Validation of biomarkers for impact evaluation of aqueous industrial waste in mesocosms

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Abstract The use of exposure biomarkers in measuring the impact of aqueous waste holds promise because such tools have short response times, are flexible in use and may give an indication about the type of pollution. However, their ecological significance has not yet been demonstrated. It is necessary to validate these responses under controlled conditions before using such biomarkers for biomonitoring. The TotalFinaElf company has developed a pilot scheme incorporating such controlled conditions. This pilot is a dynamic open mesocosm (16 channels 40 m in length supplied with river water). The research programme currently carried out in the “Pilot Rivers” aims at validating biochemical parameters (components of phases I and II (de)toxication metabolism and propionylcholinesterase activity), measured in a fresh water bivalve Corbicula fluminea as a biomarker of water quality. The comparison between biomarker responses and community ones (reference) gives information about the precocity and sensitivity of these biomarker responses. Pure substances (trichloroethylene (TCE), cadmium (CD) and anthracenic oil (AO)) have been injected during one month. Biomarker responses are as sensitive as the most sensitive community response in the presence of CD and AO. With TCE, community responses are more sensitive. Precocity of biomarker response is observed only in the presence of CD.

Keywords Artificial stream; biomarkers; biomonitoring; fresh water; impact; mesocosm

Introduction In many cases, the impact of waste in the aqueous environment is measured only by means of a physico-chemical approach whose increasing sophistication is beginning to lead to prohibitive costs. Moreover, the elements sought are not necessarily those which induce an impact on the biological component of the ecosystem. Alongside these physico-chemical systems of pollution surveillance, a biological monitoring approach making use of biological indicators of pollution is beginning to emerge. This is consistent with French and European regulations which increasingly include this notion in their texts.

In his bibliographical study, Melancon (1995) notes that while all the biochemical techniques have the merit of existing, they lack validation, particularly in situ. Generally speaking, it seems that these approaches are relatively well advanced in sea water (Narbonne et al., 1991), but less in fresh water. There are, however, works showing the feasibility of such an approach on fresh-water organisms, fish and molluscs (Livingstone, 1993; Adams et al., 1989; Van der Oost et al., 1997; Cossu et al., 1997). One of the promising organisms for this kind of application is the fresh-water mollusc, Corbicula fluminea (Narbonne et al., 1999; Milam and Farris, 1998). It should be noted that biocoenotic indicators were among the first to be considered and that the current trend is towards the development of biochemical/molecular indicators or biomarkers. These early indicators of pollution have the following advantages: short response time, more appropriate response in function of the pollution and scope for extending their application to different ecosystems.
(air, soil and water). However, it very soon became apparent that the response of the bioindicators, of whatever sort, needed to be correlated to the degree of pollution. It is also necessary to establish the ecological significance of these biomarkers. Few studies provide a comparison between the biocoenotic and cellular approaches. Taking into account all the works conducted in this sphere, it now appears necessary to validate the biomarker approach in the measurement of impact. For the purposes of this validation, it is not enough to adopt an in situ approach which, although representing a real situation, is not controlled. We therefore proposed to set up an experimental mesocosm in which control would be exercised over contamination and the exposure times of living organisms. A description of this pilot study, consisting of 16 streams, is given below. The experiment is based on works cited in the literature (Belanger, 1997; Kosinski, 1989; Rodgers et al., 1996; Pusey et al., 1994).

Material and methods

Description of the mesocosm

The Pilot Rivers are made of 16 open mesocosms in parallel supplied with freshwater, located in Lacq (Pyrénées Atlantiques region, France). The mesocosms are located outdoor, exposed to natural light and to the ambient weather conditions. The freshwater comes from a dam on the “Gave de Pau” river (France), and is gravity-fed to the 16 mesocosms (200 m³/h). This water, estimated to be of good quality according to French Water Agency criteria, is neither filtered nor treated. This supply induces a colonization (via drift) of the 16 downstream channels. Each channel or mesocosm is 40 m long, 0.5 m wide and 0.5 m deep. For the experimentation the specific volume is 4 m³. The residence time in each mesocosm is 10 minutes. The water velocity is 10 cm/s. A water-borne organism nursery has been installed upstream to the 16 mesocosms: it forms a reserve of living organisms which favours the colonization of the 16 downstream mesocosms. The water is perfectly distributed to the 16 downstream channels by a supply structure and weirs upstream to each channel installed at the same height to provide an identical flow rate in every channel. Cabinets located upstream to the 16 channels house the pumps necessary to feed in the substances whose effects are to be studied. Finally, the wastewater is released through three possible routes: directly into the river; to a macrophyte lagoon (reed bed); or connection to the TotalFinaElf Lacq plant’s water treatment facilities (Bassères and Tramier, 2001).

