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To cite this version:

Sandrine Pain, Simon Devin, Marc Parant. Biomarker versus environmental factors: seasonal variations and modelling of multixenobiotic defence (MXD) transport activity in transplanted zebra mussels. Science of the Total Environment, 2007, 373 (1), pp.103-112. 10.1016/j.scitotenv.2006.10.054 . hal-01726706

HAL Id: hal-01726706

https://hal.univ-lorraine.fr/hal-01726706v1

Submitted on 8 Mar 2018

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Biomarker versus environmental factors: seasonal variations and modelling of multixenobiotic defence (MXD) transport activity in transplanted zebra mussels.

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ABSTRACT

The occurrence of biomarker temporal variations linked to environmental factors makes it difficult to distinguish the specific effect of pollution. The present work aims to investigate the seasonal variations of the transport activity of the multixenobiotic defence (MXD), that is used as a biological tool for the monitoring of pollution in aquatic ecosystems. The MXD transport activity was monitored monthly from August 2001 to October 2002 in zebra mussels (Dreissena polymorpha) transplanted to three sites in the Moselle River. The 'efflux method' was used to evaluate functional activity of MXD by inhibition between rhodamine B and verapamil. Water physicochemical characteristics were provided by a French regulatory agency (Water Agency) that monitors river water quality. The results of a principal components analysis describe the seasonal cycle of water characteristics and demonstrate that MXD activity is subjected to significant temporal variations. These data were described with a generalised linear model that enables to link MXD variability to the seasonal variations of environmental parameters such as temperature or levels of organic contamination. This work proposes a modelling approach and highlights that the occurrence of seasonal variations in MXD response has to be taken into account in the interpretation of *in situ* monitoring studies.

KEYWORDS

Multixenobiotic defence, Biomarker, Dreissena polymorpha, Seasonal variations, Environmental parameters, Generalised linear model.

INTRODUCTION

In the field of ecotoxicology, biomarkers are widely used to evaluate the health of the aquatic environment. Biomarkers provide biological responses that are useful tools informing about pollutant exposure and their possible toxic effects in living organisms (National Research Council, 1987; Lagadic et al., 1997). Although biomarkers aim to evidence pollutant related responses, recent studies have pointed out the need to integrate other factors in the interpretation of results (Vidal et al., 2002; Bodin et al., 2004; Gillis et al., 2004; Petrovic et al., 2004). Abiotic factors (water temperature, conductivity, organic matter level, etc.) and biotic factors (age, reproduction stage, parasitism, etc.) vary throughout the year on the basis of seasonal cycles. As these variations may greatly influence biological responses, ecotoxicological researches are directed towards the evaluation of temporal fluctuations of biomarkers by field monitoring of their responses in native and/or in transplanted organisms over at least a one year period.

The general objective of the present work was to evaluate the seasonal variations of a biomarker, the activity of the multixenobiotic defence mechanism, in caged zebra mussels (Dreissena polymoprha) over a 15 months field study.

Because they are abundant in aquatic ecosystems, easy to collect and to handle (particularly for caging) and also representative of their environment (sessile), bivalve molluscs are particularly suitable for the field investigation of seasonal variations. In freshwater ecosystems, zebra mussels have received most attention for years as a sentinel species for ecotoxicological studies (de Lafontaine et al., 2000; Smolders et al., 2004; Bervoets et al., 2005; Giamberini and Cajaraville, 2005).

The multixenobiotic defence mechanism (MXD) has been proposed to constitute a relevant biomarker of exposure to pollution (Parant and Pain, 2001; Pain and Parant, 2003). Briefly, MXD acts as a first line defence system against xenobiotics by preventing cellular accumulation and enhancing cellular efflux of potentially harmful molecules that can be found in aquatic environments (Kurelec, 1992; Epel, 1998). It has been associated with the expression of a membrane glycoprotein called P-glycoprotein (Pgp) that mediates ATP-dependent efflux of a wide variety of structurally and functionally diverse compounds (Endicott and Ling, 1989; Gottesman and Pastan, 1993).

The Pgp-mediated efflux phenotype has been identified in many aquatic organisms (for a review see Bard, 2000) and especially in sentinel species as bivalve molluscs (Minier et al., 1993; Smital and Kurelec, 1997; Parant and Pain, 2001). In bivalves Pgps are mainly

expressed in tissues directly exposed to environmental pollution or in tissues involved in the detoxification of pollutants (gills, mantle, digestive gland; Kurelec and Pivcevic, 1989; Minier and Galgani, 1995). MXD has been shown to confer effective protection against deleterious effects caused by the exposure to toxic compounds in adults as well as in embryonic or larval stages (Waldman et al., 1995; McFazden et al., 2000). Finally, this defence system has also been shown to be induced in bivalves collected from polluted areas (Minier et al., 1993; Kurelec et al., 1996) and following field or laboratory exposures (Eufemia and Epel, 2000; Smital et al., 2003; Achard et al., 2004; Legeay et al., 2005). Our previous works also showed that MXD was induced in zebra mussels exposed to environmental stress when compared to control organisms (Pain and Parant, 2003).

Usually, the induction of a biological response following exposure to pollution is assessed by comparison to a control response in non-exposed organisms. This control is considered as a “normal response”, that is to say, as the physiological level of the studied function. However it must be determined whether this physiological level is constant or whether it can change, and in the latter case, it must be determined what are the factors involved in its variations. Environmental factors that can modulates parameters linked to reproduction or food supply for example are likely to influence the biology of bivalve molluscs and then to make biological response to pollutants variable (Sheehan and Power, 1999). The temporal variations of the biological defences against pollution may both affect the level of organism protection and make it difficult to distinguish between effects of pollutants and effects of other environmental factors. As a consequence, it is of primary importance to determine the normal range of variations of biomarker activities in order to propose usable and useful diagnostic tools (Bodin et al., 2004; Lau et al., 2004; Leinio and Lehtonen, 2005).

