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ORIGINAL ARTICLE

Assessment of the influence of confounding factors (weight, salinity) on the response of biomarkers in the estuarine polychaete *Nereis diversicolor*

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Abstract

The influences of salinity and body size on biochemical (activities of glutathione-*S*-transferase, lactate dehydrogenase (LDH), acetylcholinesterase and digestive enzymes amylase and CMCase), physiological (feeding and egestion rates, energy reserves) and behavioural (burrowing speed) biomarkers were examined in the infaunal polychaete *Nereis diversicolor*. Only a few biomarkers were affected, including increased egestion rate and activities of CMCase and LDH at higher salinity, and higher egestion rate in larger worms. These findings reinforce the status of *N. diversicolor* as a robust sentinel species for estuaries which are environments that are particularly productive but also particularly at risk.

Keywords: *Biochemical biomarkers; physiological biomarkers; behavioural biomarkers*

Introduction

The methodology of biomarkers has emerged from an idea derived from the diagnostic methods used in human medicine, i.e. the detection of symptoms which reveal a disease long before it becomes dangerous for the patient. Depledge (1993) defined a biomarker as 'A biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations).' A few years later, this topic of interest has been well developed and books published to propose the use of biomarkers for environmental assessment (Lagadic et al. 2000, Garrigues et al. 2001). Initiated in 1998, the BEQUALM project (Biological Effects Quality Assurance in Monitoring Programmes) aims to establish a European infrastructure for biological effects quality assurance/quality control. Despite these efforts, to date the use of biomarkers in assessing marine environmental quality is limited and it is even ignored completely in the procedures recommended in the Water Framework Directive (European Community Official Journal L327 of 22.12.2000).

This situation is partly a result of technical difficulties encountered when using biomarkers, namely the problem of confounding factors, which was well conceptualized by Cairns (1992). When a biological parameter is highly fluctuating, the occurrence of a stress may be concealed by such natural fluctuations. On the other hand, when background values are relatively stable, any change resulting from contamination factors is easily revealed. The question of confounding factors is well mastered in biomonitoring programmes based on the determination of contaminants in the tissues of bioaccumulators such as the bivalves used in 'Mussel Watch'-

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type programmes (NAS 1980). Even if this question is not so well documented in the case of biomarkers, literature data indicate that the same natural factors are at work, particularly season, temperature, size (weight/length), reproductive status, food availability and salinity in estuarine and coastal areas (Hagger et al. 2006).

For chemical concentrations, the huge quantity of data provided by 'Mussel Watch'-type programmes provides a knowledge of background values in areas where anthropogenic pressure is low, allowing us then to judge if concentrations determined in areas under investigation are abnormal or not. For biomarkers, the quantity of available data is much more restricted, and to date it is impossible to compare data obtained in a potentially impacted area to reliable background values for biomarkers. Chemical analysis of contaminant residues in both abiotic and biotic matrices could help in biomarker interpretation when comparing multiple sampling areas.

In aquatic toxicology, some investigations have shown that the use of chemical analysis of residues and biological (e.g. biomarker) endpoints results in an attractive approach to build up a weight-of-evidence matrix. Such comprehensive approaches have been applied to the assessment of the relative toxicity of estuarine sediments (Caeiro et al. 2005, Costa et al. 2005, Neuparth et al. 2005, Cunha et al. 2007). A high consistency was shown between chemical analysis and the responses of a suite of biomarkers including biochemical responses, survival, growth and reproduction (for a review see Amiard-Triquet & Dauvin 2009). However, anthropogenically contaminated estuaries are inevitably exposed to a mixture of classical contaminants (metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls), but also to many 'emerging' contaminants (polybrominated diphenyl ether, pharmaceuticals and alkylphenols) and as yet unknown organic contaminants for which limited or no analytical tools are available. It is arguably impossible to measure all potential contaminants in an organism in order to interpret biomarker responses (Amiard-Triquet & Rainbow 2009).

