EFFECTS OF ANTHROPOGENIC FACTORS ON GENETIC DIVERSITY IN THE MARINE BIVALVE CRASSOSTREA GIGAS : SEARCH FOR GENETIC MARKERS

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EFFECTS OF ANTHROPOGENIC FACTORS ON GENETIC DIVERSITY IN THE MARINE BIVALVE *CRASSOSTREA GIGAS* : SEARCH FOR GENETIC MARKERS

D. MORAGA, A. TANGUY

UMR CNRS 6539, Biologie Marine, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, 6, avenue Le Gorgeu, B.P. 809, 29285 Brest cedex, France

BIOCIDES
ENVIRONMENT
POLLUTION
ALLOZYME POLYMORPHISM
DIVERSITY
GENETIC MARKERS

ABSTRACT. – The effects of various pollutants including heavy metals, pesticides and organic contaminant on the genetic structure of the marine bivalve *Crassostrea gigas* were studied as part of an environmental biomonitoring project. This research was carried out on two natural oyster populations from the French Atlantic coast. Results indicate a differential survival of allozyme genotypes for the populations which depends on the pollutant tested. The sensitivity of allozymes to environmental stress through differential mortality reflects the adaptive nature of the surviving individuals. Moreover, it supports the hypothesis that allozymes could be used as genetic indicators in marine bivalves. Our results revealed that the six studied enzyme loci (*Aat-2, Ak, Pgdh, Cap-1, Pgi and Pgm*) involved in the physiological processes were affected by the pollutants and can therefore be considered as potential genetic indicators.

BIOCIDES
ENVIRONNEMENT
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DIVERSITE
MARQUEURS GÉNÉTIQUES

RÉSUMÉ. – Dans le cadre d'un projet de surveillance des écosystèmes, l'action de divers polluants d'origine anthropique (métaux lourds, pesticides et contaminant organique) sur la structure génétique d'un Bivalve marin *Crassostrea gigas* a été testée sur deux populations d'Huîtres creuses de la façade atlantique française. Les résultats montrent une survie différentielle des génotypes allozymiques dans les populations selon le polluant testé. La sensibilité des allozymes aux stress environnementaux, traduite par des mortalités différentielles, reflète la nature adaptative des individus et conforte l'hypothèse de l'utilisation des allozymes comme marqueurs génétiques chez les Bivalves marins. Les résultats montrent que les six gènes enzymatiques étudiés (*Aat-2, Ak, Pgdh, Pgi, Cap-1* et *Pgm*), impliqués dans des processus physiologiques sont affectés par les contaminants et peuvent à ce titre être considérés comme des marqueurs génétiques potentiels.

INTRODUCTION

The industrial revolution of the 20th century has introduced a variety of compounds into the world terrestrial and aquatic ecosystems. New forms of pollution, more or less detectable, such as contamination by hydrocarbons, heavy metals and pesticides, agricultural run-off,... have appeared. Consequently, many scientific projects have been carried out in an attempt to evaluate the impact of these different pollutants on marine fauna (Battaglia & Beardmore 1978; Bryan *et al.* 1986; Sindermann *et al.* 1982; Nevo *et al.* 1988; Rice *et al.* 1989). The results of these studies have shown that pollution induces changes in fecundity, growth, and in the genetic structure of population. When a population is faced with a polluted environment, its physiological response results from an adaptation strategy in direct relation with its genetic variability (Allendorf & Leary 1986). The genetic studies carried out to date reveal that often pollutants specifically affect loci in favouring or counter-selecting alleles and genotypes (Nevo *et al.* 1981; Ben-Schlomo & Nevo 1988; De Nicola *et al.* 1992). Monitoring programs based on the genetic signature of a sentinel species usually rely on this feature. The Pacific oyster *Crassostrea gigas*, is an appropriate species for monitoring because it is a sessile filter feeder that exhibits a high level of polymorphism at a large number of loci (Moraga *et al.* 1989). Mollusc bivalves such as *Crassostrea gigas* and *Mytilus edulis* have already been used as sentinel organisms in many monitoring projects in disturbed ecosystems (Stephenson 1991; Regoli 1992).

The monitoring program of the Bay of Brest in Brittany (France) was set up after a decrease

in marine resources that suggested a degradation in water quality. Several chemical compounds of agricultural, industrial or urban origin were identified during this program (Cann 1995; Patris *et al.* 1995; Thomas & Durand 1995). The present study deals with an attempt to detect the genetic markers (alleles and genotypes) involved in the resistance of *C. gigas* to pollution (tributyltin, pesticides and heavy metals) and, thereby, to study the survival of the Pacific oysters subject to these pollutants.

MATERIALS AND METHODS

The contamination experiments were carried out on *Crassostrea gigas* from two different sites : the "Pointe du Château" located in the Bay of Brest, and the "Matelle" in the Bay of Arcachon (Fig. 1). Samples from the first site were used in contamination experiments with tributyltin, heavy metals, *i.e.* copper and cadmium, and three of the five pesticides studied, *i.e.* alachlor, metolachlor and diuron. The impact of the two remaining pesticides (atrazine and isoproturon) was determined on populations from the second site. All the collected oysters had about the same size to avoid introducing additional parameters in the analysis. Using oysters from different sites should not be a problem in genetic indicators characterisation. To confirm this point, allelic frequencies were compared in the two populations.

Experimental conditions were the same for all of the pollutants studied. Firstly, sampled oysters were washed with fresh water to remove any epibionts, then transferred to polypropylene tanks in 50-individual sets. Oysters were subject to an 8-day conditioning period to discard the individuals damaged during the sampling procedure. For each pollutant and concentration studied, experimental sets were composed with about 80 to 100 individuals from the remaining oysters. Pollutants were added to the tanks from concentrated solutions, and aerated sea water was changed every 48 hours. This allowed the maintenance of a relatively constant level of pollutants in the tanks. Whatever the pesticide studied, the tested concentrations were the same : 0.1 and 0.2 mg/l. For heavy metals, they were 1 and 2.5 ppm for copper and 0.5 and 1.5 ppm for cadmium. Tributyltin was tested at 50 and 150 ng/l.

