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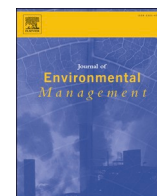
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Research article

Advancing environmental monitoring across the water continuum combining biomarker analysis in multiple sentinel species: A case study in the Seine-Normandie Basin (France)

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ABSTRACT

Nowadays, biomarkers are recognized as valuable tools to complement chemical and ecological assessments in biomonitoring programs. They provide insights into the effects of contaminant exposures on individuals and establish connections between environmental pressure and biological response at higher levels. In the last decade, strong improvements in the design of experimental protocols and the result interpretation facilitated the use of biomarker across wide geographical areas, including aquatic continua. Notably, the statistical establishment of reference values and thresholds enabled the discrimination of contamination effects in environmental conditions, allowed interspecies comparisons, and eliminated the need of a reference site.

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The aim of this work was to study freshwater-estuarine-coastal water continua by applying biomarker measurements in multi-species caged organisms. During two campaigns, eight sentinel species, encompassing fish, mollusks, and crustaceans, were deployed to cover 25 sites from rivers to the sea. As much as possible, a common methodology was employed for biomarker measurements (DNA damage and phagocytosis efficiency) and data interpretation based on guidelines established using reference values and induction/inhibition thresholds (establishment of three effect levels).

The methodology was successfully implemented and allowed us to assess the environmental quality. Employing multiple species per site enhances confidence in observed trends. The results highlight the feasibility of integrating biomarker-based environmental monitoring programs across a continuum scale. Biomarker results align with Water Framework Directive indicators in cases of poor site quality. Additionally, when discrepancies arise between chemical and ecological statuses, biomarker findings offer a comprehensive perspective to elucidate the disparities. Presented as a pilot project, this work contributes to gain insights into current biomonitoring needs, providing new questions and perspectives.

1. Introduction

Since 2000, the European Union (EU) has implemented the Water Framework Directive (WFD, Directive, 2000/60/EC) to safeguard water bodies including lakes, rivers, transitional waters, coastal waters, and groundwater. It has the advantage of serving as a single legal basis for all EU countries. However, the methods currently involved in its context are encountering certain limitations in the assessment of the aquatic environment as outlined by Milinkovitch et al. (2019). Regarding the chemical aspect, the list of priority substances is limited to 45 compounds, and the analyses do not provide insights into biological impacts or interactions between compounds (Crane et al., 2007; Kuzmanović et al., 2016). On the other hand, the ecological status is based on parameters which does not constitute early indicators of environmental quality degradation. Moreover, biodiversity indices are so integrative that they are usually not specific to the impacts of contaminant exposure (Wernersson et al., 2015), even if some studies succeed in defining parameters at high biological levels that are mainly modulated by the effects of chemical substances. For instance, a relationship has been established between the macroinvertebrate assemblages and the occurrence and bioavailability of metals in rivers (Rico-Sánchez et al., 2022). Meyer et al. (2022) also identified trait syndromes correlated with the presence of specific contaminant mixtures within diatom, macroinvertebrate, and fish communities.

Effect-based tools (EBT) offer several advantages for improving the environmental quality assessment of aquatic systems (Wernersson et al., 2015). Among them, biomarkers are defined as observed or measured changes at (sub)individual levels (e.g., DNA degradation, protein modulations), including the behavior, caused by exposure to pollutants (van der Oost et al., 2003). They are proposed as complementary tools to chemical and ecological analyses providing insights into the effects of bioavailable contaminants and consider the cocktail effects of contaminant mixtures (Lam, 2009; Wernersson et al., 2015). The results obtained can be used to better understand the relationship between environmental pressures and high-level biological impacts. As evidence, they are already implemented in the Marine Strategy Framework Directive (MSFD, 2008/56/EC). Among the various descriptors used to determine the quality status of a site and to assess the impact of human activities, this directive focuses on whether exposures to substances have adverse effects on the health of species.

One of the greatest challenges associated with the utilization of biomarkers is to clearly define their applicable conditions and interpretation, and to produce outputs which can be effectively used by water resource managers (Milinkovitch et al., 2019). A response modulation is a misinterpretation when it results from confounding factor effects (Forbes et al., 2006; Hanson, 2011). Since the 2000s, substantial efforts have been made to improve the interpretation of biomarker modulations, including a deeper understanding of their natural variability, in response to environmental and biotic confounding factors. Technically, employing controlled organisms through caging methods (i.e., active

biomonitoring) has been recognized as a suitable approach for monitoring the quality of aquatic environmental matrix (Baird et al., 2007). One of the major benefits of active biomonitoring is the reduction of the intrinsic variability of biomarkers by using standardized organisms with a same life history, eliminating the variations potentially linked to sex, age/size classes or local phenotypic characteristics of populations (Bourgeault et al., 2010; Catteau et al., 2019, 2022; Crane et al., 2007; Gouveia et al., 2017; Le Guernic et al., 2016; Oikari, 2006; Slaby et al., 2023). Moreover, it allows for research in species-poor sites and avoids sampling pressures on local populations. Moreover, another strong lever for improving the *in situ* application and interpretation of biomarkers had been the establishment of reference data set and the associated threshold values taking into account the variability induced by environmental parameters (i.e., temperature, water hardness; Barbarin et al., 2022; Barrick et al., 2016; Catteau et al., 2023; Charron et al., 2014; Coulaud et al., 2011; Erraud et al., 2018a; Lacaze et al., 2011; Lepêtre et al., 2022a; Xuereb et al., 2009). These benchmarks improve the impact assessment of contaminants by discriminating the outcome of chemical stress from the background noise and then reducing the occurrence of false negatives or false positives, and enable independence from reference sites.