Channel design

Ten centimetres of coarse sediments (20–40 mm) are layered in the channels to form the sediment. These sediments have previously been characterized by leaching test and metal analysis: pollution-free sediments were used. Before exposure, the channels were naturally colonized for two months. This minimum time necessary to stabilize the communities of aquatic organisms (benthic invertebrates and micro-algae) was determined by a previous validation study over an 8-month cycle (Bassères et al., 2001). The colonization was statistically the same in the 16 channels.

Experimental design

Trichloroethylene (TCE) was provided by SDS (Peypin France); cadmium chloride (CdCl₂, 2.5 H₂O) was purchased from Interchim (Montluçon, France) and anthracenic oil (AO) is a complex mixture of polycyclic aromatic hydrocarbons (PAH): a coal tar fraction (the most abundant being naphthalene, anthracene, phenanthrene, fluorene, fluoranthene and pyrene) provided by Chemco France (Poissy, France). Other chemicals were of the best technical grade available.

For hydrophilic substances, a stock solution was prepared using the substances diluted,
with tap water. Each substance is added to the mesocosm by diaphragm metering pumps (Prominent Gamma) from the stock solutions. The injection takes place upstream to each mesocosm directly to the stream in case of hydrophilic substances and with a mixing valve for hydrophobic substances, that continuously supplies a stable mechanical emulsion (24 h/24 h).

Two experiments were realised: the first in March 2001, the second in June 2001. The exposure lasts 30 days. At each experiment, two channels were exposed with each substance and two reference channels were considered. Previous eco-toxicological laboratory tests allow the choice of concentrations: as NOEC, 10 × NOEC and 100 × NOEC. In the first experiment, the NOEC and 10 × NOEC of substances were tested, and in the second experiment, the 10 × NOEC and 100 × NOEC were tested (cf. Table 2).

Sampling and responses

Biocoenotic responses. The benthic invertebrate communities and oligochaete communities were monitored using submerged traps (substrate sampler) placed in the sediment and which can be sampled without disturbing the substrate. These traps have nylon nets with 160 μm mesh which traps organisms larger than 160 μm. Once the samples have been taken, the coarse sediments are cleaned on a 160 μm sieve: the particles caught in the sieve are kept in formol (4%) for later identification. The samples kept in formol (4%) are then sent for identification to external specialists (Benthic invertebrates: Aquaservice; Oligochaete: BURGEAP). These fauna lists have been treated using global indicators: abundance, biodiversity (richness), and the Shannon–Weiner diversity index. The diatom communities were monitored using submerged glass plates, which served as a hard substrate adapted to their development. The diatoms are sampled by scraping and then kept in with formol (4%). They were identified by an external expert (CEMAGREF). These floristic lists have been treated using global indicators: biodiversity (taxa richness), the Shannon–Weiner diversity index and polluosensitivity index (IPS) (Coste and Prygiel, 1993), which is based on the abundance of each species, value of polluosensitivity and number of species.

Exposure biochemical indicators: biomarkers in Corbicula fluminea. Exposure indicators, in many cases biochemical indicators corresponding to an enzymatic induction (reparation) in the presence of a pollutant, were measured in the fresh-water bivalve Corbicula fluminea. The animals (15 to 20 mm), were collected from the banks of the non-polluted freshwater Cazaux-Sanguinet lake (Aquitaine, France). No sexual differences were taken into account as C. fluminea are hermaphroditic. After adequate transport and stabilization conditions (3 days at 19°C with air bubbling), the animals were then exposed in small cages (25 animals per cage, 5 cages per channel) in each stream during one month. The organisms were retrieved following the sampling schedule described below. The subcellular fraction was prepared in accordance with a methodology developed by the Laboratoire de Physico-Toxicochimie des Systèmes Naturels (LPTC) (Narbonne et al., 1991, 1999). All the steps of homogenisation were achieved at 4°C. For each experiment condition, 5 lots of five Corbicula were prepared. After the shell and the crystalline style were removed, Corbicula were rinsed in 100 mM phosphate buffer, pH 7.4, dried on absorbent paper sheets. Supernatant consisting of the submitochondrial fraction (S9) were prepared, homogenised in the same phosphate buffer (1:4 weight:volume ratio) using an Ultra-Turrax® T25 Basic (IKA® Laborteknik) and centrifuged at 9,000 g for 30 min in a BECKMAN I2-21M centrifuge. Then S9 fraction can be stored at –80°C before analysis.

Enzymatic activities were measured using a microplate analyser (Quadra 96-M320, TOMTEC) and a microplate Spectra Rainbow (TECAN) spectrophotometer. Assays were run in triplicate for each lot. Biochemical parameters studied are presented in Table 1.
Sampling kinetics. All the biological responses were monitored: before exposure (i.e.: day zero: T0), seven days after exposure (T7), 15 days after exposure (T15) and 30 days after exposure (T30).