Seasonal changes of MXD response has been evidenced in marine bivalves and these variations were linked to environmental factors such as temperature (Minier et al., 2000; Keppler and Ringwood, 2001a, 2001b). Because this evaluation is crucial for a relevant use of MXD as a biomarker, we investigated its variability in a freshwater ecosystem. For this purpose, the MXD activity was monitored monthly from August 2001 to October 2002 in zebra mussels transplanted to three sites in the Moselle River. Finally, a model linking environmental parameters to MXD levels was established.

MATERIALS and METHODS

1- Site description

The present study was carried out in the Moselle River, a tributary of the Rhine River that flows in the north-east of France. Three sites were chosen, covering approximately 100 km of the lower Moselle. A map of the river and sampling sites is presented in figure 1. La Maxe (LMX) is the site where the zebra mussels were sampled. It is located in the downstream part of the river, few kilometres downstream the town Metz, whereas Pierre La Treiche (PLT) and Liverdun (LIV) are located in the upstream part of the river.

2- Zebra mussels collection and transplantation

Zebra mussels (Dreissena polymorpha; 20 ± 2 mm in shell length) were collected in July 2001 in the Moselle River (La Maxe, figure 1). Organisms were carried to the laboratory in the water of origin. They were kept under laboratory conditions for 8 days prior to experiment, i.e. dechlorinated water (16-17°C) with continuous oxygen changed daily. Mussels were fed daily with commercial frozen food (Nauplium). Random groups of 60 'depurated mussels' were then placed in cages made of polyethylene netting with a mesh size of 4 x 4 mm, allowing free circulation of water. The dimensions of the cages were 20 x 9 x 2 cm. Forty-five cages containing 60 mussels each were then deployed *in situ*. They were transplanted in the Moselle river (15 cages per site), to PLT and LIV, and also to LMX, which was the site where the mussels were collected from. The cages were secured to the bank with a long piece of wire attached to trees present on the river bank and were allowed to float at 20 cm above the river bottom (rocky bottom; total depth of the water column at the transplanting area of about 80 cm).

Monthly monitoring of MXD activity in zebra mussels

The measurements were conducted each month from August 2001 to October 2002 (except for LIV in October 2002). One cage of mussels was collected each month at each site and carried to the laboratory in the water of origin. The MXD activity in the mussels was assessed the following day.

The efflux method was used to evaluate functional activity of MXD, that is, to assess Pgp related efflux by comparing total efflux in control organisms to that of organisms that were given the inhibitor (verapamil) (Kurelec et al., 2000; Parant and Pain, 2001). This efflux assay consists first in exposing mussels to the fluorescent dye that they accumulate in their

gills cells and second, in measuring the efflux rate of the dye with or without the inhibitor. Briefly, mussels were exposed to dechlorinated water containing 5 µM of the fluorescent dye rhodamine B in a light-protected beaker for 4 hours. After the loading period, mussels were washed three-times in dechlorinated water for 2 minutes. Mussels were then exposed either to 30 mL of dechlorinated water or to 30 mL of dechlorinated water containing 30 µM of verapamil, the MXD inhibitor. Every 5 minutes during 50 minutes, 100 µL of efflux medium were transferred in duplicate to a 96-well microplate and the fluorescence of effluxed RB was immediately measured on a Fluostar (BMG Lab Technologies). This allowed to assess the MXD related efflux rates of rhodamine B. Finally, the activity of MXD was evaluated by the difference between the efflux rates obtained in the absence and in the presence of verapamil. Each efflux measurement was conducted in triplicates.

3- Environmental parameters

Water physicochemical parameters were monthly collected by a French regulatory agency for the monitoring of river quality (Agence de l'Eau Rhin-Meuse) and were retrieved through their database (available at http://www.eau-rhin-meuse.fr/sierm/, 2006), except for water temperatures that were recorded directly during zebra mussels collection. Data on 15 parameters were finally available during the biological monitoring: oxygen saturation, chemical and biological oxygen demand, dissolved organic carbon, total nitrogen, nitrites, nitrates, ammonium, phosphorus, phosphates, suspended matter, conductivity, pH, chlorophyll a levels and temperature.

4- Statistical analyses

The analysed data are mean values calculated from the MXD activities obtained for 3 groups of 5 mussels, except for PLT in October 2002 where only two groups were used. Because the MXD responses measured each month in the three studied sites did not meet the assumptions for ANOVA (Kolmogorov-Smirnov test for normality and Levene test for variance homogeneity), the non-parametric Kruskal-Wallis ANOVA (KW) was used for comparative purposes. Pairwise comparisons were carried out with the Mann-Whitney (MW) non parametric test for two-samples. For all statistical analysis, the probability level determining significance was $p < 0.05$.

A standardised principal component analysis was performed in order to describe the seasonal variations of river physicochemical characteristics and then to investigate how these

variations could reflect and explain the variations of MXD responses in zebra mussels transplanted in the Moselle. In this aim, the PCA was first conducted using the physicochemical data (15 parameters) collected for the Moselle river during the fifteen months of the study (44 observations). Second, the MXD activity was added *a posteriori* as a supplementary variable on the correlation circle. In this way, the variable "MXD activity" did not take part in the construction of the PCA axis and this enabled to highlight the relation between this variable that we wanted to explain and the whole physicochemical explanatory variables (Philippeau, 1986). The PCA showed that the first three axis explain 67.77% of the total variance. The factorial plane F1xF3 (44.96% of the total variance) was preferred to F1xF2 (48.28% of the total variance) because the variable of interest "MXD", added *a posteriori*, was best represented.

A non parametric Spearman correlation was computed to investigate linear relationship between MXD response and water temperature.

