.

Boquene G, Galgani F, Truquet P. 1990. Characterisation and assay for use of AChE activity from several marine species in pollution monitoring. Marine Environmental Research 30:75–89.

Buege JA, Aust SD. 1978. Microsomal lipid peroxidation. Methods in Enzymology 50:302–310.

Burgeot T, Bocquéné G, Porte C, Dimeet J, Santella RM, Garcia De La Parra LM, Pfhol-Leszkowicz A, Raoux C, Galgani F. 1996. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. Marine Ecology Progress Series 131:125–141.

Chevre N, Gagne F, Gagnon P, Blaise C. 2003. Application of rough sets analysis to identify polluted aquatic sites based on a battery of biomarkers: a comparison with classical methods. Chemosphere 51:13–23.

Clairborne A. 1985. Catalase activity. In: Greenwald RA, editor. Handbook of methods of oxygen radical research. Boca Raton, FL: CRC Press. p. 283–284.

Ellman GL, Courtney KD, Andres V, Feartherstone RM. 1961. A new and rapid colorimetric determination of acethylcholinesterase activity. Biochemical Pharmacology 7:88–95.

Garrigues P, Raoux C, Lemaire P, Ribera D, Mathieu A, Narbonne JF, Lafaurie M. 1990. In situ correlations between polycyclic aromatic hydrocarbons (PAH) and PAH metabolizing system activities in mussels and fish in the Mediterranean sea: preliminary results. International Journal of Environmental Analytic Chemistry 38:379–387.

Goldberg ED, Bowen VT, Farrington JW, Harvey G, Martin J, Parker PL, Risebourg R, Robertson W. 1975. The mussel watch. Marine Pollution Bulletin 5:101–126.

Habig WH, Pabst MJ, Jakoby WB. 1974. The first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249:7130–7139.

Handy RD, Galloway TS, Depledge MH. 2003. A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. Biomarkers 12:331–343.

Huggett RJ, Kimberle RA, Mehrle PM Jr, Bergman HL. 1992. Biomarkers, biochemical, physiological and histological markers of antropogenic stress. Boca Raton, FL: Lewis.

Labrot F, Ribera D, Saint Denis M, Narbonne JF. 1996. In vitro and in vivo studies of potential biomarkers of lead and uranium contamination: Lipid peroxidation, acethylcholinesterase, catalase and glutathione peroxidase activities in three non mammalian species. Biomarkers 1:21–28.

Lafontaine Y, Gagné F, Blaise C, Costan G, Gagnon P, Chan HM. 2000. Biomarkers in zebra mussels (Dreissena polymorpha) for the assessment and monitoring of water quality of the St Lawrence River (Canada). Aquatic Toxicology 50:51–71.

Livingstone DR. 1991. Organic xenobiotic metabolism in marine invertebrates. In: Gilles R, editor. Advances in comparative and environmental physiology. Berlin: Springer. vol. 7, p. 45–185.

Lowry OH, Rosenbrough NJ, Farr AZL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:265–275.

McCarthy JF, Shugart LR. 1990. Biomarkers of environmental contamination. Boca Raton, FL: Lewis.

Michel X, Salaun JP, Galgani F, Narbonne JF. 1994. Benzo(a)pyrene hydroxylase activity in the marine mussel Mytilus galloprovincialis: a potential marker of contamination by polycyclic aromatic hydrocarbon-type compounds. Marine Environmental Research 38:257–273.

Moore MN, Wade T, Wedderburn RJ, Lowe DM, Balashov G, Buyukgun-Gor H, Daurova Y, Denga Y, Kostylev E, Mihnea PE, Moncheva S, Tabagari S, Ciocan C, Ozkoc H, Depledge MH. 1999. The UNESCO/IOC 'Black Sea Mussel Watch Pilot Study': Biological effects and contaminant residues. In: Black Sea pollution assessment. Black Sea Environmental Series Vol. 10. New York: United Nations Publ. p. 279–292.

Najimi S, Bouhaimi A, Daubeze M, Zekhnini A, Pellerin J, Narbonne JF, Moukrim A. 1997. Use of acethylcholinesterase in Perna perna and Mytilus galloprovincialis as a biomarker of pollution in Agadir marine bay (South of Morocco). Bulletin of Environmental Contamination and Toxicology 58:901–908.

Narbonne JF, Daubeze M, Clerandeau C, Garrigues P. 1999. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. Biomarkers 4:415–424.

Narbonne JF, Garrigues P, Ribera D, Raoux C, Mathieu A, Lemaire P, Salaun JP, Lafaurie M. 1991. Mixed function oxygenase enzymes as tools for pollution monitoring: field studies on the French coast of the Mediterranean Sea. Comparative Biochemistry and Physiology 100C:37–42.

Pellerin-Massicotte J. 1994. Oxidative processes as indicators of chemical stress in marine bivalves. Journal of Aquatic Ecosystem Health 3:101–111.

Prahash NT, Rao KSJ. 1995. Modulations in antioxidant enzymes in different tissues of marine bivalve Perna viridis during heavy metal exposure. Molecular and Cellular Biochemistry 146:107–113.

Regoli F, Principato G. 1995. Glutathione, glutathione-dependant and antioxidant enzymes in mussel, Mytilus galloprovincialis, exposed to metal under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquatic Toxicology 31:143–164.

Suteau P, Daubeze M, Migaud ML, Narbonne JF. 1988. PAH-metabolizing enzymes in whole mussels as biochemical test for pollution monitoring. Marine Ecology Progress Series 46:45–49.

UNEP. 1997. Report of the meeting of experts to review the MED POL biomonitoring programme. UNEP-(OCA)/MED WG.132/7. Athens: UNEP.

Wells PG, Depledge MH, Butler JN, Manock JJ, Knap AH. 2001. Rapid toxicity assessment and biomonitoring of marine contaminants. Exploiting the potential of rapid biomarker assays and microscale toxicity tests. Marine Pollution Bulletin 42:799–804.