Several studies have reported the application of biomarkers in caged organisms for monitoring aquatic environments in different water bodies, including rivers (Bonnail et al., 2019; Catteau et al., 2021; Lunardelli et al., 2018), lentic water bodies (Brodeur et al., 2021; Georgieva et al., 2022; Slaby et al., 2023), estuarine environments (Borcier et al., 2019), and marine ecosystems (de los Ríos et al., 2012; Maisano et al., 2017; Tsangaris et al., 2010). However, to the best of our knowledge, the choice of study sites is constrained by the species-specific characteristics and no attempts have been made to establish a consistent methodology across different species that would allow for the assessment of environments with significant differences in physicochemical properties such as an aquatic continuum. In fact, at the scale of a hydrographic watershed, water flow encompasses ecosystems with large variations in physicochemical properties (e.g., salinity, conductivity, hydrodynamics, turbidity). For instance, a complete network includes freshwater, transitional water, and coastal water parts, as well as lotic and lentic systems. These environments are different but form a continuum that must be fully covered in environmental monitoring programs. As a consequence, the deployment of active biomonitoring programs could take advantage of the use of multiple species in which the same biomarkers can be studied and consistently interpreted from small headwater streams to marine ecosystems. In this context, a collaborative project involving French research laboratories and a field management agency introduced an innovative strategy to address the challenges of studying continuous aquatic environments ("SASHIMI" project, funded by the Office Français de la Biodiversité, Vincennes, France, <https://www.seine-aval.fr/projet/sashimi/>). It proposed several model species (i.e., *Gasterosteus aculeatus* Linnaeus, 1758, *Platichthys flesus* Linnaeus, 1758, *Dreissena polymorpha* Pallas, 1771, *Mytilopsis*

leucophaeata Conrad, 1831, *Mytilus edulis* Linnaeus, 1758, *Gammarus fossarum* Koch, 1836, *Palaemon longirostris* Milne Edwards, 1837, and *Palaemon serratus* Pennant, 1777) to study various water body types along with biomarkers of immunotoxicity (phagocytosis efficiency) and genotoxicity (DNA damages). The results defined specific caging conditions for each species and a methodology of measure and interpretation of biomarkers based on the threshold values mobilization in order to permit interspecies comparisons along watershed continuum.

The purpose of the present study was to test a deployment of this methodology on different continua at a regional scale. This work was conducted as a part of the project “BIOSURVEILLANCE” funded by the Seine-Normandie Water Agency (Courbevoie, France, <https://www.seine-aval.fr/projet/biosurveillance/>). A total of 25 sites was studied in Seine-Normandie Basin, including three aquatic continua from the river to the sea corresponding to small and large streams spanning, as well as one coastal continuum (from west Cotentin to the Seine Bay). Different species of fish, mollusks, and crustaceans were involved in order to cover different hydrosystems. These species occupy different trophic levels and allow us to consider interspecies variations in sensitivity to contamination. The monitoring of DNA damage levels and the phagocytosis efficiency was conducted the species collection during two caging campaigns over consecutive years (in autumn). The results were interpreted using the determined reference and threshold values for the two biomarkers across the different species. This approach allowed for the comparison of responses not only between species but also across various study sites along the continuum. Furthermore, the outputs of the present study were confronted to conventional chemical and ecological status determined according to the WFD at the same location in order to assess the consistency of the biomarker approach and highlight situations in which biomarkers can provide additional insights into the evaluation process.

2. Materials and methods

2.1. Site description

Twenty-five sites located in north-western France were investigated to cover various continua from freshwater to marine water (Figs. 1 and 2). These sites were chosen because they were already monitored as part of national water quality monitoring networks supervised by the Seine Normandie Water Agency, and the French Research Institute for Exploitation of the Sea, IFREMER (Table A1). Briefly, three distinct continua can be defined: (i) the Seine River continuum with sites distributed along (SE1, SE6-7), upstream of the confluence with important tributaries (SE2-5, SE8), in the estuary of the Seine River (SE9-10), and in the English Channel near the coast (SE11-12), (ii) the Orne River continuum with sites located in the freshwater (OR1-2) and the transitional (OR3-4) parts, as well as along the littoral (OR5) of the Orne River, (iii) the Vire River continuum with sites located in the freshwater part (VI1), the transitional zone (VI2), and along the littoral (VI3) of the Vire River. Also, sites located along the Cotentin Peninsula littoral were studied (CO1-5). Detailed information regarding their precise locations is given in Supplementary Information (Tables A1). Latest chemical and ecological status established under the WFD (2022) are presented in Table 1 for all sites when available.

