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Baseline

Baseline levels of biochemical biomarkers in the endobenthic ragworm *Hediste diversicolor* as useful tools in biological monitoring of estuaries under anthropogenic pressure

Andrew Barrick[a], Jean-Marie Marion[b], Hanane Perrein-Ettajani[a], Amelie Châtel[a], Catherine Mouneyrac[a,*]

[a] Mer Molécules Sante (MMS), Université Catholique de l'Ouest, 3 place André Leroy, BP10808, 49008 Angers Cedex 01, France
[b] Laboratoire Angevin de Recherche en Ingénierie des Systèmes (LARIS), EA-7315, Université Catholique de l'Ouest, 3 place André Leroy, BP10808, 49008 Angers Cedex 01, France

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ABSTRACT

Identification of contamination in estuarine ecosystems that are impacted by anthropogenic pressures, such as the Seine estuary, is difficult to determine without considering the role environmental variation plays on the end points selected. Currently, there is interest in identifying methods in which the influence of confounding factors can be described and accounted for. In this context, the aim of this study was to define a baseline assessment criteria (BAC) for enzymatic biomarkers in ragworms (*Hediste diversicolor*) collected in a reference site (Authie). The model took into consideration the weight, temperature and salinity of the site. Values collected in the Seine estuary were analyzed with the model to determine if differences between the sites could potentially be due to contamination or were explained by environmental variation. In general, biomarker responses from the Seine estuary fell within the range of BAC, suggesting that environmental variation could explain some of the results.

The Seine estuary, located on the English Channel, is one of the most important estuaries along the French Atlantic coast. The Seine basin (78,650 km^2), concentrates 40% of the economic activity of France, a quarter of the French population (17.5 million) living within the watershed and with 85% of the area consisting of urban development (Billen et al., 2007; Blanchard et al., 2007; Thévenot et al., 2007). Consequently, a wide variety of pollutants (e.g. metals, polycyclic aromatic hydrocarbons: PAHs, polychlorinated biphenyls: PCBs, pesticides, fertilizers) inputs are present in huge quantities in the Seine estuary. As result, the Seine estuary has been identified as significantly impacted by anthropogenic activity.

It is recognized that sediments (both suspended and deposited) represent a major sink for contaminants (organic and inorganic chemicals) into aquatic environments (Hack et al., 2008). Targeting environmental risk assessments on sediment based organisms can help describe the magnitude an ecosystem is impacted by anthropogenic inputs. The annelid polychaete *Hediste diversicolor* (*H. diversicolor*) is among the most widely used sentinel organisms representative of the sedimentary compartment within an ecosystem (Durou et al., 2007; Kalman et al., 2009; Moreira et al., 2006; Pérez et al., 2004). This polychaete is a keystone species inhabiting marine and estuarine environments of European and North American coasts where it represents an important food resource for crustaceans, fish and birds, making it an ideal organisms for measuring environmental risk (Catalano et al., 2012; Thit et al., 2015).

In the environment, mixtures of contaminants are present and consequently the use of a battery of biomarkers, constituting an early warning system to identify different classes of contaminants, is recommended in environmental assessments (Amiard and Amiard-Triquet, 2008). Several core biomarkers are commonly used to achieve this. Acetylcholinesterase (AChE) activity, a key enzyme in the nervous system, is recognized as a biomarker of exposure to pesticides, metals, synthetic detergents, some components of fuel oils and algal toxins (Bocquené et al., 1990; Bocquené, 2004; Tim-Tim et al., 2009). The glutathione-S-transferases (GSTs) are a multiple-enzyme family involved in phase II detoxification processes and are used as biomarkers of several groups of pollutants including organochlorine pesticides, PCBs and petrochemical products in invertebrates (Hoarau et al., 2001; Lima et al., 2007). Catalase (CAT), an anti-oxidant enzyme, is widely used as a biomarker involved in the primary defense against oxidative damage (Amiard-Triquet et al., 2013; Bergayou et al., 2009). Thiobarbituric acid reactive substances (TBARs) reflect the state of lipid

* Corresponding author.
E-mail address: cmouneyr@uco.fr (C. Mouneyrac).

