NATURAL VARIABILITY OF BIOCHEMICAL BIOMARKERS IN THE MACRO-ZOOBENTHOS: DEPENDENCE ON LIFE STAGE AND ENVIRONMENTAL FACTORS

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Abstract: Biomarkers are widely used in ecotoxicology as indicators of exposure to toxicants. However, their ability to provide ecologically relevant information remains controversial. One of the major problems is understanding whether the measured responses are determined by stress factors or lie within the natural variability range. In a previous work, the natural variability of enzymatic levels in invertebrates sampled in pristine rivers was proven to be relevant across both space and time. In the present study, the experimental design was improved by considering different life stages of the selected taxa and by measuring more environmental parameters. The experimental design considered sampling sites in 2 different rivers, 8 sampling dates covering the whole seasonal cycle, 4 species from 3 different taxonomic groups (Plecoptera, Perlidae grandis; Ephemeroptera, Baetis alpinus and Epeorus alpicula; Tricoptera, Hydropsyche pellucidula), different life stages for each species, and 4 enzymes (acetylcholinesterase, glutathione S-transferase, alkaline phosphatase, and catalase). Biomarker levels were related to environmental (physicochemical) parameters to verify any kind of dependence. Data were statistically elaborated using hierarchical multilevel Bayesian models. Natural variability was found to be relevant across both space and time. The results of the present study proved that care should be paid when interpreting biomarker results. Further research is needed to better understand the dependence of the natural variability on environmental parameters. Environ Toxicol Chem 2017;36:3158–3167.

Keywords: Biomarker Natural variability Macro-zoobenthos Multilevel non-nested model Bayesian inference

INTRODUCTION

Since the 1970s, biomarkers have been widely used in ecotoxicology as indicators of exposure to toxicants and effects of stress factors [1–3]. The popularity of biochemical and subcellular approaches increased later on, when advances in molecular biology gave rise to new scientific disciplines, such as proteomics and genomics, dealing with cellular structure and functions [4–6]. The success of these methods as early warning indicators of undesired effects is mainly attributable to their relatively easy measurement procedures, which can be carried out in a short time, in the laboratory as well as in the field. However, the usefulness of biomarkers in ecotoxicology and, in particular, their ability to provide unambiguous and ecologically relevant information on exposure to or effects of toxicants has been challenged [7]. The debate in the scientific community on the ecological relevance of subindividual parameters, as well as their usefulness in perspective and retrospective ecological risk assessment, is still ongoing [8].

To better understand the limits of the use of subindividual approaches in ecotoxicology, one must be aware that the protection goals of ecological risk assessment are related to the maintenance of the structure and functioning of ecosystems [9,10]. It follows that ecologically relevant end points must provide information on the consequences likely to occur at higher hierarchical levels of organization (populations and communities). At present, our knowledge on the relationships between effects measured at the subindividual level and the consequences at the community level are very poor [8,11].

Moreover, an additional key problem is the assessment of reference conditions. The knowledge of the natural values of biochemical and physiological parameters and of their variability as a function of environmental conditions, not altered by anthropogenic activities, is very poor for natural populations. For freshwater macro-zoobenthos, previous studies already acknowledged that biomarker variability across time and/or space is often not fully explained by stress factors [12,13]. However, studies explicitly addressing the natural variability of biochemical parameters in aquatic populations are surprisingly scarce.

In a previous work [14], the natural variability of enzymatic levels, measured in some freshwater invertebrate taxa sampled in pristine rivers, was proven to be relevant across both space and time. Differences measured on the same taxa in different sampling points, even in the absence of any measurable stress factor, were often statistically significant. This variability was poorly explained by the monitored environmental parameters. However, it was possible to conclude that the natural variability of enzymatic responses, in the absence of stress factors, must be carefully considered when biomarkers are used as stress indicators in the environment.

Therefore, the interpretation of biomarker data may be problematic and challenging. One of the major problems for biomarker use, in ecotoxicology and in ecological risk assessment, is understanding whether the measured responses are indicators of stress or lie within the natural variability produced by environmental parameters.
The objective of the present work was to study in more detail the natural variability of biomarkers for different aquatic invertebrate species and life stages. It must be noted that the life cycle of a species can be different from site to site as a result of several factors (e.g., genetic diversity, natural environmental conditions).