2.2. Caging experiments

To encompass all ecosystems across the three continua, eight species from three different taxa were selected (Fig. 2). The list included two teleost fish, *G. aculeatus* and *P. flesus*, three bivalve mollusks, *D. polymorpha*, *M. leucophaeata*, and *M. edulis*, and three crustaceans *G. fossarum*, *P. longirostris*, and *P. serratus*. Fig. 2 illustrates the deployment locations for each species. Except at SE10 and VI2, at least two species from distinct taxonomic groups were deployed at each site.

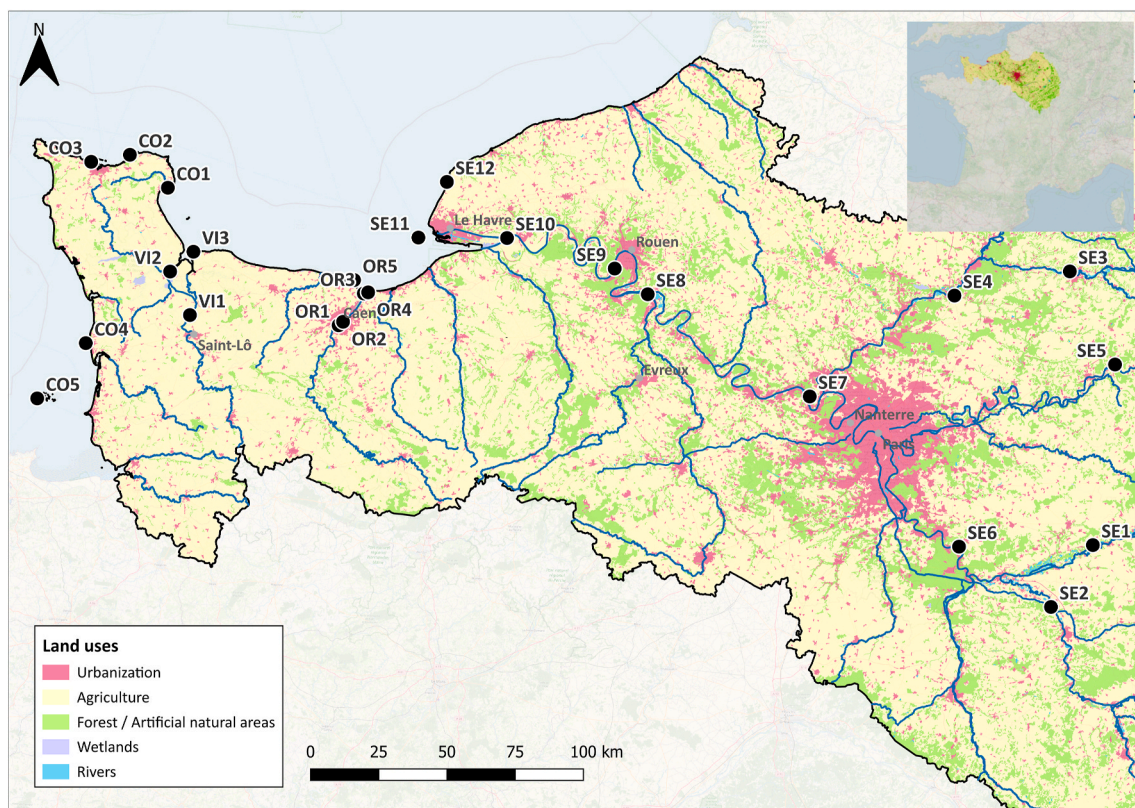


Fig. 1. Distribution of the sampling sites across the Seine (SE1-12), Orne (OR1-5), Vire (VI1-3), and Cotentin Peninsula littoral continua (CO1-5). Black dots: Sampling sites, Right corner: The Seine-Normandie Basin.