The pollutants selected were those found at the highest concentrations in the Bay of Brest. Five are herbicides and three of them, *i.e.* atrazine, alachlor and metolachlor are sprayed in fields whereas the two others, *i.e.* simazine and isoproturon, are used in road and railways maintenance by local communities. It has been shown that pesticides concentrations can be quite high in the Bay of Brest, and depend on the site examined (Thomas & Durand 1995) whereas copper and cadmium are in variable concentrations. Tributyltin was studied for it is the source of drastic ecological problems because of its toxicity. Its legal toxicity threshold set to 1 ng/l is often exceeded in some areas of the Bay (Michel & Averty 1995).

Mortality was assessed every 6 or 12 hours depending on the pollutant studied and on the rate of mortality. Each dead oyster was removed from the tank, then frozen at – 80 °C until further analysis; moreover the time of its survival was noted down. The adductor muscle and digestive gland were cut apart, ground in an extraction buffer (Tris 0.01M, EDTA 0.001M, NADP 1 %, pH = 6.8), centrifuged at 10 000 g and frozen. Depending on the loci examined, electrophoresis was carried out on a SIGMA starch gel (11 %) in either a continuous system (Tris-citrate buffer (buffer 1) : Tris 0.62 M, citric acid 0.14 M, pH = 8) or in a discontinuous system (Poulik gel (buffer 2) : Tris 0.07 M, citric acid 0.005 M, pH = 8.7; Poulik electrodes : boric acid 0.3 M, NaOH 0.06 M, pH = 8.2).

Eight enzyme systems were studied. Two isozymes, *i.e.* Aspartate aminotransferase (AAT, EC 2.6.1.1 in buffers 1 and 2) and Cytosol aminopeptidase (CAP, EC 3.4.11.1 in buffer 1 and 2), are involved in osmoregulation processes; the six others, *i.e.* Phosphoglucose isomerase (PGI, EC 5.3.1.9 in buffer 1), Phosphoglucomutase (PGM, EC 2.7.5.1 in buffer 1), Malate dehydrogenase (MDH, EC 1.1.1.37 in buffer 1), Adenylate kinase (AK, EC 2.7.4.3 in buffer 1), Phosphogluconate dehydrogenase (PGDH, EC 1.1.1.43 in buffer 1) and Malic enzyme (MDHP, EC 1.1.1.40 in buffer 1), are involved in energetic metabolism reactions. The methods and staining techniques used are those described by Pasteur *et al.* (1987).

The Fst and Fis fixation indices of Wright (1965) allow to detect genetic differentiations between the various groups of oysters by testing the homogeneity of allelic frequencies and observed heterozygosity respectively. These two indices were obtained from the processing of genetic data provided by the GENETIX 3.0 (Belkir *et al.* 1996) and BIOSYS-1 programs (Swofford & Selander 1981, 1989). Fst and Fis departures from zero were tested according to Workman and Niswander (1970) ($\chi^2 = 2NFst(k-1)$) for the Fst index and Li & Horvitz (1953) ($\chi^2 = NFis^2\ (k-1)$) for the Fis index. To adjust significance levels of multiple tests, we used the standard Bonferroni technique (Miller, 1980; Lessios, 1992); it consists in dividing the 5 % predetermined significance level, by the number of tests performed by allozyme, k, to obtain a corrected significance level $\alpha' = \alpha/k$. Possible correlation between the genotype and the survival time of the oysters was tested using Cox's proportional risk test (Cox & Oakes 1984) which is integrated into the "Survival analysis" option of the CSS program (Statistica Statsoft (1995)).

RESULTS

Survival curves

The survival curves show that the mortality of oysters exposed to 2.5-ppm copper is complete after approximately 15 days. Conversely, only 33 % of oysters subjected to 1 ppm died after 46 days of exposure. A 93 % mortality rate was observed in oysters exposed to 1.5-ppm cadmium whereas only one individual died at 0.5-ppm (Fig. 2A).