Physicochemical responses. The physicochemical parameters: pH (Fisher and Rosemount sensors and probes, pH probe model 399, with glass electrode), temperature (integrated in the pH and O₂ probes), dissolved oxygen (Fisher and Rosemount sensors and probes, current-sensing O₂ probe 499 A DO, gold cathode and silver anode) and conductivity (Fisher and Rosemount sensors and probes, immersion/insertion probe) were permanently measured by submerged sensors located at the end of the mesocosm. Water quality analyses were performed: COD, BOD₅, NH₄, NO₃, PO₄, PT, by the Lacq TotalFinaElf plant’s laboratory. The water samples were taken, kept under refrigerated conditions for a maximum of 4 h, before being analyzed. Substances analyses: TCE analyses were performed by gas chromatography coupled with mass spectrometry (GS-MS) (Hewlett-Packard Model 5973 SIM Mode). CD analyses were performed by plasma emission spectrometry ICP (Varian Spectra II). AO analyses were performed by high performance liquid chromatography (HPLC) (Hewlett-Packard Model 1090M). Water quality and substances analyses were performed according to the same schedule as previously described.

Statistical analysis
The results obtained have been processed statistically. For the global response interpretation, statistical differences between the two experiments were observed first: in the case of significant difference, a ratio was calculated for each global response, and the data of the second experiment was corrected to be comparable to the first one. For each biological response, the theoretical variation coefficient was measured for the five reference mesocosms and was used for statistical interpretation. Then to assess the effect of concentrations, the reproducibility standard deviations have been estimated using the coefficients of variation observed on the references. For each response level a standard deviation is thus evaluated theoretically. The averages are therefore compared using a “U” test (comparison of averages with theoretical standard deviation). The type 1 risks (alpha) to establish the existence of an effect are then estimated and two critical levels of 5% and 1% are produced.

Results and discussion
Physico-chemical analyses: substances
An average measured concentration is estimated for each channel. The nominal and measured concentrations were given in Table 2. The nominal concentrations will be those used in the rest of the paper. The measured concentrations were below the nominal concentrations since, in a mesocosm, there are adsorption phenomena on the sediments and plant life which develops in the mesocosm. The adsorption is more important for hydrophobic compounds (AO and TCE). Landrum et al. (1984) showed that anthracene was absorbed on sediment and periphyton. There are also differences between the stability of measured con-

Table 1  Biochemical parameters measured in mesocosm experiments. PChE, GST, NAD(P)H red were assayed as described in Vidal et al. (2001)

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH cytochrome c reductase activity (NADPH red)</td>
<td>Phase I of (de)toxication metabolism</td>
</tr>
<tr>
<td>Glutathione S-transferase activity (GST), substrate:</td>
<td>Phase II of (de)toxication metabolism</td>
</tr>
<tr>
<td>1-chloro-2,4-dinitrobenzene</td>
<td></td>
</tr>
<tr>
<td>Propionylcholinesterase activity (PChE)</td>
<td>Synaptic transmission</td>
</tr>
</tbody>
</table>
centrations of substances. TCE appeared to be the most stable over the 30 days of exposure with CV% < 28%.

**Physico-chemical analysis: pH, O₂, conductivity and temperature**

The evolution of these parameters has highlighted the fact that the substances had no effect on any of the measured parameters. During the experiments the dissolved oxygen remained in the range of 8 to 11 mg O₂/l during the first experiment (March) and 6 to 10 mg O₂/l during the second one (June). These values are quite compatible with aquatic life and do not induce stress. The temperature remained at 6–12°C in March and 15–20°C in June. The conductivity remained in the range 60–160 µS/cm² and the pH 7.5–8.

**Physico-chemical analysis: COD, BOD₅, NH₄⁺, NO₃⁻, PO₄³⁻, PT, Cl⁻**

Table 3 gives the average values observed during the 30-day exposure. No effect of the substances, at the tested concentrations, was observed on these parameters. The values are typical for natural streams.