Moreover, suitable statistical approaches have been applied to develop models capable of describing and predicting the variability of biomarker responses as a function of environmental conditions, as well as to quantify the extent to which such variability can be directly connected to spatial–temporal sources.

MATERIALS AND METHODS

Study area

Sampling was carried out at 2 pristine sites in upper Val Seriana, in the Lombardy Alps, northern Italy. The first sampling station was located in Valbondione (46°02′39″N, 10°01′10″E; altitude 950 m asl), where the River Serio is still a mountain stream. The second site was located in Valgoglio (45°58′50″N, 9°23′25″E; altitude 1100 m asl) along a tributary of the River Serio (River Goglio). Both sites are completely unpolluted because there is not any kind of human activity in the upstream areas that could alter water quality (Figure 1). In particular, the sampling sites are upstream of any human settlement, including isolated houses or farms; upstream of the sampling sites, any type of agriculture is completely absent in the watersheds that are covered almost totally by forests, with small surfaces of natural meadows; intensive animal farming is totally absent; and the presence of free animals feeding in the few meadows is occasional and sporadic. Therefore, sampling sites can be assumed to be pristine, without any type of anthropogenic impact.

The substrate in the streambed at both sampling stations is comparable and mainly composed of rocks, cobbles, and a few sandy spots.

Experimental design

The benthic organisms were sampled monthly 8 times between June 2014 and June 2015. Sampling was suspended during the winter period (November 2014–February 2015) because of the scarcity of organisms and technical difficulties in the sampling procedures, also considering that rivers were often partly frozen.

One family of stonefly (Perlidae), 2 families of mayflies (Baetidae, Heptageniidae), and 1 family of caddisfly (Hydropsychidae) were selected for the present study. One species was chosen for each taxonomic group 1 species was chosen because of its abundance and ubiquity during the sampling period: Plecoptera, Perlidae, Perla grandis Rambur; Ephemeroptera, Baetidae, Baetis alpinus (Pictet); Ephemeroptera, Heptageniidae, Epeorus alpicula (Eaton); and Trichoptera, Hydropsychidae, Hydropsyche pellucidula (Curtis). Taxonomic classification was performed using specific keys for European insect larvae [15–17]. The specimens of each species were divided into 2 to 4 empirical classes based on the dimensional range, as in Table 1. A detailed description of the life cycle and of the different developmental stages of the 4 species is not available in the literature. Therefore, dimensional classes were assumed as a proxy of the different ages of the larvae. All of the dimensional classes of the different species were not always present in the different sampling dates because of life-cycle patterns.

Four enzymatic biomarkers, frequently used as indicators of environmental stress in ecotoxicological studies [18], were selected: acetylcholinesterase (AChE), catalase (CAT), glutathione S-transferase (GST), and alkaline phosphatase (ALP). Acetylcholinesterase catalyzes the hydrolysis of the neurotransmitter acetylcholine. Its inhibition is assumed to be an indication of exposure to specific contaminants such as organophosphate and carbamate insecticides. Glutathione S-transferase is involved in conjugation reactions with a series of xenobiotics of anthropogenic origin. Catalase is an indicator of oxidative stress. Finally, the activity of ALP is affected by the presence of inorganic contaminants.

The fundamental hydrochemical characteristics of water (temperature, pH, conductivity, alkalinity, nutrients) were measured. Other indicators of pollution were never measured because of the total absence of sources of anthropogenic pollution (urban, agricultural, and industrial) in the watershed.

Sampling

The samples of zoobenthos were collected using a conic net (mesh size 500 μm) by mechanically disturbing the substrate. The animals collected were placed in trays filled with water taken from the river itself. Specimens of the 4 species selected were classified, measured with a ruler, and divided into the dimensional classes.

To keep the organisms under nonstressful conditions, specimens of the same size collected at the same site were quickly dried with blotting paper, immediately inserted in a cryovial, and placed in a container filled with liquid nitrogen. Time between collection from the stream and freezing was minimized to avoid prolonged stressing conditions, which could

![Figure 1. Location of sampling sites in the River Serio valley. Human settlements are represented by black spots. There is no evidence of human impact upstream of the sampling stations.](image-url)
alter enzymatic levels. Usually (80% of cases), 4 to 6 replicates were collected for each species and dimensional class at any date and sampling site. The number of replicates was never lower than 3.