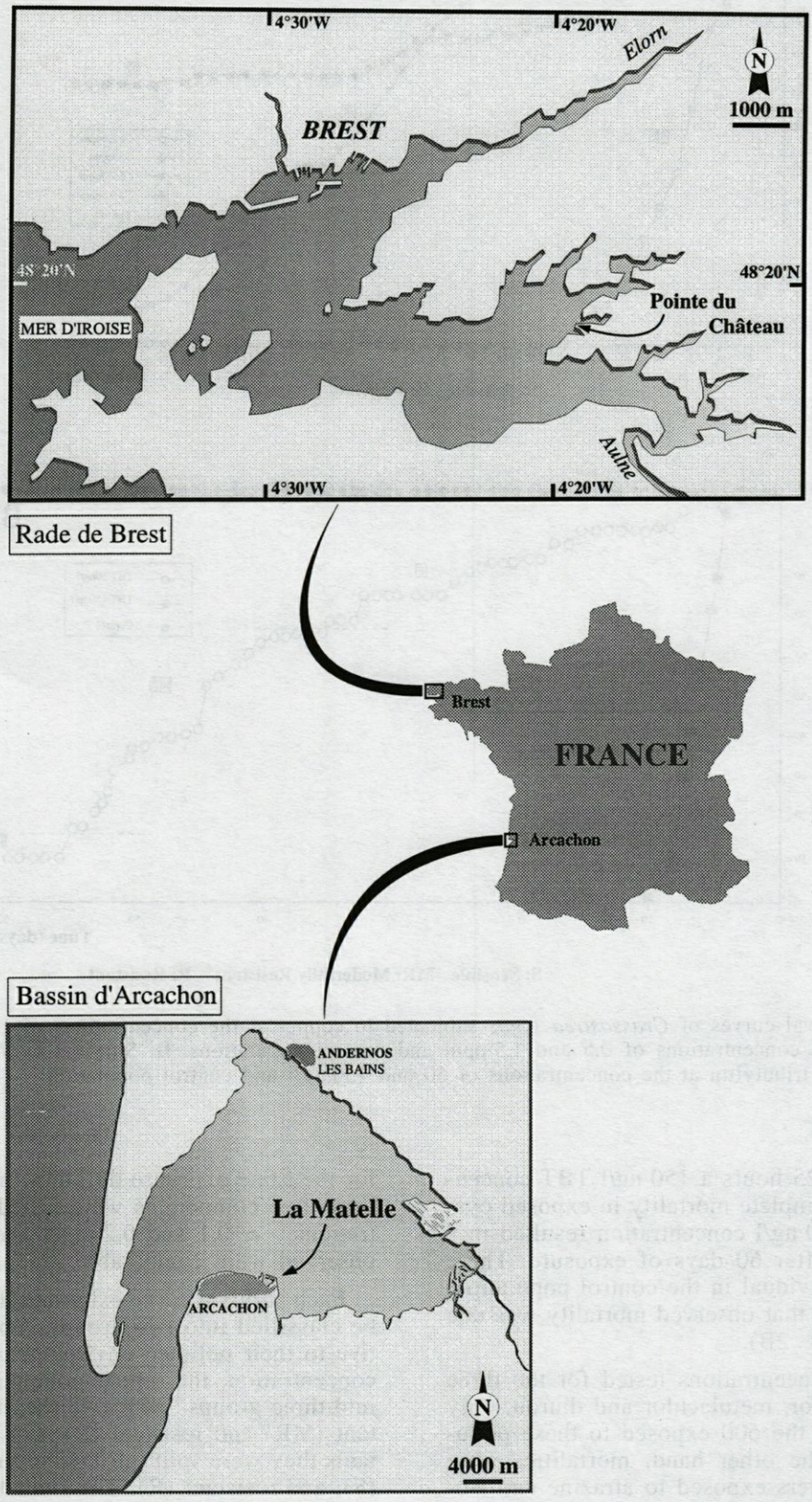

Fig. 1. – Sampling stations of *Crassostrea gigas* in the bay of Brest (Brittany, France) and in the bay of Arcachon (Aquitaine, France).

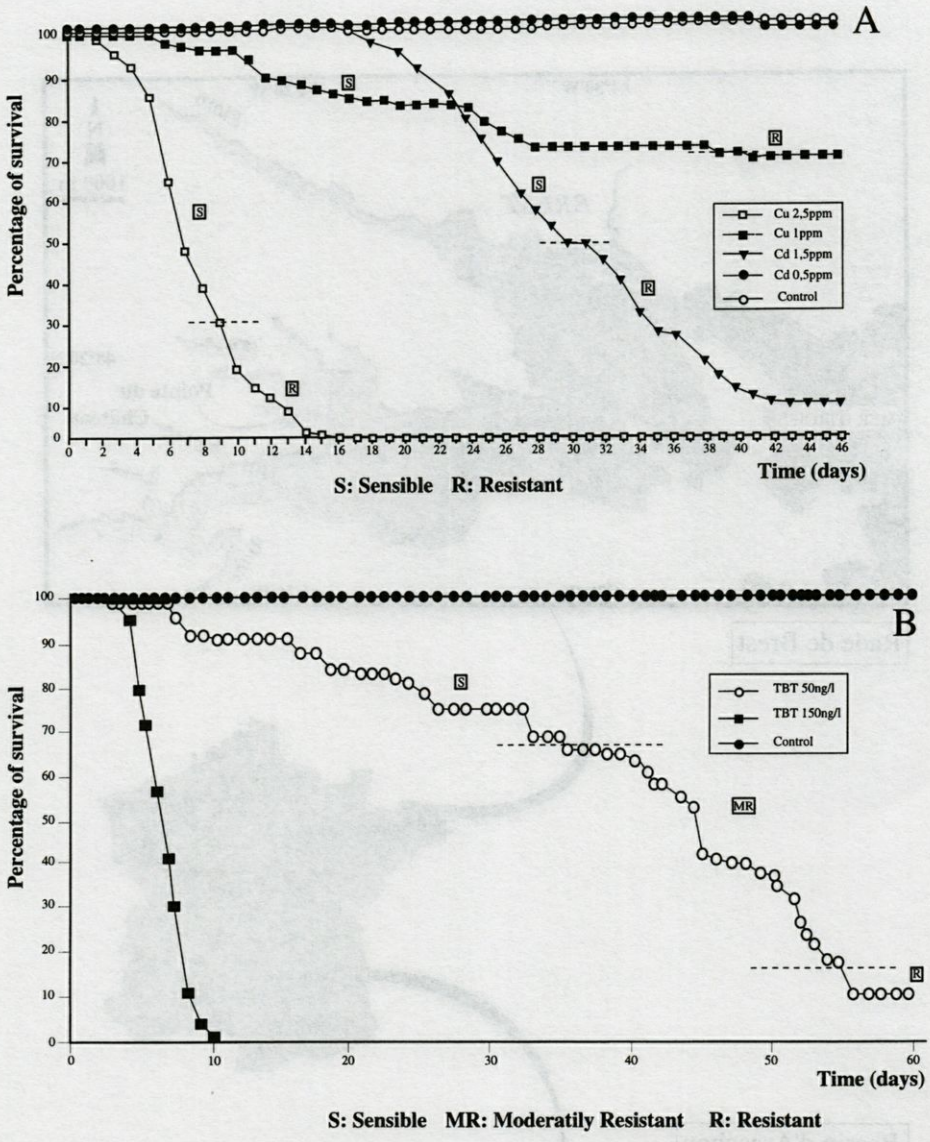

Fig. 2. – A, Survival curves of *Crassostrea gigas* subjected to copper at the concentrations of 1 and 2.5 ppm and to cadmium at the concentrations of 0.5 and 1.5 ppm and control populations. B, Survival curves of *Crassostrea gigas* subjected to tributyltin at the concentrations of 50 and 150 ng/l and control population.