A generalised linear model (GLM) (McCullagh and Nelder, 1989) was applied to analyse the dependence of MXD response on 15 variables that reflect the seasonal change of environmental conditions (see table 1 for details). Data from the three sites were pooled in order to consider a wider range of physicochemical variations. For the modelling of MXD activity, the error was assumed normal and the log link function was used. A forward stepwise procedure was adopted to build the model. At each step of the modelling process, variables can be both added or removed from the model. The significance of each variable is tested at each step to determine whether it should be entered, removed, kept in or kept out of the model. This method allowed to conserve only useful variables, and not to consider redundant or useless ones. At the end of the procedure, several parameters were controlled: the significance of variable coefficients according to the Wald' test, the contribution of each variable to the model with the loglikelihood-ratio test for cumulative effects and the overall goodness of fit according to the deviance (the parameter that measures the discrepancy between modelled and observed values in GLM). It allowed to identify variables whose elimination will not result in a significant increase in deviance, and only significant factors will be kept in the final model. The distributions of the model residuals were analysed to test the model validity.

Data were analysed using STATISTICA software (Statsoft, 1999) except for the Principal Components Analysis that was performed using ADE-4 software (Thioulouse et al., 1997).

RESULTS

1- Seasonal variability in MXD in transplanted zebra mussels

Significant differences in MXD activity measured in zebra mussels transplanted in LMX, in PLT and in LIV were observed throughout the experiment (figure 2; KW, $p<0.05$).

In LMX, the MXD activity levels were low and homogeneous (KW, $p>0.05$) from August 2001 to May 2002. From June 2002 until the end of the experiment, MXD response was increased and more heterogeneous (KW, $p<0.05$). August 2002 showed the highest level (66 pmol.RB/min/org).

In PLT, the MXD activity levels were low and homogeneous (KW, $p>0.05$) from August 2001 to January 2002. The months when MXD was significantly induced are April, June and August 2002 (MW, $p<0.05$). From March to June 2002, levels reached at PLT were higher than those obtained at LMX (MW, $p<0.05$). June 2002 showed the highest level (70 pmol.RB/min/org).

In LIV, the months when MXD was significantly induced are June and August 2002 (MW, $p<0.05$). In October 2001, March, May and June 2002, the level was higher than that obtained at LMX (MW, $p<0.05$). In January and June 2002, the level was higher than that obtained at PLT (MW, $p<0.05$). June 2002 showed the highest level (115 pmol.RB/min/org). In LIV, responses are more heterogeneous throughout the year than at the two other sites.

Note that we could not detect any MXD transport activity in December. For this measure, the dye efflux in dechlorinated water was the same as that in inhibitor solution, as if verapamil was not able to inhibit the dye efflux.

2- Seasonal variability in environmental parameters

Available physicochemical data provided by the French Water Agency for each sampling date and each site are presented in table 1. PLT and LIV, which are located in the upstream part of the Moselle River showed quite similar characteristics. LMX, located in the downstream part, generally showed higher levels for quite all the studied parameters, which denotes a water of lesser good quality with respect to the two other sites. However, the major difference between the upstream part and the downstream part of the Moselle river concerns water conductivity which was on average 3 to 3.5-fold higher in the downstream part than in the upstream part.

The principal component analysis enabled to describe seasonal variations of physicochemical characteristics of the river water. The correlation circle presented in figure 3 shows that the F1 axis is positively correlated with nitrogenous matter and negatively with phosphorous matter and dissolved organic carbon. The F3 axis is negatively correlated with the water concentrations of chlorophyll a and biological oxygen demand. In figure 4, the results of PCA are shown by the position of monthly physicochemical data on the factorial plane F1xF3. These results highlight the seasonal cycle of the physicochemical characteristics of the studied river, from the summer 2001 to the autumn 2002. Autumn and winter periods are characterised by increased levels of nitrogenous matter and on the opposite, spring and summer periods are characterised by increased temperature, pH, oxygen saturation, biological oxygen demand, concentrations of chlorophyll a, dissolved organic carbon and phosphorous matter. It can be noted that the two autumn periods and also the two summer periods (2001, 2002) are clearly distinguished in the factorial plane, indicating a marked difference in physicochemical profile between these two years.

3- Relationship between response of MXD and environmental parameters

The projection *a posteriori* of MXD activity on the correlation circle of the PCA (figure 3) showed that it was positively correlated to temperature and concentrations of chlorophyll a, and to a lesser extent to chemical oxygen demand and conductivity that were not sufficiently represented on the circle. In addition, MXD activity was correlated negatively to levels of nitrogenous matter. Levels of MXD activity were higher during summer periods than during winter periods. As other studies have highlighted the significant correlation between MXD response and water temperatures in marine ecosystems, we investigated this correlation in our freshwater ecosystem. The MXD response in zebra mussels transplanted in the Moselle River was indeed highly correlated with the temperature of the water ($r_{Spearman}$=0.493, n=132, p=1.8.10^{-9}). As water temperature is probably not the only parameter involved in MXD variations, all the environmental parameters were considered to build a generalised linear model. On the 15 physicochemical variables originally considered, only ten were finally retained, which influenced the MXD activity (Table 2). Modelled values fitted with observed ones (scaled deviance = 132, df = 121, p = 0.23), and residuals are normally distributed (deviance residuals, χ^2 = 6.1, df = 8, p = 0.63). Five variables seemed to have no influence on the biomarker activity: water conductivity, chemical oxygen demand, total nitrogen concentration and parameters related to phosphorus (phosphate and total phosphorus concentrations). The loglikelihood ratio test enabled to distinguish the variables that represent

a great weight in the model. The variables with greater weight in the model are temperature, biological oxygen demand, nitrites concentrations, and chlorophyll a levels (Table 3).