For each replicate, a suitable number of animals was collected to have a sufficient biomass to perform biochemical analysis (indicatively about 150 mg). For large Plecoptera, each replicate was represented by a single individual, whereas for small Ephemeroptera, each replicate was represented by a pull of approximately 10 to 15 individuals. Some specimens of different size or each species were collected and preserved in ethanol for careful taxonomic control.

**Analytical methods**

**Hydrochemical data.** Physicochemical characteristics of the water were measured on all sampling dates at the 2 sites. Water temperature, pH, and conductivity were measured in the field using a probe. For dissolved oxygen, samples were collected in glass bottles, fixed in the field, and measured within 24 h according to the American Public Health Association, the American Water Works Association, and the Water Environment Federation [19]. Oxygen saturation was calculated considering on-site measurements of water temperature. Nutrients (orthophosphate phosphorus, total phosphorus, ammonia, nitrite, nitrate, total nitrogen) were analyzed according to the American Public Health Association, the American Water Works Association, and the Water Environment Federation [19].

**Enzymatic parameters.** For each set of analyses, insect larvae, collected as described in the Sampling section, were homogenized with 9 volumes of 50 mM mannitol, HEPES-Tris 10 mM, pH 7.5, in a Potter-Elvehjem Teflon pestle and glass tube apparatus at 3000 rpm for 10 cycles. Crude homogenate was centrifuged at 15 000 g at 4 °C for 30 min. The resulting supernatant was used to measure enzyme activities. For the AChE assay, the homogenization buffer contained 1% Triton X-100. Catalase was assayed according to Bergmeyer and Grassl [20] using 12 mM H2O2 as substrate. Glutathione S-transferase was measured according to Habig et al. [21]. Acetylcholinesterase was assayed at 412 nm in the presence of 0.5 mM acetyl-tio-coline iodide as substrate as reported by Berra et al. [22]. Alkaline phosphatase was assayed at 405 nm at pH 8.1 using p-nitrophenylphosphate as substrate. Assays were performed at a temperature of 25 °C in a Cary50 spectrophotometer (Agilent) at saturating substrate concentration as determined by kinetic analysis; each determination was the mean of 2 replicates obtained with 10 to 40 μL of sample homogenate in a 1-mL cuvette test. Enzyme activities were analyzed by Cary Win UV application software for Windows XP, expressed as international units in micromoles per minute, and referred to protein concentration as determined by the method of Bradford [23] using bovine serum albumin as standard.

**Statistical methods**

The collected data show a complex structure which can be more easily interpreted in a hierarchical perspective. Indeed, for a given enzymatic biomarker (AChE, GST, CAT, ALP) measured on 1 of the 4 taxa of freshwater benthic invertebrates (i.e., for each statistical unit), we have measurements carried out at 2 geographical sites on 8 different dates.

Thus, we can distinguish between first-level units, represented by the sampled invertebrates, and second-level units, which can be identified with sites and times. This means that the first-level units can be grouped together with respect to the second-level ones, no nested (hierarchical) relation existing between the latter. Moreover, some covariates were measured on the first-level units (e.g., life-cycle), whereas others were site- and time-specific (i.e., measured on the second-level units; group level). Figure 2 illustrates the model structure together with all such covariates (inside the arrows and distinguished by unit level).

Ignoring the described hierarchical data structure, a standard linear model on the log-transformed enzymatic biomarker (dependent variable) can be fitted, including life-cycle stage, sampling time, and sampling site as individual covariates. Despite its simplicity, the linear model completely ignores the hierarchical data structure and rules out the inclusion of the covariates on the second-level units listed in Figure 2. To overcome these limitations, a suitable class of models can be resorted to. In light of the inherent hierarchical structure of the data and of the non-nested relation between the 2 possible groupings, the most appropriate methodological tool is a non-nested multilevel model. The latter is itself characterized by a hierarchical structure, and it allows accounting for the covariate effect. Indeed, the peculiarity of hierarchical models is that the model at the first level is defined as a classic regression model in which, however, some parameters, rather than being fixed, are themselves modeled by normal distributions with unknown variance (second-level model). A second important advantage of hierarchical models is the possibility of conjointly including covariates at the individual level (i.e., first level) and at the group level (i.e., second level).