In less than 225 hours a 150 ng/l TBT concentration caused complete mortality in exposed oysters whereas a 50 ng/l concentration resulted in a 90% mortality after 60 days of exposure. There was no dead individual in the control population, which confirmed that observed mortality was due to tributyltin (Fig. 2B).

At the two concentrations tested for the three pesticides, alachlor, metolachlor and diuron, only 5 oysters among the 600 exposed to these pollutants died. On the other hand, mortalities were recorded for oysters exposed to atrazine and isoproturon (Fig. 3). The survival curves obtained for these two last pollutants at the two concentrations tested look alike. However, it seems that isoproturon had faster and stronger effects than atrazine for the same exposure duration. In addition, when these two compounds were tested at two concentrations, *i.e.* 0.1 and 0.2 mg/l, the mortality rates observed were comparable.

Each survival curve exhibited that oysters could be classified into two groups, more or less sensitive to their polluted environment. For each TBT concentration, the whole population was divided into three groups : sensitive (S), moderately resistant (MR) and resistant (R). For the other pollutants they were split into two groups, *i.e.* sensitive (S), and resistant (R). The limit between the two groups was determined from the point of inflexion in survival curves. The genetic characteristics of these groups expressed as allelic and genotypic frequencies, heterozygosity rate were thus compared.

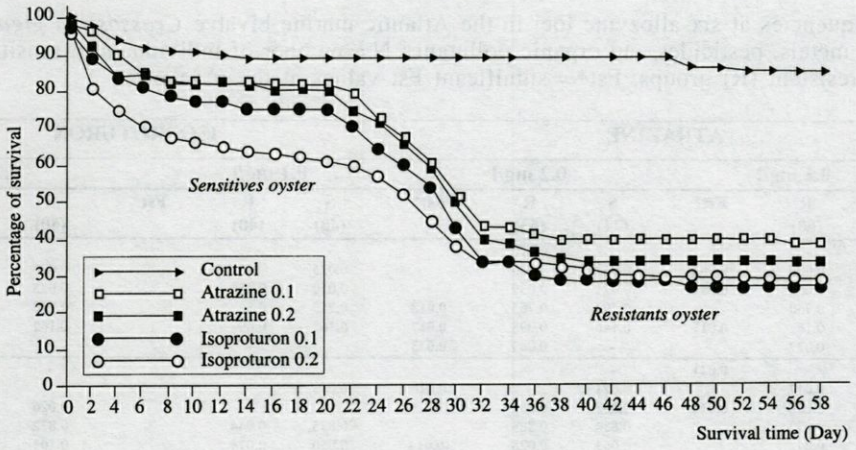

Fig. 3. – Survival curves of *Crassostrea gigas* subjected to atrazine and isoproturon at the concentrations of 0.1 and 0.2 mg/l and control population.

Genetic data analysis

Allelic frequencies at a given locus were compared in the two populations, *i.e.* Pointe du Château and La Matelle, and did not present any significant difference. For each pollutant and concentration, allelic frequencies were analysed with the Fst test in the S, MR and R populations. More or less noticeable differences were observed in the distribution of several alleles. This is illustrated by the significant values listed in Table I. It should be noted that, sometimes, the same allele can be counter-selected or favoured depending on the pollutant concentration. In the present work, alleles with the same trend were considered as probable genetic markers. However, this choice must be strengthened by a comparison of allelic frequencies in the different groups. Very low frequencies were found for some alleles that were thus excluded. The different genetic indicators retained are given in Table II.

The genetic data obtained in heavy metals environment were processed with the Cox model; this exhibited that the genotype had no significant influence (at the 5% level) on the survival rate of oysters whatever the concentrations tested.

The Cox model highlighted that, for 50-ng/l tributyltin, only the *Aat-2* locus had a significant statistical Chi-square value; this suggests that oyster genotype significantly affects its survival time. The analysis of the survival curves obtained with this model evidenced that the 90/100 genotype is at a higher frequency in the tributyltin resistant oysters (Fig. 4). The genetic data from the two oyster groups exposed to tributyltin were pooled and showed that the locus had a significant Chi-square value ($\chi^2 = 56.36$, df = 5, p = 0.011). This highly confirms the role played by some genotypes in oyster survival. A similar correlation was observed for the 100/102 genotype at the locus although the probability at this locus was not significant.

Only the *Pgm* locus at 0.1 mg/l of isoproturon showed a significant χ^2 value ($\chi^2 = 18.65$, df = 4, p = 0.0009). From the survival curves analysis (Fig. 5) it is obvious that the 98/105 and 105/105 genotypes survive longer than other genotypes. All the genetic data from subjects exposed to isoproturon were pooled; this showed that some genotypes at the *Pgm* locus significantly affected oyster survival. The 105/105 genotype seems to confer a better capacity to resist isoproturon whereas the 100/100 and 98/98 genotypes are more common in susceptible individuals. For atrazine, a correlation was found for the *Pgm* locus when all the data from oysters exposed to atrazine were gathered although the probability be significant only at the 10% level.

At the population level oysters from the Bay of Arcachon exposed to atrazine and isoproturon presented significant heterozygote deficiencies at the *Ak* (Fis = 0.131, $\chi^2 = 32.12$, df = 6), *Cap-1* (Fis = 0.328, $\chi^2 = 142.87$, df = 4) and *Pgm* (Fis = 0.243, $\chi^2 = 142.60$, df = 7) loci. For the *Cap-1* and *Pgm* loci, these deficiencies were extremely high. Oysters sampled from the Bay of Brest also exhibited significant deficiencies in heterozygotes at the *Ak* (Fis = 0.124, $\chi^2 = 35.67$, df = 4), *Pgm* (Fis = 0.094, $\chi^2 = 35.87$, df = 7) and *Pgdh* (Fis = 0.167, $\chi^2 = 64.70$, df = 6) loci (Table III).