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peroxidation of the membrane and are used as evidence of damage due to of oxidative-stress (Knight et al., 1988).

To effectively use biomarkers to measure responses to chemical stress, it is necessary to distinguish fluctuations of biological responses due to the influence of biotic (e.g. weight, reproductive cycle) and abiotic (temperature, salinity) factors as well as the fluctuations attributed to pollutants (Amiard and Amiard-Triquet, 2008). According to the fundamentals of biochemistry, temperature is known to influence the activity of enzymes by changing the protein's structure, altering their catalytic efficiency or binding capacity (Burgeot et al., 2010). This influence is important to characterize for the methodology of biomarkers as many sub-individual markers are based on enzymatic activities.

In estuarine and coastal environments, salinity another important source of variability for numerous biomarkers, as previously shown in endobenthic invertebrates such as *Scrobicularia plana* and *H. diversicolor* (Fossi Tankoua et al., 2012; Kalman et al., 2010). The influence of the size/weight/age complex on biomarker responses to toxicants is at least partly influenced by the differences in the ability to uptake contaminants. This effect has been well documented by previous studies on contamination for biomonitoring species such as the bivalves used in Mussel Watch programs (NAS, 1980). Thus, the influence of these confounding factors creates uncertainty in the interpretation of results (Amiard-Triquet et al., 2015; Kalman et al., 2010). In this perspective, it is essential to define baseline data of biomarkers that may distinguish between natural variability (noise) and contaminant induced stress (signal), a concept well conceptualized in (Cairns, 1992).

In the framework of the National Program of Ecotoxicology (French Ministry of Ecology and Sustainable Development), a multi-disciplinary approach was conducted during 2-year time period (February 2003 to November 2004) to assess the health status of the Seine estuary (Amiard-Triquet et al., 2007) compared to the Authie estuary (Amiard-Triquet and Rainbow, 2009; Durou et al., 2007). The Authie estuary can be considered as a reference site as there is a very weak anthropogenic influence with metal concentrations, herbicides and pesticide reported in very low concentrations. This site is also located at the same latitude near the Seine estuary making it an ideal reference area for comparison (Durou and Mouneyrac, 2007). A multi-biomarker (CAT, GST, AChE, TBARs) approach was applied to *H. diversicolor* collected from both sites (Authie, Seine) with four sampling campaigns (winter, spring, summer, autumn) per year with the aim of identifying the influence of confounding factors (temperature, salinity, weight of worms) on biomarker responses.

One method for the determination of baseline values is the application of statistical analysis such as a multiple polynomial regression model (MPR) initially developed in Barrick et al. (2016) allowing for standardization of the influence of confounding factors (salinity, weight of worms, temperature) on energy reserves (lipids, glycogen) and defining a baseline representative of reference conditions for *H. diversicolor*.

The present study has two aims: i) the determination of baseline assessment criteria (BAC) and environmental assessment criteria (EAC) of four biomarkers (CAT, GST, AChE, TBARs) using *H. diversicolor* from the reference Authie estuary and ii) the comparison between published historical data on biomarker responses between worms collected in the multi-polluted Seine estuary and the reference Authie site (Durou et al., 2007) and these baseline values estimated with the MPR model.

Individuals of *H. diversicolor* were collected in the intertidal zone at low tide in the reference Authie site (50°22.217′ N and 1°36.484′ E) and the multi-polluted Seine estuary (49°27′17.52″ N and 0°11′19.57″ E). Samplings were carried out on four occasions each year (February 2003 to November 2004). Temperature and salinity of water remaining on the mudflat surface were recorded using a field thermometer (CG867) and salinometer (WTW LF 196). Biomarker values (CAT, GST, AChE, TBARs) measured in *H. diversicolor* were used from a previous study (Durou et al., 2007). Details of biomarkers methods have been fully described in Durou et al. (2007). Seasonal temperature and salinity measurements and weight of worms collected from the reference Authie site are shown in Table 1.