One last relevant advantage of multilevel models is the possibility of expressing the variability of each random component by means of an ad hoc variance parameter (σ² for the dependent variable; σ² time and σ² site for the random effects). Consequently, we are able to evaluate the effect of site and time variability on the total by calculating the intraclass correlation (ICC),

\[
\text{ICC}_{\text{Time}} = \frac{\sigma^2_{\text{Time}}}{\sigma^2_{\text{Time}} + \sigma^2_{\text{Site}}} = \frac{\sigma^2_{\text{Time}}}{\sigma^2_{\text{Site}} + \sigma^2_{\text{Site}}} 
\]

which expresses the fraction of total variation in the data that is accounted for by between-group variation and ranges from 0 to 1.

The model estimation can be fulfilled using a Bayesian approach, which, given a prior distribution for all parameters, leads to a posterior one (i.e., a final distribution updated in the light of data). Such posteriors cannot be computed analytically.
for complex models; thus, the simulation-based method Markov chain Monte Carlo was implemented. A more formal definition of the model as well as all estimation issues can be found in the Supplemental Data.

RESULTS

Physicochemical parameters

The values of the main hydrochemical data and the concentrations of nutrients at the 2 sampling stations are shown in Table 2. The higher temperature at the Valgoglio station is attributable to the different sampling hours (i.e., morning in Valbondione, early afternoon in Valgoglio). For the other measured physicochemical parameters, the values may be different for the 2 stations because of differences in the characteristics of the drainage basin. However, in general, they are typical of the water hydrochemistry of pristine alpine stream, with high oxygen content (always higher than 9.6 mg/L) and low nutrient concentrations. The only exception is the peak of nutrients (both nitrogen and phosphorus) in the Valgoglio sample of May 2015. The only explanation for this outlier is the runoff of nutrients in a sporadic episode from a cattle pasture in the mountain meadow, the only possible human activity in the watershed.

Enzymatic parameters

The results of enzymatic analyses are shown in Figures 3 to 7. The complete data set is reported in the Supplemental Data (Table S1). In Figure 3, the averages of the data from different sites and times for the different species and life stages are reported. These data allow description of the differences between species and life stages, excluding space and time variability. Substantial differences are evident both in absolute values and in the trends from younger to older life stages. For Perla, a decreasing trend appears for all enzymes (with a partial exception for ALP). Lower values are always typical of older specimens. Such a trend common to all enzymes does not appear in other species. In some cases enzymatic values increase at the oldest stages (e.g., CAT or ALP in Baetis), or a clear trend is not evident (e.g., CAT in Epeorus).

All enzymes show a large variability in the 4 species. However, the variability range is different among the enzymes and the species. The ratio between maximum and minimum values measured for each enzyme in each species (all life stages) ranges between 4 (AChE, Baetis) and 97 (AChE, Hydropsyche) (see Supplemental Data, Table S2). Both AChE and GST show generally higher values in Ephemeroptera, particularly in Epeorus, whereas lower values were measured in Perla and even lower values in Hydropsyche. On the contrary, for CAT and ALP, Ephemeroptera shows generally lower values in comparison with other taxa. The highest values of CAT were measured in Perla, whereas the highest values of ALP were in Hydropsyche.

More detailed comparisons can be made on the data of Figures 4 to 7, where the variability in space and time is also shown. For the same enzyme and species, measured values were substantially different at the different sampling times (see, e.g., in Figure 5, the high values of GST for Baetis in March 2015). This was expected because the metabolism of the animals could be affected by the seasonal variability of many environmental parameters such as temperature, light and length of the day, and food availability. In some cases, however, comparable seasonal
values are not repeated in different years. For example, the high GST values measured in June 2014 in all dimensional classes of *Epeorus* are not repeated in June 2015.