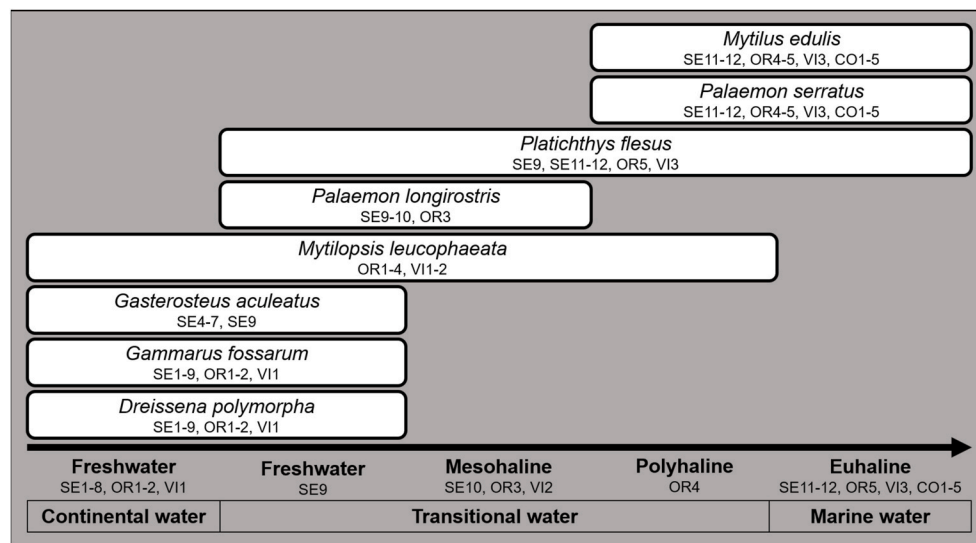


Fig. 2. Species distribution by study sites classified according to water salinity. SE1-12: Seine river continuum, OR1-5: Orne river continuum, VI1-5: Vire river continuum, CO1-5: Contentin Peninsula littoral continua.

Table 1

Chemical and ecological assessments in the context of the WFD (2022) of the study sites.

EU Waterbody ID	Site ID	Chemical status		Ecological status	
		Status with uPBT	Reason for failure to achieve good status	Status	Reason for failure to achieve good status
FRHR34-F2150600	SE1	Good	None	Moderate	Multi-metric invertebrate index (i2m2)
FRHR70A	SE2	Failing to achieve good*	Benzo[a]pyrene, Perfluorooctanesulfonate	Good	None
FRHR211	SE3	Failing to achieve good*	Benzo[a]pyrene, Mercure, Perfluorooctanesulfonate	Moderate	Biological Diatom Index (IBD)
FRHR216C	SE4	Failing to achieve good*	Benzo[a]pyrene, Perfluorooctanesulfonate	Good	None
FRHR137	SE5	Failing to achieve good	Benzo[a]pyrene, Mercure, Perfluorooctanesulfonate	Good	None
FRHR73A	SE6	Failing to achieve good*	Benzo[a]pyrene, Perfluorooctanesulfonate	Moderate	Macrophyte index (IBRM)
FRHR230A	SE7	Failing to achieve good	Benzo[a]pyrene, Benzo[g,h,i]perylene, Fluoranthene, Perfluorooctanesulfonate, C10–C13 chloroalkanes	Moderate	Biological Diatom Index (IBD)
FRHR261	SE8	Good	None	Good	None
FRHT01	SE9	Failing to achieve good	4-Nonylphenol, Aclonifen, Bis(2-Ethylhexyl) Phthalate, Dichlorvos, Heptachlor, Lindane, PAH, Perfluorooctanesulfonate, Tributyltin, Trichlorobenzene	Bad	Fish
FRHT03	SE10	Failing to achieve good*	Heptachlor, PAH, PCB (52/101/118/138/153), Tributyltin	Moderate	Fish, Invertebrate
FRHC16	SE11	Failing to achieve good*	PCB (52/101/118/138), Tributyltin	Good	None
FRHR361	SE12	Failing to achieve good*	PCB (52/101/118/138), Tributyltin	Good	None
FRHR361	OR1	Failing to achieve good*	Benzo[a]pyrene	Moderate	Biological Diatom Index (IBD)
FRHT04	OR2	NA	NA	Moderate	Intertidal macroalgae
FRHC14	OR3	NA	NA	Moderate	Intertidal macroalgae
FRHC14	OR4	NA	NA	Moderate	Intertidal macroalgae
FRHC14	OR5	Failing to achieve good*	PCB (101/118/138)	Good	None
FRHR317	VI1	Failing to achieve good	Benzo[a]pyrene, Fluoranthene	Moderate	Fish
FRHT06	VI2	Failing to achieve good*	PCB (118)	Moderate	Biological Diatom Index (IBD)
FRHT06	VI3	Failing to achieve good*	PCB (118)	Moderate	Biological Diatom Index (IBD)
FRHC09	CO1	Failing to achieve good*	PCB (118)	Moderate	Angiosperms, Intertidal macroalgae
FRHC07	CO2	Good (2019) ¹	NA	Good	None
FRHC61	CO3	Failing to achieve good*	PCB (118), Tributyltin	Good	None
FRHC03	CO4	Good	None	Moderate	Angiosperma
FRHC01	CO5	Good	None	Good	None

uPBT: ubiquitous, Persistent, Bioaccumulative and Toxic.

*Good chemical status if uPBT are not considered.

¹Data from the 2019 report.

Life cycle stage used, organism size, and exposure duration for each species are presented in Table 2. All details regarding caging conditions are provided in Table A2 of Supplementary Information. This includes information such as sex ratio (e.g., controlled or not), population origin, optimal caging period and duration, density, cage specifications, immersion depth, salinity, and hydrodynamics. The use *M. leucophaeata* and *D. polymorpha* were done with caution as both are naturalized species. The first record of *M. leucophaeata* in France was in the canal from Caen to the Channel (north-western France) in 1898 (Chemin, 1911). Regarding *D. polymorpha*, it was recorded for the first time in the Seine river in 1855 (Prié, 2023). Consequently, these species were only caged in sites where they were already present in the associated catchment.