At the selected concentrations, it was difficult to obtain an increasing dose-response

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**Table 2** TCE, CD and AO nominal and measured concentrations in water samples from channels during two experiments

<table>
<thead>
<tr>
<th>Concentration</th>
<th>First experiment</th>
<th>Second experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCE CD AO Control</td>
<td>TCE CD AO Control</td>
</tr>
<tr>
<td>Nominal</td>
<td>10,000 100,000 50 500</td>
<td>800 8,000 0 0</td>
</tr>
<tr>
<td>Measured</td>
<td>2,208 20,207 29 374</td>
<td>427 4,729 0 0</td>
</tr>
<tr>
<td>CV%</td>
<td>28 28 11 42</td>
<td>36 10 0 0</td>
</tr>
</tbody>
</table>

**Table 3** Monthly average concentrations of the physicochemical parameters measured in the water

<table>
<thead>
<tr>
<th>mg/l</th>
<th>NH₄⁺</th>
<th>NO₃⁻</th>
<th>PO₄³⁻</th>
<th>PT</th>
<th>Cl⁻</th>
<th>BOD₅</th>
<th>COD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.011</td>
<td>4.56</td>
<td>0.4</td>
<td>0</td>
<td>3.9</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Std dev</td>
<td>0.029</td>
<td>0.95</td>
<td>0.06</td>
<td>0</td>
<td>0.9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mean</td>
<td>0.1</td>
<td>4.00</td>
<td>0.30</td>
<td>&lt;0.2</td>
<td>2.9</td>
<td>&lt;5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Std dev</td>
<td>0.05</td>
<td>0.89</td>
<td>0.08</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 1** Evolution of NADPH reductase activity in function of the anthracenic oil concentration, in *Corbicula fluminea* (**p ≤ 0.001; **p ≤ 0.01; *p ≤ 0.05, comparing exposed to control**).
curve for biomarkers responses. Hormetic response was observed (Figure 1). When exposed to AO, first, there is an increase in NADPH red., in accordance with results with Vidal et al. (2001a,b), then a decrease of activity. AO concentration above 800 µg/l is nearly lethal. Corbicula’s death was observed after 30 days of exposure at 8,000 µg AO/l. An hypothesis is that, at such concentrations, their metabolism is damaged, and the enzymatic activity decreases. In this experiment, it was shown that biomarker responses may have an increasing dose-response curve only below the lowest observed effect concentration (LOEC) of the communities. As soon as the concentration is above the LOEC’s, then the metabolism of Corbicula fluminea seems to be damaged. Figure 2 gives information in accordance to previous results (Vidal et al., 2001a,b): when exposed to CD, first an decrease of the GST, then an induction. In such case, to obtain increased dose-responses curve may be difficult. This renders it important to carry out a multimarker approach to benefit from a global interpretation. The biomarker of exposure can give information on the presence of substances but it would be difficult to quantify their effect.

To test the sensitivity and precocity of this set of biomarker responses, we compared the LOEC for biomarker and biocoenotic responses. In the case of TCE, the invertebrates communities generally gave more precocious and sensitive responses than diatoms and biomarkers, particularly when considering invertebrate abundance and structure of oligochaete assemblages (Figure 3a). In the case of CD, if the LOEC is observed at 5 µg/l for both biocoenetic and biomarkers responses, the GST exhibits earliest response (1 day of exposure) and oligochaete react after 7 days of exposure (Figure 3b). For AO (Figure 3c), biomarkers’ responses are as sensitive as biocoenotic one and the earliest responses are given by oligochaete assemblages (7 days of exposure). The sensitivity of oligochaete to cadmium and PAH (Prygiel et al., 1999) seems to be supported by our data; sensitivity to TCE is insufficiently known.

Conclusions
The biomarkers exhibit contrasted responses to the selected substances: each substance having its dedicated biomarkers (TCE:GST; CD:PChE-GST; AO:PChE-NADPH), justifying a multimarker approach eventually specific to a substance. A discriminant analysis needs to be carried out to demonstrate this. Communities react to every substance, with responses generally as precocious as biomarker ones, except for CD. Biomarkers’ responses are as sensitive as ecosystem responses (except for TCE). The biomarkers are alarm indicators, indicating the presence of substances at concentrations that do not necessarily induce noxious effects in the field, except if the concentration increases. As hormetic doses–response curves were determined, this set of biomarker responses could not be used...
to quantify an effect. These results also demonstrate the interest of using integrated biomonitoring methods when assessing noxious substance effects in the field (Lafont et al., 1991).

These preliminary results are encouraging but do not allow us to draw conclusions with certainty. The research is continuing and focusing on other substances, including various substances mixtures, other biomarkers responses (oxidative stress, genotoxicity). Consideration of the ecology of the diatom and oligochaete species is also planned.

**Figure 3** a, b, c: LOECs (µg/l) for each biological response observed the earliest, with TCE (3a), cadmium (3b) and anthracenic oil (3c) substances. Biomarkers’ responses: GST, NADPH, PChE; biocoenotic responses: Oligo:oligochaete; INV:invertebrates; DIAT:diatoms; abd:abundance; bdv:biodiversity, shan:shannon index; ips:Polluosensitivity index. ND: non-determined LOEC (if there is a LOEC for these responses, than these are up to the high concentration tested)
References