Heterozygosity at different loci was considered with respect to pollutant class and concentration; this exhibited that its rates varied at locus level. As a rule, a 150 ng/l tributyltin concentration favoured the survival of individuals heterozygous at *Pgdh*, *Aat-2*, *Cap-1* and *Pgm* loci. On the other hand, a 50-ng/l concentration favoured individuals homozygous at the *Pgi* and *Pgdh* loci.

Whatever isoproturon concentration used, heterozygotes were counter-selected at any locus. With the two atrazine concentrations studied, heterozygotes were favoured at *Ak* and *Cap-1* loci.

Table I. – Allele frequencies at six allozyme loci in the Atlantic marine bivalve *Crassostrea gigas* due to controlled pollutions by heavy metals, pesticides and organic pollutant : N = number of individuals in sensitive (S), moderately resistant (MR) and resistant (R) groups. Fst* = significant Fst values at the a' level.

		ATRAZINE						ISOPROTURON					
		0.1 mg/l			0.2 mg/l			0.1 mg/l			0.2 mg/l		
	N	S (25)	R (60)	Fst*	S (24)	R (63)	Fst*	S (40)	R (40)	Fst*	S (40)	R (53)	Fst*
Ak	90	-	-		-	0.010		-	-		-	-	
	95	0.080	0.009	**0.062**	0.042	0.020		0.025	-		0.025	0.011	
	98	0.040	0.054	**0.016**	0.021	0.039		0.050	0.056		0.025	0.078	**0.016**
	100	0.760	0.750		0.792	0.765	**0.013**	0.750	0.833		0.788	0.789	
	102	0.100	0.161	**0.017**	0.146	0.098	**0.017**	0.162	0.097		0.162	0.100	**-0.014**
	105	0.020	0.027		-	0.069	**0.033**	-	-		-	0.022	
Pgi	75	0.021	-	**0.021**	-	-		-	-		-	-	
	80	-	0.017		0.021	-	**0.018**	0.013	-		-	-	
	90	0.042	0.008	**0.016**	0.063	0.065		-	-		0.026	0.060	
	100	0.875	0.932		0.854	0.889		0.875	0.944		0.872	0.890	
	105	0.063	0.042		0.063	0.028	**-0.014**	0.050	0.014		0.103	0.040	
	110	-	-		-	0.009		0.013	-		-	0.010	
Cap-1	95	0.020	-	**0.019**	-	0.033		-	-		0.013	0.010	
	98	0.040	0.058		0.104	0.100		0.090	0.063		0.075	0.051	
	100	0.860	0.858		0.854	0.767	**0.019**	0.821	0.875		0.775	0.847	
	102	0.040	0.067		0.021	0.050		0.051	0.050		0.125	0.061	
	105	0.040	0.017		0.021	0.050		0.038	0.013		0.013	0.031	
Pgm	90	0.021	-	**0.021**	0.042	-	**0.056**	-	0.026		-	0.020	
	95	0.083	0.066	**-0.014**	0.104	0.059		0.050	0.066		0.103	0.038	**0.020**
	98	0.104	0.197		0.229	0.186		0.138	0.289	**-0.048**	0.180	0.298	**0.025**
	100	0.333	0.484	**0.025**	0.229	0.500	**0.120**	0.400	0.487		0.282	0.442	**0.038**
	102	0.063	0.032	**0.046**	0.104	0.034	**0.026**	0.025	0.039	**-0.012**	0.077	0.029	**0.024**
	105	0.292	0.189		0.229	0.153		0.100	0.079	**-0.015**	0.231	0.144	**0.012**
	110	0.083	0.025	**0.022**	0.021	0.008		0.15	-	**0.133**	0.077	0.019	**0.045**
	115	-	-		0.042	-	**0.056**	0.100	-	**0.083**	0.051	-	