Classically, biological monitoring may be used to compare temporal and spatial variations of the biological effects of contaminants. In the first case, the objective is to assess any degradation or improvement of the health status of that site over time – for instance following remediation work or the cessation of pollutant inputs. In the second case, the objective is to assess the health status of a given site against information for other sites in order to gain an incontrovertible insight into what are the 'normal' or subnormal values of the biological response concerned. This is the approach ruled in the EC Water Framework Directive which lays out the requirement to determine the health status of water masses by comparison with 'undisturbed' conditions. In both cases, it is necessary to design a sampling procedure, limiting to the best degree any intersite differences in natural factors able to act as confounding factors, among which size and salinity may be important as explained below.

In polluted areas, bioenergetics may be affected either directly or indirectly through effects on prey species resulting in decreased food availability. Direct effects include decreased feeding rates (Moreira et al. 2006, Shipp & Grant 2006, Kalman et al. 2009) and impairment of enzymes involved in food assimilation (Dedourge et al. 2008, Kalman et al. 2009). Allometric differences might be found when comparing polluted and reference sites because of the indirect effects of pollutants on predator species (Chapman 2004).

In developed countries, pristine sites do not any longer exist and even relatively clean sites are scarce, being generally restricted to small estuaries, more likely to be spared from urbanization and industrialization. As a consequence, sites that may be used as reference sites have a number of natural characteristics that may differ from those in bigger estuaries, particularly salinity during the tidal cycle (Amiard-Triquet & Rainbow 2009). In addition, coastal areas are often less impacted by human activities than internal estuaries but salinity differences are important.

Because sediments constitute the main reservoir for most of the chemicals introduced into aquatic environments by human activities, the infaunal polychaete *Nereis diversicolor* is currently used as a biological monitor species in European countries (Pérez et al. 2004, Moreira et al. 2006, Shipp & Grant 2006, Durou et al. 2007a, b, Kalman et al. 2009, Solé et al. 2009) as well as the congeneric species *Nereis virens* in other parts of the world. Moreover, the use of a suite of biomarkers covering different levels of biological organization is a useful strategy when employing pollutant-linked biomarkers (Hagger et al. 2006). Thus in the present study, we used *N. diversicolor* to investigate the influence of salinity and size on different classes of biomarkers: biochemical (activities of glutathione-*S*-transferase (GST), lactate dehydrogenase (LDH), acetylcholinesterase (AChE) and digestive enzymes), physiological (feeding and egestion rates, energy reserves as glycogen, lipids and proteins) and behavioural (burrowing speed).

Materials and methods

Collection

The influence of worm size on biomarkers was investigated in individuals collected from an estuarine mudflat in the mesohaline area of the Loire estuary (2° 07′ 59.76′ W, 47° 16′ 29.66′ N), a moderately polluted estuary on the French Atlantic coast in July 2008. To study salinity effects, worms were collected in the Goyen estuary on

the south coast of Brittany, France (precise locations: 4° 29′16′ W, 48°02′17′ N and 4° 31′ 24′ W, 48° 02′18′ N) in April 2008. This estuary receives minimal anthropogenic pressure, so avoiding any interactions of contaminants with salinity effects on the biochemistry, bioavailability and potential effects of contaminants (Turner & Millward 2002, Tremblay et al. 2005, Luoma & Rainbow 2008). Information on chemical contamination in the sampling sites was available in the framework of the French 'Mussel Watch' Programme (RNO 2006). Salinity was measured *in situ* using the water remaining at the surface of the mudflat at low tide.

At each site, individuals of *N. diversicolor* were hand-picked gently at low tide from intertidal mudflats. After collection, individuals were transported to the laboratory with wet algae or sediment from the site of origin without seawater in cold containers. Ten individuals from each sampling site were immediately rinsed and frozen at –80°C until biochemical measurements. Specimens used for behavioural assays were maintained in aerated containers filled with the natural filtered seawater adjusted to the salinity of the site of origin for less than 1 week before testing.

Behavioural tests

All the behavioural tests were set up in a dark, temperature-controlled room (16°C) and conducted within 1 week of collection.