DISCUSSION

MXD seasonal variations have already been studied in marine bivalves (Minier et al., 2000, Keppler and Ringwood, 2001a, 2001b; Bodin et al., 2004). However, to our knowledge, this aspect has not been investigated in freshwater bivalves. Whereas the previously cited works were based on Pgp expression levels, we monitored the functional activity of the multixenobiotic defence in transplanted zebra mussels throughout a fifteen months study.

The monitoring of MXD activity in transplanted zebra mussels showed the occurrence of significant seasonal changes throughout the year. Though there were differences between the three studied sites, the global tendency was for the measured levels of MXD activity to be higher and significantly induced in the second part of our study, in spring and summer 2002. In 2002, MXD induction occurred during the warmest months as it was previously shown in marine bivalves by measuring the enhancement of Pgp expression (Minier et al., 2000; Keppler and Ringwood, 2001a, 2001 b). Indeed MXD induction in zebra mussels was significantly correlated with the water temperature in our study as well as in the previously cited works. Some authors even showed induction of MXD activity in marine bivalves exposed in laboratory to heat shock, suggesting a possible direct role of temperature in MXD induction (Eufemia and Epel, 2000). However, no induction was observed in transplanted zebra mussels during the warmest month in 2001 (August), which presented a similar value for water temperature. As this unexpected result was obtained for the first month of the experiment, difficulties linked to acclimation of mussels to caging conditions can not be excluded. However, a second hypothesis can be proposed. Regarding the position of the warmest months (August 2001 and June, July, August 2002) in the factorial plane F1xF3 of the PCA, it can be seen that they were clearly distinguished in the plane, indicating that they presented different physicochemical characteristics. This result suggests that the difference in MXD response between the warmest months of the two years may be explained by marked difference of physicochemical characteristics of the studied ecosystem (chlorophyll a levels, oxygen saturation…see table 1). Indeed, although the generalised linear model allowed to confirm that water temperature positively influences MXD activity, it also revealed that MXD is influenced by other environmental parameters that have to be taken into account in field

survey protocol. As a consequence, even if the water temperature is a predominant factor whose variations seem to reflect those of MXD response, other parameters are involved.

Among the 15 physicochemical parameters that were taken into account in our analysis, 10 were found to be significantly involved in the description of MXD temporal variations (biological oxygen demand, temperature, nitrites, suspended matter, chlorophyll a, pH, dissolved organic carbon, ammonium, nitrates oxygen saturation; tables 2 and 3). The model gives the relative contribution of each parameter to the global phenomenon. So it appears difficult to isolate the role of each environmental parameter. However, the contribution of biological oxygen demand and dissolved organic carbon is quite easy to consider. Both parameters describe level of organic matter in water. As a wide variety of organic molecules (natural toxins, algal metabolites, microbial degradation products, synthetic musk fragrances, environmental contaminants; see Smital et al., 2004 for a review) were shown to interact with MXD, it seems logical that the variations of MXD response could be explained in part by factors that describes organic matter levels. Moreover, biological oxygen demand corresponds to the fraction of organic matter that can be biologically degraded. This biodegradable part of organic matter may be preferentially transported by MXD, then explaining the preponderant place of BOD_5 in the description of MXD temporal variations.

Some authors had shown that algal metabolites could induce MXD defence (Eufemia et al., 2002). As a consequence, it has been hypothesised that the phytoplankton production could sometimes explain the high levels of MXD response in warm seasons (Minier et al., 2000). One can be surprised of the negative correlation, although it is very low, of chlorophyll a levels in the description of MXD variability in our study. However, even if the correlation coefficient is lowly negative, the weight of this parameter in the model is high, confirming its real contribution in the variability of MXD. The contribution of other factors (pH, oxygen saturation…) appears also difficult to explain. A direct involvement of these parameters in MXD variability is probably not to consider, supporting the idea that one isolated parameter can not explain all the complexity of seasonal variation of a biological response. However it is important to consider environmental factors on the whole and to model their interaction with the biota in order to better understand biological responses in realistic exposure conditions.

A number of environmental and physiological factors may influence biomarker responses that may fluctuate throughout the year (Sheehan and Power, 1999). Usually, biomarker responses are higher during summer period than in winter period (increased temperature, primary production, metabolism, stress, decreased oxygenation). The modification of these

parameters contributes to increasing environmental stress for living organisms, and results in activation of biomarkers of stress. The occurrence of such variations renders more complex the interpretation of biomarker response during in situ monitoring studies. As MXD activity has been proposed as a suitable biomarker of organic contamination in aquatic ecosystems, it seems essential to take into account the occurrence of the variability in the interpretation of results. In this aim, it would be of interest to determine whether the seasonal variations are similar from one year to the next. Moreover it will be interesting to compare the present investigation with monitoring studies of mussels transplanted in contaminated areas in order to determine how the addition of the "contamination" parameter could modify our model.

ACKNOWLEDGEMENTS

This research was supported by a fellowship from the French Ministry of Research and Technology. The authors are grateful to Jean-Nicolas BEISEL (Laboratory of Biodiversity and Ecosystem Functioning – University Paul Verlaine of Metz) for his valuable help with statistical analysis.

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Table 1: Physicochemical data obtained from the French Water Agency for the monitoring of river water (http://www.eau-rhin-meuse.fr/sierm/) for each month and site, except for water temperature which was recorded during mussels sampling (O_2%: oxygen saturation, COD and BOD_5: chemical and biological oxygen demand, DOC: dissolved organic carbon, N_{tot}: total nitrogen, NO_2: nitrites, NO_3: nitrates, NH_4: ammonium, P: phosphorus, PO_4: phosphates, SM: suspended matter, Cond: conductivity, pH, Chla: chlorophyll a levels and T: temperature).