The caging experiments were conducted during two successive years, in Autumn (2020); 2021. Test dates for each site and species are available in Supplementary Information (Table A3). This timing ensured a natural food supply and avoided periods of unfavorable climatic and hydrodynamic conditions such as heatwaves, droughts, and heavy flooding. Additionally, it fell outside the breeding period of the *G. aculeatus*, *D. polymorpha*, *M. edulis*, and *M. leucophaeata*. This last consideration holds less significance for other species, given that only juvenile *P. flesus* were used, and that crustacean model reproduction is distributed throughout the year and their sexual phenotype can be easily calibrated before the exposure.

2.3. Biomarker measurements

Phagocytosis is a conserved immune defense mechanism in the living (Yutin et al., 2009). The phagocytosis efficiency, defined as the proportion of cells that have internalized three or more fluorescent beads, was determined using flow cytometer analyses. The methodology was adapted from Marchand et al. (2017), Barjhoux et al. (2020), Diop et al. (2022), and Gendre et al. (2023). Genotoxic impacts were assessed by measuring the DNA integrity. It was conducted using the alkaline comet assay which enables the detection of DNA double-strand breaks, single-strand breaks, and alkali-labile sites (Singh et al., 1988). The results were expressed as the proportion of DNA located in the tail (% tail DNA), which is an indicator of DNA damage. The comet assay procedure was homogenized across the species of interest based on the methodologies described by Lacaze et al. (2011), Erraud et al. (2018b), Borcier et al. (2019), and Cant et al. (2022). Tables 3 and 4 describe the key steps involved in both procedures for each species.

2.4. Animal welfare

All experiments were conducted in accordance with the European directive 2010/63/EU. For *G. aculeatus*, experimental protocols were conducted at INERIS (registration number E60-769-02), were submitted and reviewed by a French nationally recognized ethical committee (CREMEAPS, registration number 96) and were approved by the French ethics committee in animal experimentation (APAFIS project number #20,760). For *P. flesus*, the study was conducted in accordance with European Commission recommendation 2007/526/EC, on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Both species were anesthetized using tricaine mesylate at a concentration (100 mg.L⁻¹ for *G. aculeatus* and 10 mg.L⁻¹ for *P. flesus*), and then euthanized by cervical dislocation.

2.5. Application of threshold values for the interpretation of biomarkers

A reference value, defined as the baseline response under “normal” conditions (including individual variability) along with induction and inhibition thresholds were established for all combinations of biomarkers and species (Table 2). These values enable the comparison of sites using different model species and eliminate the need for defining a reference site. Their determination depended on the size of the dataset and the available knowledge about biomarker variability.

For all species, only the induction of DNA damage was interpreted for the assessment of a toxicological impact and the environmental quality. It differed for the phagocytosis marker for which induction and/or inhibition of the response were considered. The interpretation of an impact on organism immunity was influenced by the dataset available for each species, leading us to consider only the inhibition of responses for *D. polymorpha* and *M. leucophaeata* and the induction of responses for *M. edulis*, due to the high dispersion of the measures and/or elevated baseline levels.

For *D. polymorpha*, *M. edulis*, and *P. serratus* the statistical method of evaluation of the reference values and associated thresholds is adapted from Besse et al. (2013) and was depicted in Leprêtre et al. (2022) and Catteau et al. (2023). Concerning *P. longirostris*, the phagocytosis efficiency lacked a sufficient dataset to establish reference values and thresholds. Nevertheless, those determined for the DNA damage biomarker in *P. serratus* were applied to *P. longirostris*, as the transferability was previously demonstrated (Erraud et al., 2019). For *G. fossarum*, the reference value, the induction threshold, and inhibition threshold were established as the median, the upper, and the lower

Table 2
Caging conditions and interpretation guidelines.

	Caging conditions			Reference value [Inhibition threshold, Induction threshold]		
	Stage	Size	Exposure duration	Phagocytosis efficiency (%)	DNA damage (% of tail DNA)	Interpretation grid (x = % of individuals exceeding the threshold)
<i>Dreissena polymorpha</i>	Adult	22–25 mm	21 days	56 [41, NA]	4.2 [NA, 8.7]	- x ≤ 25%: No effect -25% < x ≤ 75%: Effect - x > 75%: Strong effect
<i>Mytilus edulis</i>	Adult	40–50 mm	15 days	17.5 [NA, 37]	2.4 [NA, 6.3]	
<i>Mytilopsis leucophaeata</i>	Adult	>15 mm	28 days	22.4 [15, NA]	9.2 [NA, 12.3]	
<i>Gammarus fossarum</i>	Adult	8–10 mm	7 days	31.4 [17.5, 58.2]	10.1 [NA, 18.5]	
<i>Palaemon longirostris</i>	Adult	30–40 mm	15 days	NA	4.7 [NA, 11.4]*	
<i>Palaemon serratus</i>	Adult	55–65 mm	15 days	18.4 [8.4, 30.4]	4.7 [NA, 11.4]	
<i>Gasterosteus aculeatus</i>	Adult	35–45 mm	21 days	Model size	7.61 [NA, 17.6]	- x ≤ 5%: No effect -5% < x ≤ 20%: Effect - x > 20%: Strong effect
<i>Platichthys flesus</i>	Juvenile	80–90 mm	15 days	38.7 [35.9, 40.5]	32.3 [NA, 33.5]	- x ≤ 40%: No effect -40% < x ≤ 80%: Effect - x > 80%: Strong effect

* Reference value and thresholds obtained with *P. serratus*.