Burrowing experiments

These were carried out in individual containers as described by Bonnard et al. (2009). Twenty worms were submitted to each test. Animals were tested in the presence of the sediment of their site of origin. In addition, for specimens originating from two areas differing in salinity in the Goyen estuary, cross-tests were performed using the sediment of the other area. For each condition, the curves representing the percentages of organisms remaining unburrowed at each time of observation were linearized using natural logarithms.

Feeding rate

The feeding rate of *N. diversicolor* was quantified according to the methodology of Moreira et al. (2006) and previously applied to specimens from contaminated and reference sites in France (Kalman et al. 2009). Twenty worms from each site were placed into plastic beakers containing about 30 ml seawater adjusted to the salinity of the site of origin and left for 24 h without disturbance. The water was changed and the animals were allowed to feed (food ration = 100 larvae of *Artemia salina*) for 1 h. At the end of the test, the remaining larvae were counted and results were expressed as the number of larvae fed per worm per hour.

Egestion rate

Egestion rate experiments were performed according to the methodology of Kalman et al. (2009). Twenty worms from each site were recovered from the sediment and rinsed with seawater several times to remove sediment particles from the body surface. They were then placed individually in 100 ml of natural filtered seawater adjusted to the salinity of the site of origin and left for 2 h without disturbance. The faeces were collected by filtering water through preweighed 3 μm cellulose nitrate membrane filters (Whatman®; GE Healthcare, Amersham, UK), and dried for 24 h at 60°C. Egestion rate was expressed as g faeces produced per 2 h.

Determination of enzyme activities

Ten worms from each site were analysed for each biochemical marker. In all cases, homogenization was carried out at +4°C. Results were expressed in terms of total protein concentration, determined as described below.

AChE/ChE activity

AChE/ChE activity was determined using the method of Ellman et al. (1961) as adapted for a microplate reader by Galgani and Bocquené (1991) and modified for analysis in *N. diversicolor* (Scaps & Borot 2000). Briefly, organisms were homogenized in three volumes of ice-cold Tris buffer (Tris 50 mM, NaCl 150 mM, pH 7.4, 0.1 mM anti-protease cocktail) using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenates were centrifuged (30 min, 4°C, 15 000 *g*). Measurements were performed on the supernatant using UV-visible UVIKON xs and ELx800uv spectrophotometers (BioTek Instruments, Winooski, VT, USA), respectively.

Amylase and carboxymethylcellulase activities

Amylase (E.C.3.2.1.1.) and carboxymethylcellulase (CMCase – 3.2.1.4.) activities were assayed according to the method developed by Bernfeld (1955) and adapted for *N. diversicolor* by Kalman et al. (2009). Organisms were homogenized in deionized water on ice at a ratio of 100 mg ml^{-1} to 50 mg ml^{-1} (w/v) for CMCase and amylase, respectively, using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 4°C for 30 min at 15 000 *g* and the supernatant was used for subsequent enzyme analyses. The substrate solutions (starch 1% for amylase; carboxymethylcellulose 1% for CMCase) were prepared in 20 mM of phosphate buffer (containing 6 mM NaCl) pH 7 and pH 6.5, respectively. The reaction mixtures, composed of the enzyme extract (125 μl), the appropriate substrate (125 μl) and a few drops of toluene, were incubated at 35°C for 30 min (amylase) or 2 h (CMCase). The reaction was stopped by adding 250 μl of dinitrosalicylic acid (DNS) and heating the reaction mixtures for 5 min in a boiling water bath;

mixtures were then cooled in cold running water for at least 10 min. Two millilitres of distilled water was added to the samples and the optical density was determined at 540 nm. The blanks were prepared by adding the enzyme extract after the DNS reagent. Maltose (concentration range 0–3 µmol) was used for the calibration curve.

GST activity

GST activity was determined with a microplate reader as described by Frasco and Guilhermino (2002). The activity was analysed in triplicate at 25°C using 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1 mM GSH as substrates and measured at 340 nm each 20 s for 5 min. The enzymatic activity was expressed in µM min^{-1} mg^{-1} protein.

LDH activity

Measurements of LDH activity were determined with a microplate reader as described by Diamantino et al. (2001).