For the same enzyme, species, sampling site, and date, measured values may be substantially different in the different life stages. For example, on several sampling dates, AChE in *Perla* is significantly higher at younger life stages. On the contrary, in the same species, ALP is frequently higher in oldest specimens (Figure 4). In *Epeorus*, AChE and ALP are frequently higher in younger specimens, whereas GST is, in
many cases, higher at the oldest life stages (Figure 6). This variability is justified by the physiological and metabolic differences that may characterize the different life stages of the insect larvae. However, the results do not show, in any species, a regular relationship between enzyme values and life stage at all sampling sites and times. For the same enzyme, species, and date, measured values may be substantially different at the 2 sampling sites, even in the same life stage. For example, AChE for Perla life stages 1 and 2 is much higher in Valbondione than in Valgoglio in June and July 2014 and several other cases (Figure 4). Comparable differences were observed for AChE in Epeorus in September and October 2014 (Figure 6). This variability is more difficult to explain considering the conditions of the sampling sites, apparently characterized by the absence of stress factors, either anthropogenic or natural, and by substantially comparable environmental conditions.

**Statistical elaboration**

At first, we fitted the standard linear model and estimated the regression parameters with a least squares approach. To evaluate differences among groups (namely, life-cycle stages, sampling sites, and sampling times), we performed an analysis of variance.
The results presented in Table 3 show that differences among sampling times are statistically significant in each model. Furthermore, differences between life-cycle stages are statistically significant in almost every model, apart from models for AChE and GST enzymes in Hydropsyche, where p values are >0.05. Finally, note that differences among sampling sites are statistically significant only for the following models: GST in Baetis, AChE and GST in Epeorus, AChE and GST in Perla, and CAT in Hydropsyche. In many models, therefore, empirical evidence emerges supporting significant differences in enzymatic response between sampling sites and times.

Focusing on the more refined multilevel model, for each taxa and each enzyme separately, we fitted a model without group (i.e., second-level) covariates as well as another with group intercepts and covariates. With respect to the latter model, we selected the water temperature, the oxygen level (mg/L), the total amount of nitrogen (µg N/L), and the percentages of N-NH4, N-NO2, and N-NO3 as covariates for the time effect. Moreover, we chose conductivity, pH, and total amount of phosphorus as covariates for the site effect. All covariate values are reported in Table 3.

Table 4 reports the estimated ICCs defined by Equation 1 for both models. In almost all of the models, both the sampling site and the sampling time have a notable effect on the total amount of variability. The effect of the sampling site seems to be particularly relevant in the models with group covariates, for all

(Figure 7. Measured values of the 4 enzymes in 2 different life stages (classes 1 and 2) of Hydropsyche at the 2 sampling sites from July 2014 to July 2015. AChE = acetylcholinesterase; ALP = alkaline phosphatase; CAT = catalase; GST = glutathione S-transferase; VB = Valbondione; VG = Valgoglio.

(Figure 8. For each sampling time, life-cycle stage is reported on the x axis and the acetylcholinesterase (AChE) levels on the y axis for Epeorus (EH). Black dots represent the observed values; the solid line is the estimated regression line for the Valbondione site, while the dashed line is the estimated regression line for the Valgoglio site.)
Natural variability may cover effects of stress factors.

AChE = acetylcholinesterase; GST = glutathione S-transferase; CAT = catalase; ALP = alkaline phosphatase; ICC = intraclass correlation.

The taxa. By way of example, for AChE in Baetis, ICC_Time and ICC_Site are similar, thus suggesting that the sampling time and the sampling site both determine a similar amount of variability on the total one.

A deeper inspection highlights that, in all of the models with group covariates, the total proportion of variability attributable to time plus site is greater than the same proportion in the corresponding models without covariates (i.e., the explanatory variables help in highlighting the effect of time and site on the total variability). Moreover, for all of the estimated models without covariates, ICC_Time is greater than the corresponding ICC_Time estimated in models with group covariates, an inverse relation emerging for ICC_Site. In other words, by adding covariates to the second-level model, the proportion of total variance attributable to the site increases at the expense of the proportion owing to time, but the overall variability proportion attributable to time plus site increases.