Table 3
Key steps of the phagocytosis efficiency measurement.

	<i>D. polymorpha</i>	<i>M. leucophaeata</i>	<i>M. edulis</i>	<i>G. fossarum</i>	<i>P. serratus</i>	<i>G. aculeatus</i>	<i>P. flesus</i>
Cell sampling	Hemocyte, Post. adductor muscle 7-10 ind.	Hemocyte, Post. adductor muscle 9-10 ind.	Hemocyte, Post. adductor muscle 9-20 ind.	Hemocyte, Dorsal puncture 4 pools of 5 ind.	Hemocyte, Cardiac puncture 5-13 ind.	Leukocyte, Spleen 25-30 ind.	Leukocyte, Spleen 5-14 ind.
Numeration and quality assessment	Hoescht 33,342 and PI stains Fluo. microscopy	Microscopy	Hoescht 33,342 and PI stains Flow cytometry	Hoescht 33,342 stain Fluo. microscopy	PI stain Flow cytometry	Microscopy	Microscopy
Exposure conditions	10 ⁵ cells/well 96-well microplate Beads Ø: 2 µm Cell:bead = 1:50 4 h, 16 °C, dark	10 ⁵ cells/250 µL Microtube Beads Ø: 1 µm Cell:bead = 1:100 1 h, 17 °C, dark	10 ⁵ cells/well 96-well microplate Beads Ø: 2 µm Cell:bead = 1:50 2 h, 15 °C, dark	Bead adjustment 96-well microplate Beads Ø: 2 µm Cell:bead = 1:200 4 h, 16 °C, dark	8 × 10 ⁴ cells/well 96-well microplate Beads Ø: 1 µm Cell:bead = 1:250 2 h, 15 °C, dark	8 × 10 ⁴ cells/well 96-well microplate Beads Ø: 1 µm Cell:bead = 1:250 2 h, 15 °C, dark	2 × 10 ⁵ cells/well 96-well microplate Beads Ø: 1 µm Cell:bead = 1:150 1 h, RT, dark
Flow cytometry analysis	Triple labelling: Hoescht, PI, beads	Double labelling: Hoescht, beads	Double labelling: PI, beads	Double labelling: Propidium iodide, beads	Double labelling: PI, beads	Single labelling: Beads	Single labelling: Beads

Ind.: Individuals, PI: Propidium iodide, Fluo.: Fluorescent, Beads: Yellow-green fluorescent polystyrene microspheres.

Table 4
Key steps of the comet assay for the DNA damage measurement.

	<i>D. polymorpha</i> <i>M. leucophaeata</i> <i>M. edulis</i>	<i>G. fossarum</i>	<i>P. longirostris</i> <i>P. serratus</i>	<i>G. aculeatus</i> <i>P. flesus</i>
Cell sampling	Hemocyte, Post. adductor muscle 8-15 ind.	Spermatozoa, Testis 4-5 pools, 5 ind.	Spermatozoa, Spermatophore 8-10 ind.	Erythrocyte, Blood ¹ 5-30 individuals
Cell density and viability	6 × 10 ⁵⁻⁶ cells/mL Survival rate threshold: ≥85%			
Inclusion	0.5% low-melting point agarose-type VII in PBS (10 mM pH 7.4, 37 °C) on microscope slide			
Lysis	2.5 M NaCl, 100 mM Na ₂ EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10 1 h	18 h	1 h	
Denaturation	300 mM NaOH, 1 mM Na ₂ EDTA, pH > 13, 30 min			
Electrophoresis	300 mA, 20 V, 24 min, Standard Comet assay tank (20 slides)			
Neutralization	0.4 mM Tris, pH > 7.5, 2 × 10 min			
Dehydration	100% EtOH, 5 min			
Coloration	SYBR green (1X), 5 min			
Quantification	Image acquisition: Fluo. Microscopy Scoring: 100 nucleoids/gel (Comet assay IV software, Perceptive instrument, Suffolk, UK)			

¹ Cryopreserved and rapidly defrosted (37 °C, 18 s) prior to the analysis, Ind.: Individuals, Fluo.: Fluorescent.

quartiles, respectively, of distributions derived from results obtained with 4 pools of 5 individuals each from 20 contrasted study sites for phagocytosis efficiency and 10 pools of 3 individuals from a control condition for DNA damage. For *G. aculeatus*, a linear model was previously developed to obtain reference values and thresholds for the phagocytosis efficiency according to the organism size and sex (Marchand et al., 2019). Finally, the reference value for *P. flesus* was determined as the mean of the results obtained from caged organisms in control conditions at three distinct periods, and the thresholds were set as 95% confidence interval.