Determination of energy reserves

Ten worms from each site were homogenized in liquid nitrogen. The powder obtained was then homogenized with 1.5 ml citrate buffer at pH 5.0 for analysis for glycogen and lipids. Total lipids were determined by a sulpho-phosphovanillin reaction according to the method of Frings et al. (1972). Glycogen concentrations were estimated in two aliquots of the homogenate, one of which was submitted to enzymatic digestion by amyloglucosidase according to Carr and Neff (1984). Olive oil and glycogen from oyster (Type III; Sigma, St Louis, MO, USA) were used as standards for each method, respectively.

Prior to protein analysis, worms were ground in Tris buffer (0.1 M, pH 7.5). The homogenate obtained was centrifuged at 9000 *g* for 20 min at 4°C. The quantity of proteins in the supernatant was determined according to the method of Bradford (1976) using bovine serum albumin as the protein standard.

Statistical analysis

The kinetic curves for burrowing activity were log-transformed in order to linearize these data, and then compared by analysis of covariance (ANCOVA) between regression coefficients of the least-square best-fit regression lines. Otherwise, results are presented as mean ± SD. To define significant differences, normally distributed data were analysed by the *t*-test (between two groups), one-way ANOVA (multiple comparisons of means) followed by Tukey's HSD post hoc method. In other cases, the non-parametric Kruskal–Wallis and Mann–Whitney comparison tests were used. The level of significance was established at $p < 0.05$. Statistical analyses were performed using a standard statistical package (STATISTICA, version 6).

Results

Behavioural tests

Burrowing speed

No difference in burrowing behaviour was shown between small (0.04 ± 0.02 g wet weight (ww)) and large (0.20 ± 0.06 g ww) specimens (Figure 1A).

When worms from the Goyen estuary were allowed to burrow in their sediment of origin, no significant inter-site differences in the burrowing speed were observed depending on salinity, 13 or 30 (Figure 1B). In cross-tests, ragworms from sediment with low salinity (Goyen 13) exposed to sediment with higher salinity (Goyen 30) did not show significant changes of their burrowing activity. Conversely, worms from Goyen 30 exposed to sediment from Goyen 13 exhibited higher activity than in the presence of their sediment of origin, but the difference was not significant (Figure 1B).

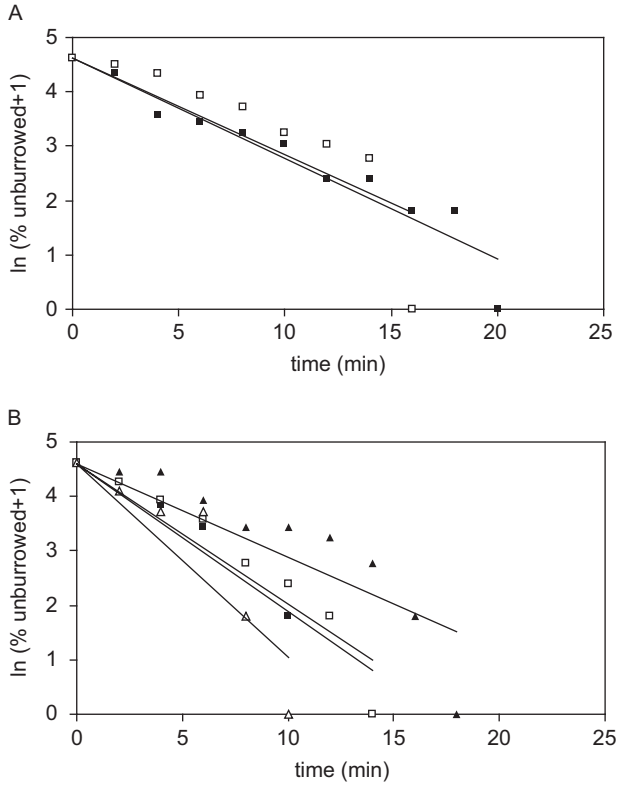

Figure 1. Influence of weight and salinity on burrowing behaviour. (A) Ragworms from the Loire estuary selected according to their weight (■ small; □ large). (B) Ragworms from two sites in the Goyen estuary differing by salinity allowed to burrow in their sediment of origin (filled symbols, ■ Goyen 13; ▲ Goyen 30) or in the sediment from the other site (open symbols, □ Goyen 13; △ Goyen 30).