		CUIVRE						CADMIUM			TRIBUTYLTIN							
		2.5 ppm			1 ppm			1.5 ppm			50 ng/l				150 ng/l			
	N	S (63)	R (27)	Fst*	S (30)	R (60)	Fst*	S (48)	R (42)	Fst*	S (32)	MR (58)	R (10)	Fst*	S (14)	MR (68)	R (18)	Fst*
Aat-2	90										0.030	0.052	0.200	**0.027**	0.036	0.081	0.083	
	100										0.773	0.578	0.500	**0.055**	0.786	0.603	0.500	**0.029**
	110										0.197	0.368	0.300	**0.057**	0.178	0.316	0.417	
Ak	90	-	0.027	**0.131**	-	-		-	-		-	-	-		-	0.008	-	
	95	0.059	0.107		0.033	0.008	**0.015**	0.010	0.012		-	0.035	0.050		-	0.029	0.028	
	98	-	-		0.100	0.042		0.042	0.107	**0.017**	0.106	0.043	-	**0.018**	-	0.015	-	
	100	0.735	0.705		0.800	0.825		0.854	0.702	**0.054**	0.742	0.833	0.700		1	0.808	0.916	**0.051**
	102	0.206	0.152		0.050	0.125	**0.018**	0.083	0.155		0.152	0.087	0.250	**0.027**	-	0.139	0.056	**0.028**
	105	-	0.009	**0.017**	0.017	-		0.010	0.024		-	-	-		-	-	-	-
Pgi	80	-	-		-	-		0.021	0.024		0.030	0.008	-		-	0.014	-	
	90	0.029	0.036		0.083	0.033		0.021	0.060		0.015	0.026	-		0.036	0.045	0.056	
	100	0.897	0.946		0.883	0.925		0.906	0.845		0.894	0.921	0.900		0.928	0.919	0.944	
	105	0.044	0.018		0.034	0.042		0.042	0.071		0.015	0.035	-		-	-	-	
	110	0.029	-	**0.027**	-	-		0.010	-		0.045	0.008	0.100	**0.034**	0.036	0.022	-	
Pgdh	95	0.015	-		-	-		-	0.012		0.015	-	-		-	-	-	
	98	0.029	0.044		-	0.033	**0.013**	0.031	0.012		0.015	0.008	0.088		-	-	-	
	100	0.691	0.789		0.783	0.775		0.771	0.821		0.864	0.900	0.912		1	0.860	0.778	**0.041**
	102	0.103	0.134	**0.075**	0.066	0.117	**-0.016**	0.104	0.059	**-0.014**	-		-		-	0.074	0.138	
	105	0.132	0.053	**0.041**	0.100	0.067		0.062	0.071		0.106	0.092	-		-	0.051	0.028	
	110	0.029	0.009		0.050	0.008	**0.027**	-	0.012		-	-	-		-	0.015	0.056	
	115	-	-		-	-		-	0.012									
Cap-1	95	-	-		0.016	0.025		-	-		0.015	0.017	-	**0.027**	-	0.007	-	
	98	0.147	0.071	**0.027**	0.133	0.125		0.104	0.071		0.091	0.123	0.150		0.036	0.104	0.111	
	100	0.794	0.776	**0.014**	0.750	0.750		0.844	0.881		0.818	0.754	0.750		0.964	0.787	0.778	**0.024**
	102	0.044	0.143	**0.014**	0.100	0.083		0.031	0.047		0.061	0.079	0.100		-	0.088	0.027	
	105	0.015	0.009		-	0.0167		0.021	-		0.015	0.026	-		-	0.014	0.084	
Pgm	90	-	-		0.017	-		-	0.012		0.015	-	-		-	0.007	0.027	
	95	0.073	0.116	**0.013**	0.083	0.067		0.083	0.097		0.091	0.052	0.100		0.036	0.058	0.083	
	98	0.132	0.107	**0.022**	0.050	0.067		0.042	0.134		0.076	0.087	0.050		0.036	0.126	0.139	
	100	0.588	0.518		0.567	0.625		0.646	0.524		0.667	0.623	0.500		0.786	0.604	0.584	
	102	0.088	0.125		0.100	0.075		0.104	0.097		0.030	0.079	0.050		0.071	0.139	0.111	
	105	0.103	0.107		0.167	0.116		0.083	0.61		0.091	0.157	0.300	**0.024**	0.071	0.066	0.056	
	110	0.147	0.027		0.016	0.050		0.041	0.073		0.030	-	-					

Copper and cadmium had opposite effects on heterozygosity at some loci. Indeed, copper favoured individuals homozygous at the *Pgi, Ak* and *Pgdh* loci and heterozygous at the *Cap-1* locus. On the other hand, cadmium had the opposite effect at the same loci (Table III).

Table II. – Potential genetic indicators in *C. gigas* for the studied pollutants : (S) allele counter-selected by the pollutant; (R) allele favoured by the pollutant.

	TRIBUTYLTIN	COPPER	CADMIUM	ATRAZINE	ISOPROTURON
GENETIC INDICATORS	Aat-2^{90} (R)	Pgdh^{102} (R)	Ak^{98} (R)	Ak^{95} (S)	Ak^{98} (R)
	Aat-2^{110} (R)	Pgdh^{105}(S)	Pgdh^{102} (S)	Ak^{98} (R)	Ak^{102} (S)
	Ak^{98} (S)	Cap-1^{98} (S)		Pgi^{105} (S)	Pgm^{98} (R)
	Pgm^{105} (R)			Pgm^{95} (S)	Pgm^{105} (R)
				Pgm^{102} (S)	Pgm^{110} (S)
				Pgm^{110} (S)	

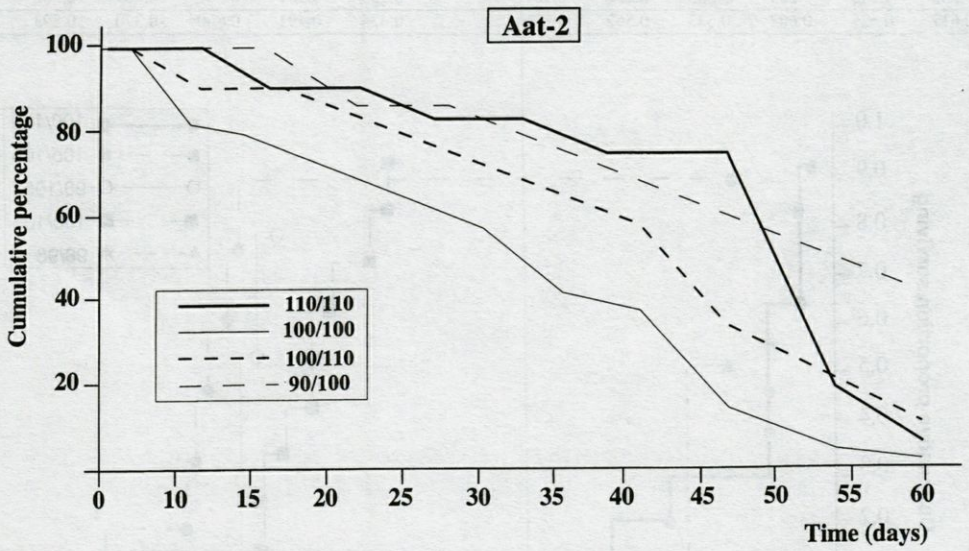

Fig. 4. – Cox's model : curves of cumulative percentage of survival of the main genotypes at the *Aat-2* locus in tributyltin contamination experiments at the concentration of 50 ng/l.