Table 2 provides an overview of the interpretation guidelines established for all biomarker-species pairs. These guidelines were defined according to statistical approaches and expert statements, taking into consideration variations in methods and species. Their purpose is to ensure clarity for a non-specialist audience and to be used by stakeholders. Three distinct levels were established: “no effect”, “effect”, and “strong effect” based on the number of individuals outside the thresholds. The numbers of individuals used to define levels are provided in Tables A.4-A11. To determine the environmental quality of a study site, the precautionary approach known as the “one out–all out” strategy, similar to the approach in the WFD, was employed. In the present work, this implies that the worst level determined by any biomarker-species pairs defined the overall quality assessment of the site.

3. Results & discussion

3.1. Methodological readiness

First, the results highlighted that biomonitoring using biomarkers measured in caged species at the continuum scale is technically feasible. In fact, only 7 of the 131 (5.3%) cages deployed during the experimental campaigns were lost due to either siltation, water level drop, or degradation by other site users (Table 5). This represents a low percentage and did not affect the overall implementation of all the monitoring program. The survival rates of organisms were also satisfactory for all species during the two campaigns (Table 5, *D. polymorpha*: 93.8 ± 5.4%, *M. leucophaeata*: 89.8 ± 16.9%, *M. edulis*: 90.9 ± 8%, *G. fossarum*: 84.3 ± 6.6%, *P. longirostris*: 78.4 ± 7.7%, *P. serratus*: 59.8 ± 11.5%, *G. aculeatus*: 94 ± 9.3%, and *P. flesus*: 84.7 ± 16.3%, mean ± SD). The lower survival rates observed for *P. longirostris* and *P. serratus* could be attributed to cannibalistic behavior within the cage, despite the inclusion of refuges such as polyvinyl chloride modules and grid substrates. In the future, modifications of caging conditions for these species could be implemented by individualizing the organisms. A very low survival rate of 38% was observed for *M. leucophaeata* placed at OR4 in 2021 thus, biomarker measurements from this specific condition were excluded from the subsequent analysis. In contrast, all other cages containing this species exhibited notably higher rates (≥85.1%).

Table 5

Survival rates (%) and cage recoveries per site during both campaign years.

ID	<i>D. polymorpha</i>		<i>M. leucophaeata</i>		<i>M. edulis</i>		<i>G. fossarum</i>		<i>P. longirostris</i>		<i>P. serratus</i>		<i>G. aculeatus</i>		<i>P. flesus</i>	
	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021
SE1	98.3	91	–	–	–	–	68.6	80	–	–	–	–	–	–	–	–
SE2	99.2	88.5	–	–	–	–	76.4	87.8	–	–	–	–	–	–	–	–
SE3	99.2	83.5	–	–	–	–	83.6	89.4	–	–	–	–	–	–	–	–
SE4	99.2	Loss	–	–	–	–	88.6	82.2	–	–	–	–	96	93	–	–
SE5	Loss	89	–	–	–	–	87.1	92.8	–	–	–	–	Loss	Loss	–	–
SE6	99.2	91	–	–	–	–	85.7	85.6	–	–	–	–	100	100	–	–
SE7	97.5	91.5	–	–	–	–	85.7	86.7	–	–	–	–	100	100	–	–
SE8	99.2	92	–	–	–	–	82.1	91.1	–	–	–	–	–	–	–	–
SE9	96.7	82	–	–	–	–	81.4	76.7	Loss	75.6	–	–	73	90	93.3	93.3
SE10	–	–	–	–	–	–	–	–	Loss	Loss	–	–	–	–	–	–
SE11	–	–	–	–	95	82.5	–	–	–	–	46	42	–	–	93.3	53.3
SE12	–	–	–	–	85	77.5	–	–	–	–	56	64	–	–	100	66.7
OR1	98.3	92	97.7	93.5	–	–	82.9	90.6	–	–	–	–	–	–	–	–
OR2	99.2	87.5	95.5	93.5	–	–	80	91.1	–	–	–	–	–	–	–	–
OR3	–	–	96.7	89.2	–	–	–	–	72.5	87.2	–	–	–	–	–	–
OR4	–	–	85.1	38	82.5	75	–	–	–	–	58	62	–	–	–	–
OR5	–	–	–	–	92.5	87	–	–	–	–	50	52	–	–	100	66.7
VI1	95	94	100	91.9	–	–	71.4	95.6	–	–	–	–	–	–	–	–
VI2	–	–	100	97	–	–	–	–	–	–	–	–	–	–	–	–
VI3	–	–	–	–	100	82.5	–	–	–	–	60	54	–	–	93.3	86.7
CO1	–	–	–	–	97.5	100	–	–	–	–	39	83	–	–	–	–
CO2	–	–	–	–	95	97.5	–	–	–	–	77	66	–	–	–	–
CO3	–	–	–	–	95	89.5	–	–	–	–	73	60	–	–	–	–
CO4	–	–	–	–	95	100	–	–	–	–	63	74	–	–	–	–
CO5	–	–	–	–	–	97.4	–	–	–	–	53	64	–	–	–	–

3.2. Biomarker interpretation

3.2.1. Geographical pattern

Overall, even if the sites were not selected to represent a gradient of anthropogenic pressures, an improvement of the environmental quality was observed in downstream sites across all continua and in the littoral areas (Table 6 & A.4-A.11). This improvement is particularly noticeable in taxa deployed all along the continua (i.e., mollusks and crustaceans) whether in 2020 or in 2021. It could be attributed to a potential dilution effect, suggesting lower-contamination levels. In the case of the Seine River, this observation is confirmed by other studies. Dendievel et al.