Egestion rate

The quantity of egested sediments is depicted in Figure 2. In the larger specimens (mean wet weight 0.18 g), the quantity of egested sediment was significantly higher than in smaller specimens (mean wet weight 0.08). However, the influence of the weight class of ragworms is limited as the ratio between mean animal weights reached 2.25 while the ratio between egested quantities was only 1.16. The influence of salinity was much more marked, the egested quantity being 1.76 times higher in specimens originating from the marine area (salinity 30) compared with the brackish area (salinity 13).

Post-feeding rates

For both weight classes, the individual variations of the post-feeding rate were very important. However, considering the performance of the 20 specimens tested for each weight class, the patterns were very similar (Figure 3).

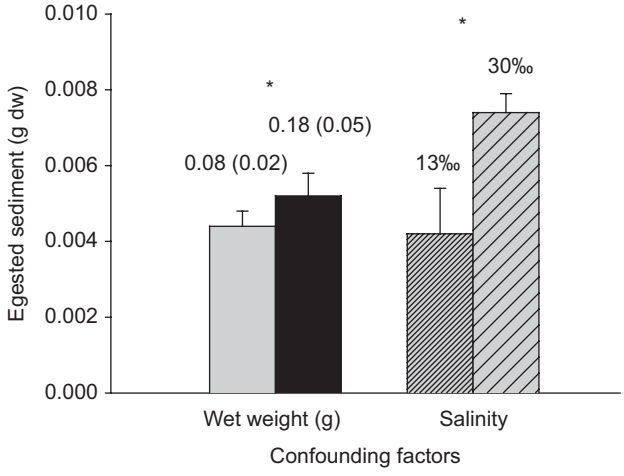

Figure 2. Influence of weight (mean and SD of wet weights of 20 individuals are presented (over each bar)) and salinity (brackish site 13; marine site 30) on egestion rate. *Difference between paired bars significant at the 95% level.

Determination of enzyme activities

The influence of weight was studied using samples showing a large range of weights (ratio between mean minimum and maximum reaching 3.75) (Table 1). Despite this cautious approach, the activities of enzymes involved in energy metabolism (amylase, CMCase and LDH) did not differ significantly, as also observed for GST. Among the five enzymatic activities examined in worms originating from a marine and a more brackish area in the Goyen estuary, only LDH showed a significant influence of salinity, the activity being about twofold higher in specimens originating from the marine area (Table 1).

Energy reserves

The influence of weight on energy resources was carefully studied in a previous work (Durou et al. 2007a), whereas the influence of salinity was not so well documented (Durou et al. 2007b). In complementarity with this previous work, glycogen, lipid and protein levels were determined in ragworms originating from the marine and brackish areas in the Goyen estuary (Table 1). No

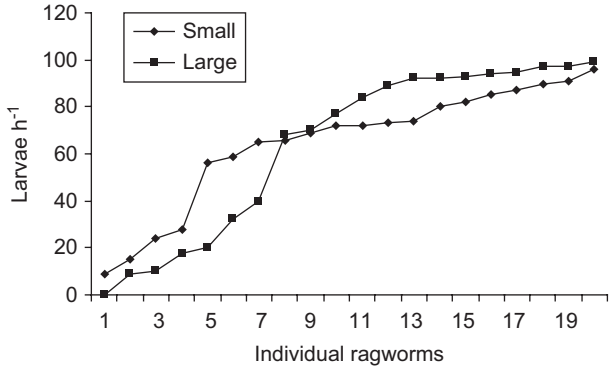

Figure 3. Influence of weight on post-feeding rates expressed as the number of *Artemia* larvae captured within 1 h. Small: 0.04 g wet weight on average (SD 0.03); large: 0.15 (0.04).

Table 1. Influence of weight and salinity on bioenergetics and biochemical markers, mean (SD). Means with the same superscript symbol differed significantly at the 95% level.