Table 3. Results from an analysis of variance

| Taxon    | Enzyme | Cycle | Time | Site |
|----------|--------|-------|------|------|
| Perla    | AChE   | 0*    | 0*   | 0*   |
|          | GST    | 0.006*| 0*   | 0*   |
|          | CAT    | 0*    | 0*   | 0.728|
|          | ALP    | 0*    | 0*   | 0.236|
| Baetis   | AChE   | 0*    | 0.714|
|          | GST    | 0*    | 0*   | 0.004*|
|          | CAT    | 0*    | 0*   | 0.348|
|          | ALP    | 0*    | 0*   | 0.008|
| Epeorus  | AChE   | 0*    | 0*   | 0*   |
|          | GST    | 0*    | 0*   | 0*   |
|          | CAT    | 0*    | 0*   | 0.126|
|          | ALP    | 0*    | 0*   | 0.279|
| Hydropsyche | AChE | 0.057 | 0* | 0.174 |
|          | GST    | 0.099 | 0*   | 0.938|
|          | CAT    | 0.0003* | 0* | 0*   |
|          | ALP    | 0*    | 0*   | 0.215|

*Significant (at 1% level) p values of F tests.

Table 4. Estimated intraclass correlations given by Equation 1 for time and site effects without and with covariates

| Taxon    | Enzyme | ICC_Time No group covariates | ICC_Site No group covariates | ICC_Time Group covariates | ICC_Site Group covariates |
|----------|--------|-------------------------------|------------------------------|---------------------------|---------------------------|
| Perla    | AChE   | 0.214                         | 0.479                        | 0.005                     | 0.958                     |
|          | GST    | 0.566                         | 0.233                        | 0.108                     | 0.845                     |
|          | CAT    | 0.524                         | 0.055                        | 0.448                     | 0.419                     |
|          | ALP    | 0.254                         | 0.083                        | 0.215                     | 0.646                     |
| Baetis   | AChE   | 0.669                         | 0.050                        | 0.448                     | 0.438                     |
|          | GST    | 0.790                         | 0.077                        | 0.167                     | 0.764                     |
|          | CAT    | 0.642                         | 0.065                        | 0.620                     | 0.322                     |
|          | ALP    | 0.404                         | 0.160                        | 0.229                     | 0.649                     |
| Epeorus  | AChE   | 0.767                         | 0.119                        | 0.525                     | 0.440                     |
|          | GST    | 0.657                         | 0.179                        | 0.218                     | 0.741                     |
|          | CAT    | 0.751                         | 0.080                        | 0.514                     | 0.413                     |
|          | ALP    | 0.683                         | 0.078                        | 0.651                     | 0.282                     |
| Hydropsyche | AChE | 0.578                         | 0.079                        | 0.297                     | 0.579                     |
|          | GST    | 0.684                         | 0.038                        | 0.674                     | 0.252                     |
|          | CAT    | 0.291                         | 0.155                        | 0.157                     | 0.822                     |
|          | ALP    | 0.400                         | 0.152                        | 0.268                     | 0.662                     |

AChE = acetylcholinesterase; GST = glutathione S-transferase; CAT = catalase; ALP = alkaline phosphatase; ICC = intraclass correlation.

Figure 8 plots the estimated regression functions for the model with group covariates about Epeorus, given the logarithm of AChE as a dependent variable. In each plot, we reported the observed values at a single sampling time. It clearly emerges that the site effect is relevant and the estimated lines are well distinguished at all sampling times. Comparable comments may be drawn from the estimated regression functions for the remaining models, reported in the Supplemental Data (Figures S1–S16). Note that the site effect seems to be modest (the estimated lines are almost overlapping) only with respect AChE in Baetis, CAT in Perla, and GST in Hydropsyche.

DISCUSSION AND CONCLUSION

The results obtained in the present study clearly demonstrate that, even in the absence of anthropogenic stress factors, enzymatic biomarkers show a natural variability capable of producing differences that in many cases are highly statistically significant. The variability of enzymatic parameters has been observed also in cases that are apparently comparable (same species, same life stage, same seasonal period). Therefore, it is not justified by metabolic patterns that may be explained by different climatic conditions (temperature, light cycle, etc.) or by different stages of the life cycle.

These results confirm those already described, in the same geographic area and for comparable taxa, by Ippolito et al. [14]. Moreover, the present work overcomes some biases of that study [14]: the selected taxa were taxonomically classified at the species level, and possible differences attributable to different stages of the life cycle have been taken into account and quantified. A comparison with the values measured in the present work with those measured by Ippolito et al [14]. shows that, on average, comparable values have been observed for the same taxonomic groups. However, no comparable seasonal trend seems evident. This confirms that the observed variability is determined by a complex combination of factors.