DISCUSSION

For a clear understanding of the present study goals, a precise definition of "allelic indicator" should be given. It can be described as an allele whose frequency of occurrence will be modified in a predictable way by the presence of a given pollutant in the environment. This allele is either counter-selected by pollution, and thus the term "pollution sensitivity indicator" can be used, or favoured by this pollutant, and there, the term "resistance indicator" is appropriate. Monitoring these two types of indicators is of the utmost interest as the fluctuations in frequency of these alleles depend on the improvement or degradation in environment quality. As a rule, the allele frequencies of sensitivity indicators increase with a decrease in the concentration of a given environmental pollutant. With increasing pollution levels, individuals carrying the alleles favoured by pollutants have a better chance of survival.

When tested concentrations differed by only 2 or 3, genetic data analysis showed that, whatever the pollutant added, a same allele could be counter-selected or favoured. This reduces the choice among alleles potentially useful as specific genetic indicators. When the two concentrations give the same pattern of evolution and/or when the Fst values confirm a significant difference between the frequency distributions, we can conclude that this allele is a good indicator.

Indicators characterisation evidenced that each pollutant has a specific impact on several gene loci. This specificity has already been observed in many species. Ben Schlomo & Nevo (1988) demonstrated that in *Palaemon elegans* some genotypes at the *Pgm* locus could be counter-selected or favoured with cadmium or mercury which led them to suggest genotype adaptability. In other

Table III. – Heterozygosity rates in the S, MR and R groups and Fis values for the studied loci in the two populations (* : significant at the 5% level).

	ATRAZINE				ISOPROTURON				
	0.1 mg/l		0.2 mg/l		0.1 mg/l		0.2 mg/l		
	S	R	S	R	S	R	S	R	Fis
Ak	0.404	0.408	0.350	0.399	0.408	0.293	0.352	0.361	**0.131***
Pgi	0.228	0.129	0.262	0.205	0.229	0.106	0.229	0.203	**0.065**
Cap-1	0.255	0.255	0.259	0.396	0.315	0.228	0.378	0.275	**0.328***
Pgm	0.774	0.686	0.817	0.685	0.774	0.666	0.816	0.692	**0.243***

	CUIVRE				CADMIUM		TRIBUTYLTIN						
	2.5 ppm		1 ppm		1.5 ppm		50 ng/l			150 ng/l			
	S	R	S	R	S	R	S	MR	R	S	MR	R	Fis
Aat-2							0.333	0.491	0.800	0.349	0.530	0.569	**-0.022**
Ak	0.412	0.393	0.300	0.283	0.250	0.404	0.393	0.280	0.600	-	0.325	0.156	**0.124***
Pgdh	0.441	0.321	0.333	0.250	0.250	0.287	0.212	0.193	0.200	-	0.252	0.372	**0.167***
Pgi	0.206	0.089	0.200	0.150	0.187	0.262	0.212	0.158	0.200	0.135	0.153	0.105	**-0.008**
Cap-1	0.265	0.286	0.233	0.383	0.250	0.167	0.333	0.421	0.500	0.069	0.362	0.375	**0.067**
Pgm	0.617	0.625	0.667	0.533	0.562	0.536	0.424	0.491	0.800	0.370	0.593	0.617	**0.094***

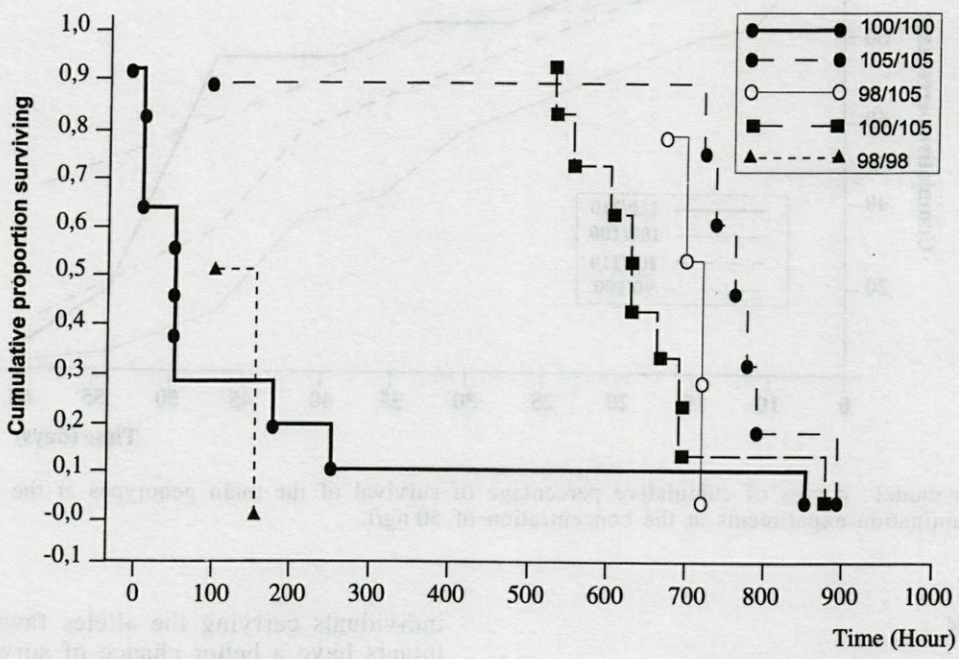

Fig. 5. – Cox's model : curves of cumulative percentage of survival of the main genotypes at the *Pgm* locus in isoproturon contamination experiments at the concentration of 0.1 mg/l.

studies (Lavie & Nevo 1986) performed on the same heavy metals, similar results were obtained for the *Pgdh* and *Pgi* loci which carried alleles and showed genotypes characteristic of metal-resistance or -sensitivity. Our results underline that the allele of *Ak* and *Pgm* loci seem to be the most susceptible to contamination by cadmium or copper. At 1.5 ppm cadmium concentration, the results obtained are comparable to those of De Nicola *et al.* (1992). These authors also evidenced a counter-selection of heterozygotes for the Pgm locus which confirms our results on cadmium in *Crassostrea gigas*. Homozygosity favoured at the *Pgm* locus was also demonstrated in our experiments carried out with atrazine and isoproturon; however, opposite results were obtained with tributyltin. De Nicola *et al.* (1992) did not establish a relationship between cadmium and the counter-selection of heterozygotes at the *Pgi* locus in *Idotea baltica*. Others authors (Lavie & Nevo 1986; Chagon & Guttman 1989), however, have shown a counter-selection of heterozygotes at the *Pgi* locus in experiments with cadmium. Our results underline that homozygotes were favoured at the same locus under copper contamination conditions. The work carried out by Mortimer & Hughes (1991) on the crab *Heloecius cordiformi* showed that individuals heterozygous at the *Pgi*

locus were favoured by an organophosphorous larvicide. We obtained similar results at the *Pgi* locus when atrazin and isoproturon were administered.