	Size		Salinity	
	0.04 (0.03) g ww	0.15 (0.04) g ww	13	30
Glycogen (mg g^{-1} ww)	n.a.	n.a.	1.9 (0.5)	1.5 (0.5)
Lipids (mg g^{-1} ww)	n.a.	n.a.	4.5 (1.9)	3.0 (0.9)
Proteins (mg g^{-1} ww)	n.a.	n.a.	134 (27)*	48 (10)*
LDH (μM min^{-1} mg $prot^{-1}$)	0.36 (0.11)	0.30 (0.08)	0.05 (0.01)†	0.12 (0.02)†
GST (μM min^{-1} mg $prot^{-1}$)	0.009 (0.004)	0.008 (0.003)	0.007 (0.003)	0.008 (0.003)
AchE (μM min^{-1} mg $prot^{-1}$)	n.a.	n.a.	0.013 (0.006)	0.015 (0.006)
CMCase (μM maltose min^{-1} mg $prot^{-1}$)	1.9 (1.0)	2.5 (0.7)	1.7 (0.8)‡	3.0 (1.6)‡
Amylase (μM maltose min^{-1} mg $prot^{-1}$)	16 (9)	13 (8)	114 (39)	150 (62)

n.a., not analysed; ww, wet weight; LDH, lactate dehydrogenase; GST, glutathione-*S*-transferase; AChE, acetylcholinesterase; CMCase, carboxymethylcellulase.

differences occurred for glycogen and lipid concentrations in the worms whereas protein concentrations were significantly higher in specimens from the brackish area.

Discussion

Based on our state of knowledge (Amiard-Triquet & Rainbow 2009) and in line with the approach described in the EC Water Framework Directive to assess the status of water masses, in order to study the ecotoxicological status of an estuary, it is essential to compare results against those for a reference site. The choice of the latter must be decided considering the highest possible similarities for abiotic factors, particularly temperature and salinity. Either for intersite comparisons or time-course monitoring in a contaminated site, the biological characteristics of the relevant sentinel species, particularly their size, must be carefully taken into account.

In the case of temperature, this objective is generally achieved easily by selecting both reference and site under investigation within a sufficiently restricted geographical area. This is the reason why the potential influence of temperature has not been examined in the present study. Many literature reports have previously documented this question. An increase of activity levels of enzymatic biomarkers with increasing temperature is highly probable, independently of any chemical stress in non-contaminated environments. In invertebrates such as mussels and shrimps, increased AChE activities in parallel with increasing temperature have indeed been observed (Pfeifer et al. 2005, Menezes et al. 2006). No seasonal change in the GST activity was observed in another polychaete worm *Laeonereis acuta*, collected from an unpolluted site (Geracitano et al. 2004). In the case of *N. diversicolor*, in a reference site on the continental coast of the English Channel (Authie estuary), no clear seasonal trends were observed over a 2-year sampling period for AChE, GST and catalase activities determined in specimens belonging to different size classes and the same was true for concentrations of thiobarbituric acid reactive substances (TBARS) (Durou et al. 2007a). Because other natural factors (e.g. food availability, reproductive status) may influence biomarker responses, it is sometimes difficult to draw definite conclusions from field studies (Pfeifer et al. 2005). The influence of temperature and salinity on AChE activity levels in *N. diversicolor* was tested experimentally (Scaps & Borot 2000). At a salinity of 15, they did not find any effect of temperature on AChE activity, whereas at 30, AChE activity tends to decrease with increasing temperature. The authors concluded that salinity changes produced more pronounced effects than temperature changes. Concerning energy reserves, no seasonal pattern was identified in *N. diversicolor* from the Authie estuary (Durou et al. 2007a). Experimental heat shocks (ragworms collected at 5°C in the field and maintained for 4 weeks at 22°C in the laboratory; or collected at 20°C and maintained at 7°C) did not induce any changes in lipid concentrations whereas for glycogen, increased temperature induced a depletion which only became significant after 2 weeks of exposure (Durou 2006). Over a large range of temperatures (5–30°C), a strong influence of this factor was shown on postexposure feeding (Moreira et al. 2005).