Explaining the origin of the observed variability is not easy. Besides the variability among the different life stages, which may be assumed as intrinsic to the species considered, the statistical models developed were able, by respecting the hierarchical structure of the data, to describe the effect of the “site” and “time” variables on the enzymatic parameters. However, finding some precise rules between the parameters characterizing the site and time variability and the biomarker variability was not possible.

Obviously, site and time characteristics are determined by a combination of parameters. Some of these are environmental factors that have been described only in part in the present study. Ecologically relevant factors, such as food availability, competition, and other ecological interactions, should be better investigated. Moreover, intrinsic biological factors of the species considered may also be relevant. For example, genetic variability of different populations may play a relevant role even at sampling sites relatively close, considering the strong isolating factors present in the mountain environment. In this regard, the observed variability has been divided into a part explained by chemical and physical parameters characterizing the basic hydrochemistry of the selected sites and a residual part interpretable as an irreducible amount of natural variability. More detailed studies accounting for additional chemical characteristics (e.g., naturally occurring metals and metalloids) or more complex ecological conditions (type and amount of food available, habitat structure, etc.) could be helpful for better explaining the role of the site and time variables.
Some experimental evidence in the literature confirms that the variability of enzymatic parameters across time and/or space is often not only explained by stress factors [12,13,24,25]. Unfortunately, studies addressing the natural variability of biochemical parameters are very scarce, and most of them were related to marine biota [24–29]. Cravo et al. [25], measuring several biomarkers (including AChE and GST) in the marine clam *Ruditapes decussates*, concluded that natural variability may act as a confounding factor and must be carefully accounted for in ecotoxicological studies. In freshwater ecosystems, Berra et al. [22] studied the environmental variability of a pool of enzymes across a wide range of taxa, taking into account seasonal and spatial variability. The focus of their work was on the different enzymatic response among taxa; however, spatial and temporal variations were also recorded within the same taxon. Schmidt et al. [27] studied the temporal variation of a pool of biomarkers in mussels sampled at 1 pristine site in Ireland. Significant fluctuations in biomarker levels, observed among seasons, have been explained by the authors as naturally linked to the reproductive cycle or to limited food availability.

Changes of environmental conditions such as temperature or salinity may have an influence on enzymatic levels [28,29], especially when coupled with exposure to a chemical stressor [30,31]. Indeed, the importance of physicochemical characteristics of water (even in the absence of pollutants) in determining *Daphnia* enzymatic levels has been already proven under laboratory conditions [32].

Nevertheless, attempts to link variability in biomarkers to environmental parameters in the field under natural conditions are rather scarce. Olsen et al. [33] used an in situ system to expose for 48-h fourth-instar *Chironomus riparius* larvae, coming from the same laboratory source, at 13 uncontaminated river sites across southeast England. Both GST and AChE significantly varied almost by 2-fold across sites. Despite the authors’ attempt to use multiple and simple linear regression to examine relationships between physicochemical variables and biomarker activity, no clear relationships could be established. This confirms the findings of the present study. Biomarker variability is reasonably determined by the concurrence of several factors, including environmental parameters, ecological interactions, and biological characteristics of the population studied.

These data strongly support the hypothesis that the use of enzymatic biomarkers as indicators of stress originated by anthropogenic pollution, without a precise knowledge of the range of natural variability, may lead to serious mistakes.

In human medicine, the measurement of biochemical parameters represents an indispensable tool for health assessment. Measured data are compared with a well-defined range of “normal” values. These values are the result of epidemiological studies based on a huge amount of information. Getting a comparable amount of information in environmental studies is inconceivable. Nevertheless, more attention must be paid to the range of natural variability of all types of subindividual end points (biochemical, physiological, genetic) commonly used as biomarker indicators of environmental quality. Specific studies should be developed at least on the species most frequently used as bioindicators. Without this kind of information, any hypothesis on the possible damages at the individual level and, even more, at the population level cannot be supported. Therefore, the use of these tools for ecological risk assessment may be seriously misleading.

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**Data Availability**—All detailed data are included in the Supplemental Data.

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