		O_2%	BOD_5	COD	DOC	N_{tot}	NH_4	NO_2	NO_3	P	PO_4	SM	T	COND	pH	Chl a
SITES	DATES	%	$mgO_2.L^{-1}$	$mgO_2.L^{-1}$	$mgC.L^{-1}$	$mg.L^{-1}$	$mgNH_4.L-^{1}$	$mgNO_2.L^{-1}$	$mgNO_3.L^{-1}$	$mg.L^{-1}$	$mgPO_4.L^{-1}$	$mg.L^{-1}$	°C	$\mu S.cm^{-1}$		$\mu g.L^{-1}$
LMx	Aug 01	74	2.0	16	3.6	1.5	0.20	0.08	5.6	0.19	0.43	15	23	1130	7.4	4.9
	Sep 01	76	2.0	19	3.4	2.0	0.50	0.23	6.4	0.22	0.21	16	16	1490	7.7	3.0
	Oct 01	88	2.0	20	2.0	2.7	0.02	0.12	9.6	0.15	0.06	10	16	1360	7.7	2.7
	Nov 01	95	3.0	20	3.1	3.0	0.30	0.27	9.3	0.15	0.06	19	7	1350	7.8	1.7
	Dec 01	93	2.0	12	2.9	6.0	0.49	0.25	11.9	0.14	0.09	7	3	1450	7.8	1.1
	Jan 02	93	3.0	18	2.8	3.0	0.41	0.11	9.9	0.06	0.09	22	4	1550	7.9	2.2
	Feb 02	95	3.5	18	2.6	2.5	0.24	0.11	8.5	0.13	0.03	54	8	1205	7.9	3.2
	Mar 02	98	3.0	20	3.4	3.5	0.22	0.15	12.0	0.19	0.08	78	11	843	7.9	4.1
	Apr 02	115	3.3	18	2.3	2.3	0.32	0.15	7.1	0.10	0.03	14	12	1213	8.1	43.9
	May 02	122	3.0	23	3.6	2.5	0.07	0.22	8.5	0.12	0.06	17	18	1510	8.1	52.4
	Jun 02	102	4.0	27	4.3	1.5	0.10	0.40	4.0	0.17	0.09	16	26	1635	7.8	28.7
	Jul 02	103	3.0	21	3.6	1.0	0.06	0.11	1.1	0.21	0.07	19	23	1485	7.7	30.2
	Aug 02	68	3.0	19	2.8	1.5	0.26	0.10	5.5	0.21	0.33	10	25	1225	7.4	3.2
	Sep 02	91	3.0	29	2.5	2.5	0.13	0.15	6.1	0.26	0.18	15	20	1450	7.8	24.9
	Oct 02	88	3.0	22	4.6	2.1	0.20	0.12	6.4	0.17	0.16	12	15	1467	7.7	2.7
PLT	Aug 01	68	2.0	15	3.4	1.0	0.13	0.07	3.3	0.14	0.28	14	23	390	7.3	8.1
	Sep 01	79	2.0	28	3.0	1.0	0.19	0.03	2.5	0.19	0.18	14	14	500	7.5	6.5
	Oct 01	83	2.0	12	2.6	2.0	0.08	0.03	6.0	0.10	0.09	3	15	292	7.5	1.6
	Nov 01	99	2.0	9	2.7	2.0	0.09	0.03	4.7	0.12	0.15	8	5	371	7.8	0.7
	Dec 01	94	2.0	18	2.4	2.0	0.07	0.05	8.4	0.10	0.03	10	1	262	7.6	1.1
	Jan 02	93	3.0	11	1.5	3.0	0.18	0.04	8.9	0.09	0.15	12	2	543	8.0	1.1
	Feb 02	101	3.0	10	2.2	2.0	0.05	0.03	7.3	0.10	0.06	8	6	309	7.9	1.1
	Mar 02	94	3.0	6	1.3	2.0	0.02	0.03	8.0	0.09	0.06	4	10	345	8.1	3.8
	Apr 02	108	3.0	9	1.4	2.0	0.04	0.03	4.9	0.05	0.03	8	10	399	8.6	22.7
	May 02	93	3.0	12	3.2	2.0	0.05	0.05	4.9	0.09	0.06	5	16	285	7.9	10.8
	Jun 02	86	3.0	13	3.1	2.0	0.13	0.07	4.9	0.22	0.12	12	25	405	7.7	14.0
	Jul 02	131	3.0	15	3.1	1.0	0.04	0.03	1.7	0.13	0.06	9	22	515	8.1	27.0
	Aug 02	81	3.0	12	3.3	1.0	0.13	0.04	1.5	0.18	0.15	10	25	475	7.9	28.1
	Sep 02	97	3.0	20	3.4	1.0	0.07	0.04	3.0	0.02	0.01	5	19	450	8.0	24.3
	Oct 02	106	3.0	7	4.3	1.7	0.07	0.03	5.0	0.13	0.09	16	13	197	7.1	1.1
LIV	Aug 01	79	2.0	16	3.2	1.0	0.15	0.07	4.3	0.15	0.23	7	22	405	7.6	2.2
	Sep 01	87	2.0	12	2.9	1.0	0.12	0.04	3.9	0.16	0.11	11	14	354	7.5	1.9
	Oct 01	86	2.0	9	2.3	2.5	0.07	0.08	7.2	0.11	0.13	4	14	389	7.5	0.6
	Nov 01	91	2.0	13	2.7	2.0	0.05	0.05	8.0	0.09	0.11	9	6	475	7.8	1.1
	Dec 01	90	2.0	38	2.3	2.0	0.21	0.07	8.7	0.10	0.09	2	2	420	7.8	2.2
	Jan 02	93	3.0	15	2.8	3.0	0.15	0.07	10.8	0.12	0.09	11	3	576	8.0	1.4
	Feb 02	94	3.0	11	1.9	2.0	0.08	0.04	7.7	0.08	0.05	8	7	409	7.8	0.8
	Mar 02	100	3.0	21	3.1	3.0	0.24	0.09	12.1	0.22	0.12	105	9	325	7.9	7.3
	Apr 02	118	3.0	13	2.2	3.0	0.04	0.04	8.2	0.08	0.03	13	11	389	8.3	29.2
	May 02	110	3.0	13	3.0	2.0	0.07	0.08	5.8	0.13	0.09	9	16	456	7.9	18.4
	Jun 02	103	3.0	14	3.7	1.5	0.13	0.04	2.8	0.14	0.09	9	25	436	7.9	11.4
	Jul 02	96	3.0	15	3.1	1.0	0.07	0.03	1.7	0.14	0.08	10	22	427	8.0	26.5
	Aug 02	84	3.0	15	3.3	1.0	0.10	0.05	2.8	0.13	0.22	7	25	436	7.8	11.0
	Sep 02	89	3.0	13	3.9	2.0	0.06	0.04	3.8	0.18	0.20	5	19	479	7.8	4.4