In the present study – despite a large difference between weight classes selected from a population of a brackish site in the Loire estuary – no influence of weight was shown on enzymatic biomarkers (Table 1) nor on behavioural responses (burrowing and post-feeding rate). The egestion rate was significantly higher in larger individuals but the influence of weight was limited, the ratio between egested quantities being only 1.16.

In the Authie estuary already mentioned, the influence of size on physiological (energy reserves) and biochemical markers (AChE, GST and catalase activities, TBARS concentrations) was examined in ragworms collected on eight sampling dates from February 2003 to November 2004 (Durou et al. 2007a). No consistent trend was revealed in the case of protein concentrations, whereas lipid concentrations were significantly higher in large specimens on five sampling dates and glycogen concentrations on six sampling dates (among eight). In agreement with the present study, size generally had no effect on GST activity. Except on two occasions, catalase activity did not differ with the size of ragworms and no clear trends were shown for catalase activity and TBARS concentrations. On the other hand, on half of the sampling dates, AChE activity was higher in the small specimens than in the larger ones.

The ragworm *N. diversicolor* is a very euryhaline species, for which the onset of water content regulation, including the stability of the body volume, is 25–35% seawater and the critical low salinity is 1–2% seawater (Ogleby 1978). Thus, in the current case, ragworms were living far from their lower salinity tolerance range. In agreement with these assumptions, *N. diversicolor* living at different salinities in the Seine and Authie estuary had identical water contents (Poirier et al., personal communication). However, salinity had a clear effect on several biomarkers examined in the present study, namely the egestion rate, the concentration of proteins, the LDH activity, and to a lesser degree ($p = 0.044$) the activity of CMCase. Concerning the egestion rate, in the euryhaline polychaete *Capitella* sp. I, a significant reduction of fecal pellet production was observed below salinity of 20 (Pechenik et al. 2000). The methodology of postexposure feeding was not available at the time when ragworms were collected from the Goyen, but Moreira et al. (2005) found a significant but limited influence of salinity on

feeding. Contrary to the findings of the present study, in another reference estuary (Authie estuary), Durou et al. (2007b) did not show any significant differences in protein concentrations along a salinity gradient (19–33). The main difference between these two studies is the period at which ragworms were collected (April in the Goyen; September in the Authie). Interpretation of energy reserves as biomarkers is not a simple task because, in addition to nutritional factors and potential effects of pollutants, physiological changes are strongly related to the reproductive status (Durou & Mouneyrac 2007). Osmoregulation represents a key physiological response in estuarine organisms which could be responsible for the higher concentrations of proteins in ragworms collected from the brackish area in the Goyen estuary.

In the cases of some widely used biomarkers such as AChE and GST, salinity differences had no influence on activities in ragworms from brackish and marine areas in the Goyen estuary. Laboratory experiments at salinities (15 and 30) nearing those encountered in our field study (13 and 30) showed only a transient increase of AChE activity at the beginning of maintenance that was interpreted as a short-term stress effect (Scaps & Borot 2000). In the closely related species *Laeonereis acuta*, GST activity was determined in sites with different salinities but no clear effect was demonstrated, perhaps because salinity was low and not highly variable (0–6) (Geracitano et al. 2004). No salinity effect was shown in the brown shrimp *Crangon crangon* for both AChE and GST activities (Menezes et al. 2006). In mussels, a negative relationship between salinity and AChE activity has been reported, but the variability was mainly observed for salinities below 10 (Pfeifer et al. 2005). In *Eurytemora affinis*, a copepod living in the oligo-mesohaline zones in estuaries, a maximum expression of AChE and GST was shown at salinity 5–15 (Cailleaud et al. 2007). The fact that no consistent trends may be derived from the literature dealing with AChE and salinity in invertebrates is most probably due to the preferred salinity characteristic of each species. Depending on this preference, different species are not able to cope identically when exposed to the same salinity. The mechanisms probably underlying the salinity-induced alterations in AChE activity (metal ion binding effect and secondary ionic strength effect) have been briefly reviewed (Pfeifer et al. 2005).