The *Pgi* and *Pgm* loci are often involved in heavy metal contamination experiments and have been extensively studied. The mechanisms of interactions between these enzyme systems and the metal ions have also been dealt with in many studies on marine species. Heavy metals such as copper, cadmium, zinc or mercury act on the active sites of membrane proteins, and more particularly, on the sites involved in the cellular transport of calcium (Gnassia-Barelli *et al.* 1995). Dixon & Webb (1971) and Gottlieb & Greve (1981) have shown that the catalytic efficiency of isozymes at the *Pgi* locus depends on metallic ion interactions with the thiol groups of proteins. The *Pgm* locus has been extensively studied by Milstein (1961a, b, c) who demonstrated a strong *Pgm* competitive inhibition between the usual metallic ion involved at the protein active site (ion Mg^{2+}) and pollutant ions. This ion substitution blocks the active site and inactivates the enzyme by "poisoning" it. *Palaemon elegans* tissues were histologically studied with staining techniques by Ben-Schlomo & Nevo (1988) who confirmed these results. In addition, Milstein studies (*op. cit.*) continued by Boyer (1970) have highlighted that competition reactions depend on pollutant nature.

TBT action mechanisms differ from those observed on heavy metals : this organic pollutant is fixed by both membrane proteins and phospholipids, and thus functionally and structurally alters the plasma membrane. This destabilization of the membrane structure entails changes in cellular permeability (Moore 1985). Very high TBT concentrations sometimes lead to the disruption of the plasma membrane and to the release of cellular content (Byington *et al.* 1974). These results suggest that the *Aat-2* locus may be an appropriate TBT pollution indicator as it codes an enzyme involved in osmoregulation. In mollusc bivalves and certain fish species, TBT acts preferentially on immunocytes whose chemiluminescence activity decreases proportionally with increasing TBT concentrations (Fisher *et al.* 1990; Dunier & Siwicki 1993). Tributyltin has various effects such as growth alterations in *Crassostrea gigas* juveniles at 5 ng/l concentration (Nell & Chvojka 1992), problems of shell thickening in oysters at 2 ng/l (Alzieu *et al.* 1981-1982, 1986), and female sex changes in a number of gastropod species (Bryan *et al.* 1986; Ellis & Pattisina 1990).

The survival capability of individuals relies on the pollution resistance mechanisms at their disposal to withstand pollution. These detoxification processes differ and depend on the pollutant nature. During tributyltin detoxification, TBT is transformed into dibutyltin (DBT) and, subsequently, into monobutyltin (MBT) (Maguire 1987). It has been shown that DBT and MBT are 50 to 1 000 times less toxic than TBT itself (Wong *et al.* 1982; Wester & Canton 1987); this toxicity depends on the species studied. The resistance to heavy metals relies on other detoxification processes. Okasaki & Paniertz (1981) demonstrated that detoxification activity was relatively high in *C. gigas* and that metal concentrations decreased more rapidly in internal tissues such as the digestive gland than in external tissues. These authors showed that detoxification activity was particularly intense for heavy metals such as copper, cadmium, zinc, mercury or iron. Others studies have revealed that, even though heavy metal concentrations were high in *C. gigas* tissues, their toxicity was partially neutralized by sequestering metals into the blood amoebocytes (George *et al.* 1978; Thomson *et al.* 1985).

Atrazine is an herbicide which acts on the process of photosynthesis by inhibiting electron transport in photosystem II. It indirectly affects stomata opening, transpiration, ion transport, reactions involving energy supply and plant hormone- and ion-balances (Ebert & Dumford 1976). Atrazine toxicity has been widely studied in invertebrates using the LC 50 methodology and the results obtained depend on species and exposure times. The LC 50 of *C. gigas* is about 30 mg/l for a 48-hour exposure time, but it is reduced to 1 mg/l when this time is extended to 96 hours (Maecek *et al.* 1976). Exposure time seems to be a more crucial factor than concentration for the survival of oysters. Our results showed that LC 50 was observed after a 30-day exposure at 0.1 and 0.2 mg/l atrazine concentrations. In other invertebrates, toxicity values for atrazine ranged within 94 µg/l and 1 g/l (Eisler 1989; Premazzi & Stecchi 1990). Though pesticides be known to alter the genetic structure at the population level (Tsakas & Krimbes 1970; Pasteur & Sinegre 1975, Hughes *et al.* 1991), only few studies dealing with pesticides impacts on genetic populations have been carried out up to now. However, none of them concerned isoproturon although it seems to behave like atrazine. Nowadays, alachlor and metolachlor replace atrazine, strictly forbidden in EEC in maize cultivation. From the results presented here these two herbicides seem less toxic than atrazine; however, their toxicity may increase at higher concentrations.

Our experiments carried out on different pollutants revealed, through the examination of six allozyme loci, the selective action of these biocides on the genetic structure of the Pacific oyster *C. gigas*. These pollutants have repercussions on the mortality levels; they can alter the development and survival of the populations in their natural environment. These results are in general

agreement with those obtained by other authors on other species. Identifying these biological indices is the first step to implement a monitoring program in littoral ecosystems.

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