Table 2: Generalised linear model results. *P-values* refer to the significance of coefficient according to the Wald' test.

	Coefficient	Standard Error	95% Confidence Interval		*P-value*
pH	3.232	0.464	2.322	4.142	<0.001
NH_4	2.934	0.72	1.522	4.345	<0.001
BOD_5	1.269	0.248	0.783	1.755	<0.001
DOC	0.359	0.095	0.172	0.545	<0.001
NO_3	0.158	0.049	0.062	0.254	0.001
T	0.144	0.015	0.115	0.174	<0.001
O2%	-0.018	0.005	-0.029	-0.008	<0.001
SM	-0.02	0.006	-0.033	-0.008	0.001
Chla	-0.03	0.006	-0.042	-0.018	<0.001
NO_2	-4.761	0.955	-6.633	-2.889	<0.001

Table 3: Results of the loglikelihood ratio test for cumulative effects. Variables were added sequentially in the generalised linear model, and the resulting increase in loglikelihood was tested with a chi-square test. Variables are presented in decreasing order of importance in the model.

	χ^2	*P-value*
T	52.337	<0.0001
BOD_5	32.846	<0.0001
NO_2	28.964	<0.0001
Chla	20.984	<0.0001
NO_3	16.139	0.0001
SM	11.04	0.0009
NH_4	6.725	0.0095
pH	6.035	0.014
DOC	5.097	0.024
O2%	0.029	0.8648

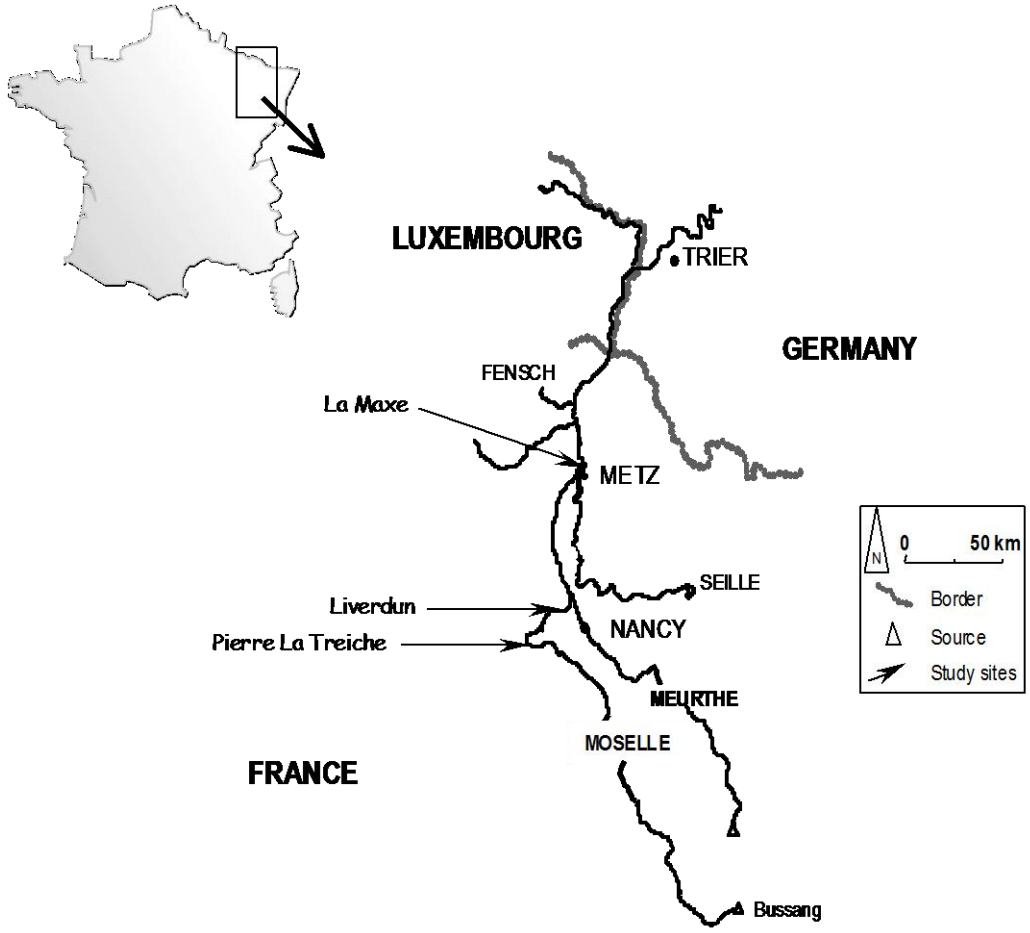

Figure 1: Location of the study sites in the Moselle River.