Table 1
Means of wet weight (± SD) of worms and temperature (°C) and salinity (psu) in situ measurements at each sampling periods during 2003 and 2004.

Sampling month	Wet weight ± SD (g)	Temperature	Salinity
February 2003	0.124 ± 0.017	−1	26.9
May 2003	0.21 ± 0.034	14	25.8
November 2003	0.095 ± 0.031	4.5	26.6
February 2004	0.094 ± 0.045	4.8	23.5
May 2004	0.113 ± 0.037	12.2	25.8
August 2004	0.129 ± 0.023	29	15.6
November 2004	0.106 ± 0.031	15.6	19.7

Based on the biomarkers (AChE, GST, TBARs and GST) of Authie data from 2003 and 2004, a series of observations were simulated according to mean values and standard deviation, assuming normal distribution. The numbers of these simulations were in accordance to the monthly sample size of the collected data (24 individuals per month). Confounding factors (temperature, salinity, weight) and biomarker values of ragworms collected in the reference site (Authie estuary) were used to identify the formula describing the relationship between each biomarker (CAT, GST, AChE, TBARs) and confounding factors.

Statistical analysis was conducted using the statistical software “R 3.2.1” to conduct pairwise analysis to identify which equation (linear, polynomial, etc.) best described the relationship between each biomarker and the confounding factors used in the study. This established a polynomial regression formula. To address collinearity in the model orthogonal polynomial regression was used to obtain new regression models with uncorrelated variables and achieved a final, stable polynomial regression equations for each respective biomarker:

$$\begin{aligned}\text{AChE} = {} & -14,964.36 - 588.74(\text{weight}) + 4029.99(\text{weight})^2 \\ & - 8190.42\,(\text{weight})^3 - 4.09(\text{temperature}) - 0.28(\text{temperature})^2 \\ & + 0.04(\text{temperature})^3 + 1917.60(\text{salinity}) - 81.11(\text{salinity})^2 \\ & + 1.14(\text{salinity})^3\end{aligned}$$

Adjusted $R^2 = 0.79$

$$\begin{aligned}\text{GST} = {} & -14,749.93 - 8.58(\text{weight}) + 75.83(\text{weight})^2 - 188.97(\text{weight})^3 \\ & + 3.46(\text{temperature}) - 0.88(\text{temperature})^2 + 0.05(\text{temperature})^3 \\ & + 1900.11(\text{salinity}) - 81.27(\text{salinity})^2 + 1.16(\text{salinity})^3\end{aligned}$$

Adjusted $R^2 = 0.72$

$$\begin{aligned}\text{CAT} = {} & -24,274.60 + 16.65(\text{weight}) - 59.59(\text{weight})^2 \\ & + 4.02(\text{temperature}) - 1.03(\text{temperature})^2 + 0.07(\text{temperature})^3 \\ & + 3062.86(\text{salinity}) - 128.20(\text{salinity})^2 + 1.78(\text{salinity})^3\end{aligned}$$

Adjusted $R^2 = 0.67$

$$\begin{aligned}\text{TBARs} = {} & 562.40 - 0.55(\text{weight}) + 4.34(\text{weight})^2 - 9.95(\text{weight})^3 \\ & - 0.16(\text{temperature}) + 0.03(\text{temperature})^2 \\ & - 0.001(\text{temperature})^3 - 73.27(\text{salinity}) + 3.17(\text{salinity})^2 \\ & - 0.05(\text{salinity})^3\end{aligned}$$

Adjusted $R^2 = 0.81$

Based on the previous regression equations, predicted values were calculated for each biomarkers and residuals obtained by subtracting from simulated values.

The residuals, which measure a departure from the model, were clustered into three groups using k-means clustering following a previously described method (Barrick et al., 2016). Cluster groups were