In conclusion, by using a battery of biomarkers, it is possible to take into account many chemicals which affect more or less specifically certain biomarkers; the use of such a battery is a promising way to link different levels of biological organization from molecular/biochemical responses (with great potential as a sensitive and early alert system) to biomarkers with added ecological value (e.g. revealing threats to reproductive success with expected effects on the fate of the population). In *N. diversicolor*, many biomarkers which have been shown as sensitive biological responses to exposure to chemical contaminants at environmental levels are not affected or are only slightly affected by the so-called confounding factors (Table 2). This is the case for individual weight and salinity studied in the present work and the literature also indicates a limited effect of temperature. With some exceptions, it is generally possible to sort animals of equivalent size from different sites, even if from a functional point of view, it may be better to collect a sample representative of the whole population living at a given site (Durou et al. 2007a). When collection of identical

Table 2. Responsiveness of different classes of biomarkers, determined in *Nereis diversicolor*, to estuarine contamination and to confounding factors.

	Contamination	Temperature	Weight class	Salinity
Water content	ND[1]	No seasonal pattern[1]	NS[1]	NS[2]
Burrowing	Decreased speed[3,4]	ND	**NS[5]**	**NS[5]**
Egestion rate	Decreased[3]	ND	**> in larger worms[5]**	**> at higher S‰[5]**
Post-feeding rate	Decreased[5,6,7]	Strong effect[6]	**NS[5]**	Moderate effect[6]
Glycogen	Decreased[8]	No seasonal pattern[8]	Generally higher inlarge specimens[8]	NS[9]
Lipids	Decreased[8]	No seasonal pattern[8]	Generally higher inlarge specimens[8]	NS[9]
Proteins	Decreased[8]	No seasonal pattern[8]	No consistent trend[8]	NS[9] or **> at low S‰[5]**
LDH	Increased[7]	ND	**NS[5]**	**> at higher S‰[5]**
GST	Increased[7,8,10,11]	No seasonal pattern[8,12] Limited effect[13]	**NS[5]**	**NS[5]**
AChE	Decreased[3,8,10,11]	No seasonal pattern[8]	≥ in small specimens[8]	**NS[5]** or transient effect[13]
CMCase	Decreased[3]	ND	**NS[5]**	**> at higher S‰[5]**
Amylase	Decreased[3]	ND	**NS[5]**	**NS[5]**

ND, not documented; NS, no significant difference.

[1]Durou et al. 2008; [2]Poirier et al. pers. comm; [3]Kalman et al. 2009; [4]Mouneyrac et al. 2009; [5]**Present study**; [6]Moreira et al. 2005; [7]Moreira et al. 2006; [8]Durou et al. 2007a; [9]Durou et al. 2007b; [10]Pérez et al. 2004; [11]Solé et al. 2009; [12]No seasonal pattern in *Laeonereis acuta* after Geracitano et al. 2004; [13]Scaps & Borot 2000.

specimens in identical conditions is impossible, the use of correction factors is recommended. Those which were designed initially to adjust metal concentrations (Poirier et al. 2006) may be adapted for biomarkers expressed as concentrations (energy reserves, TBARS) or activities (enzymes). More generally, by determining the variation of a given biomarker along a gradient (salinity, temperature), it is possible to adjust field data as proposed by Moreira et al. (2005) for postexposure feeding rates.

The interspecific variability of biological responses that may be used as biomarkers of exposure to, or effects of, chemicals represents a serious challenge for environmental risk assessment as different species are variably tolerant to the presence of toxic compounds in their medium. This variability can be linked to differences in exposure (for instance through feeding habits), in bioaccumulation pattern – some species being well recognized as strong accumulators, or in intrinsic biological characteristics, such as physiological preferences for temperature and salinity. In addition to its representativeness of the intertidal estuarine mudflats and to its key role in the structure and functioning of estuarine ecosystems, this relatively low influence of confounding factors reinforces the status of *N. diversicolor* as a robust sentinel species for estuaries, which are environments that are particularly productive but also particularly at risk.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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