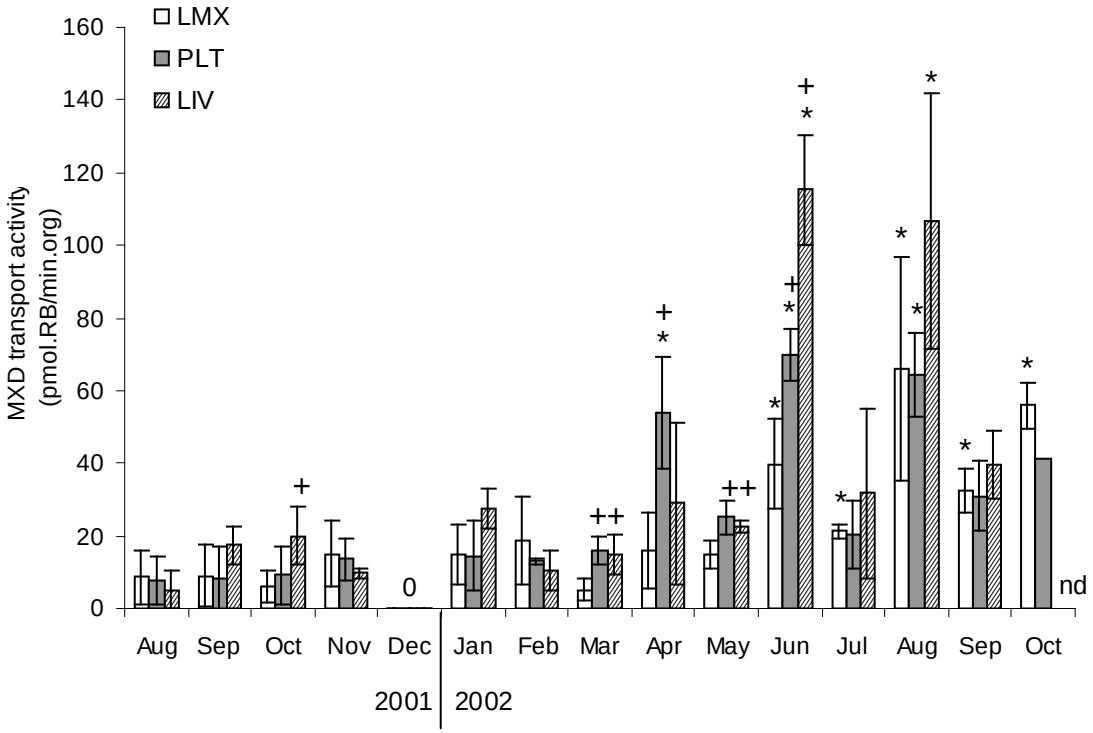

Figure 2: MXD transport activity (pmol.RB/min/org) in zebra mussels transplanted in LMX, PLT and LIV between August 2001 and October 2002. Data are mean ± SD of results obtained from 3 groups of 5 mussels at each month between August 2001 and September 2002. In August 2002, the MXD activity was not determined for LIV (nd) and the bar presented for PLT corresponds to the mean of 2 groups (29.6; 53.3 pmol.RB/min/org) instead of 3; that is why no SD appears in the graph. In December 2001, the MXD levels were null at all sites (0). * indicates months for which MXD level was significantly induced (Mann Withney , $p<0.05$) with respect to the other months for a given site. + indicates months for which MXD level was significantly induced with respect to the level measured in LMX which is the site where zebra mussels were sampled (Mann Withney , $p<0.05$).

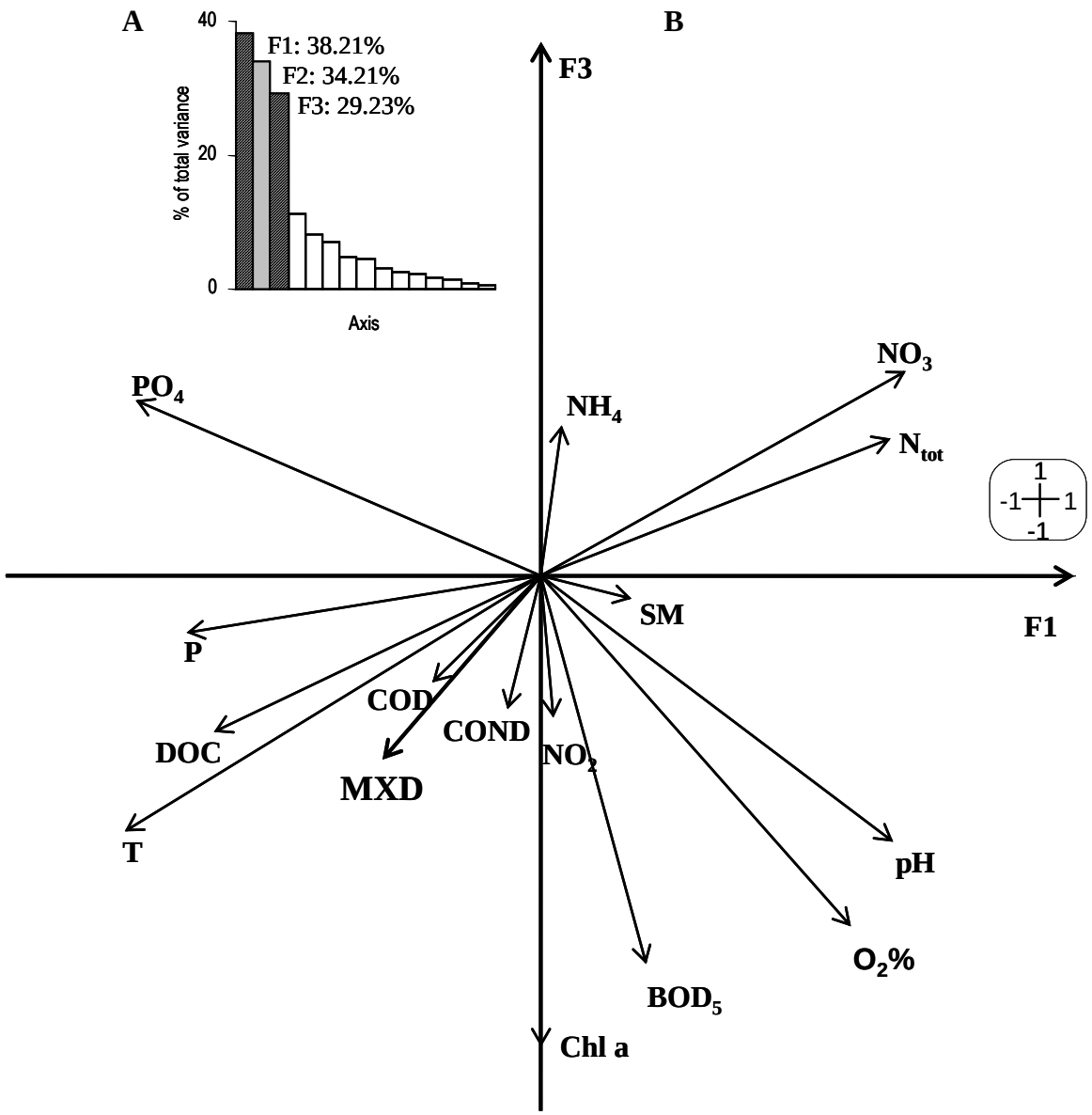

Figure 3: Eigenvalues (A) and correlation circle (B) of the principal component analysis performed using the physicochemical characteristics (15 parameters, 44 observations). The MXD activity was added *a posteriori* on the correlation circle.

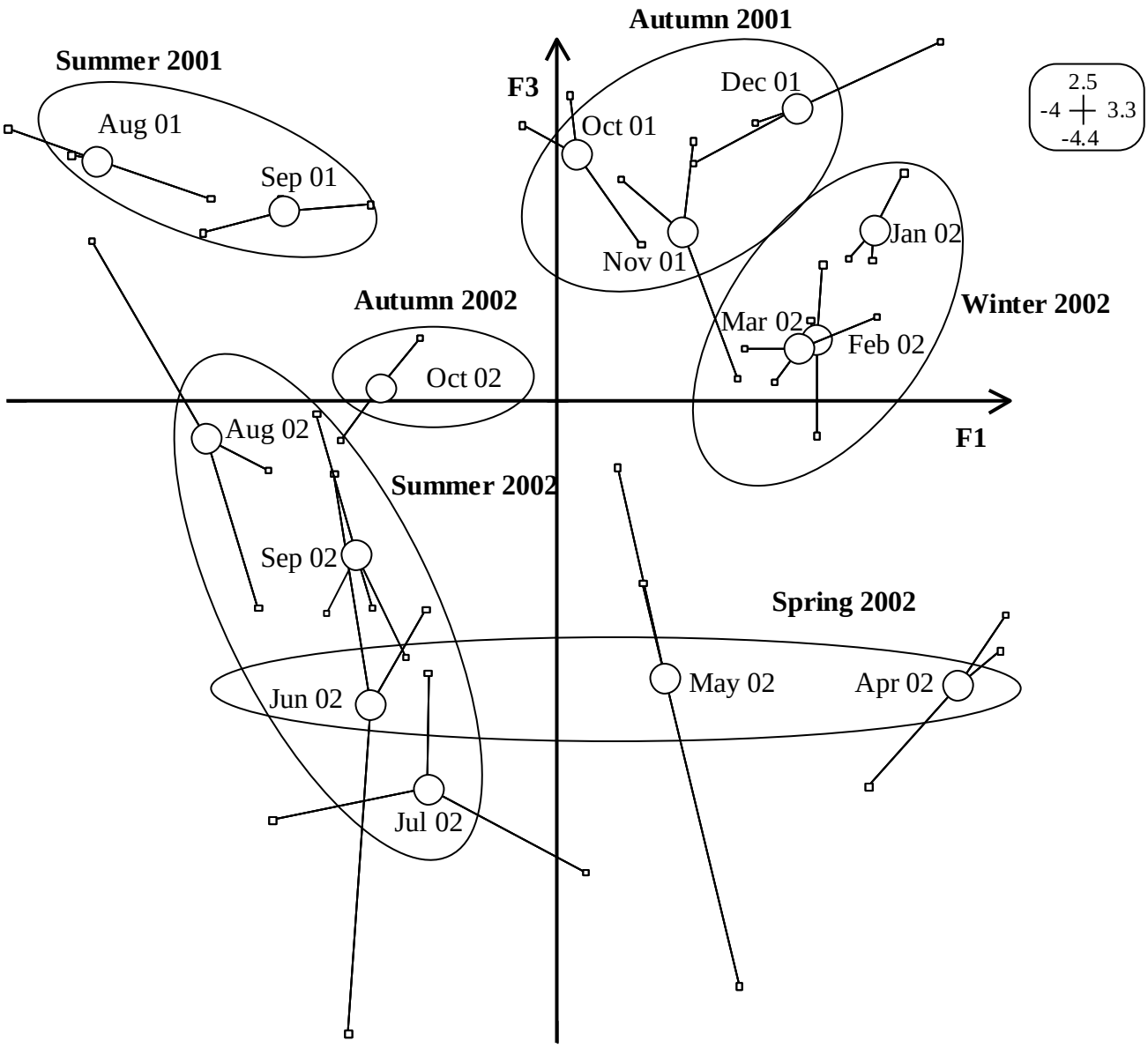

Figure 4: Factorial plane F1xF3 of the principal component analysis showing the position of the physicochemical data recorded for each studied month. Sampling dates were grouped by drawing arbitrary ellipses representing each studied season.