(2022, 2020) showed that the sediment contamination with PCB and metals increases with the strong influence of anthropic activities occurring in Paris and Rouen, but then decreases towards the estuary. In contrast, this trend was not confirmed in the sites where *P. flesus* was caged, as a “strong effect” level was principally identified in the respective sites (discussed in Section 3.2.3).

3.2.2. The benefit of studying two biomarkers

The use of different biomarkers enables the integration of various modes of action of contaminants, thereby enhancing the assessment of environmental quality (Catteau et al., 2022; Slaby et al., 2023; Yancheva

Table 6

Quality status determined using the biomarker approach (phagocytosis efficiency or DNA damage) according to species in 2020 and 2021. Sites are presented from upstream to downstream for the Seine, Orne, and Vire continua.

	<i>D. polymorpha</i>		<i>M. leucophaeata</i>		<i>M. edulis</i>		<i>G. fossarum</i>		<i>P. longirostris</i>		<i>P. serratus</i>		<i>G. aculeatus</i>		<i>P. flesus</i>	
	PE DD		PE DD		PE DD		PE DD		PE DD		PE DD		PE DD		PE DD	
	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021
SE1																
SE2																
SE3																
SE4																
SE5																
SE6																
SE7																
SE8																
SE9																
SE10																
SE11																
SE12																
OR1																
OR2																
OR3																
OR4																
OR5																
VI1																
VI2																
VI3																
CO1																
CO2																
CO3																
CO4																
CO5																

No effect Effect Strong effect Cage loss

PE: Phagocytosis efficiency, DD: DNA damage, NA: Not analyzed.

et al., 2022). In this work, a degraded status determined by one biomarker was often associated with a degraded status by the second biomarker. In fact, the determined quality level was consistent between both biomarker analyses for a single species in $75.1 \pm 20.2\%$ of sites ("no effect" vs. "effect" and "strong effect"). Nevertheless, interspecies variations were observed. Both biomarker analyses derived either an "effect" or "strong effect" levels in $78.8 \pm 8.6\%$ of sites for *D. polymorpha*, $51.7 \pm 22.4\%$ of sites for *G. fossarum*, $56.7 \pm 16.5\%$ of sites for *M. leucophaeata*, $87.5 \pm 8.8\%$ of sites for *G. aculeatus*, and $87.5 \pm 8.8\%$ of sites for *P. flesus* (across the two campaign periods). For *P. serratus* and *M. edulis*, it never happened ($0 \pm 0\%$ of sites) because, as shown in Table 6, the majority of the concerned sites were classified with a "no effect" level by both biomarker analyses in 2020 and 2021 ($74.6 \pm 2.2\%$ and $81.9 \pm 4.9\%$, respectively).

When the effect levels were different between both biomarker analyses in a species, DNA damage analyses led to a worst evaluation in 66.7% of sites. As approximately one-third of environmental contaminants are classified as genotoxic (Claxton et al., 1998), this result could be expected. Nevertheless, it does not suggest that DNA damage is more suitable to detect environmental contamination as in several cases the phagocytosis efficiency analyses led to an "effect" or a "strong effect" level while the genotoxicity biomarker reported a "no effect" level.

3.2.3. Implementation of several species per site

Considering multiple species when using biomarkers to assess environmental quality is of major importance. As demonstrated by Cateau et al. (2022), including various species in biomarker approaches enhanced the accuracy of pollution assessments. In the present study, variations were observed between species at a specific site during a single caging period (Table 6 and A4-A.11). Indeed, the species involved in this study had distinct physiological and behavioral characteristics, leading to differences in bioavailability and sensitivity to contaminants. Notably, they do not occupy the same level in the food chain. Thus, the responses obtained with the different species can either corroborate the findings or provide additional information about the contamination.

Herein, the assigned quality levels differed between species for 52.4% and 68.4% of sites for the phagocytosis efficiency measurements and for 63.2% and 54.5% of sites for the DNA damage measurements in 2020 and 2021, respectively. However, as the available knowledge regarding biomarker modulations and the type of dataset used to determine reference values and thresholds were not consistent across all species, the comparison between species caged at one site during the same period should be done with caution. In this study, certain species require the acquisition of more extensive datasets to enhance the interpretation grid in the present study. Herein, it seemed more appropriate to compare the changes in environmental quality among species over geographical or temporal scales. By doing so, the use of multiple species enabled us to identify trends (Table 6). For example, improvements in the gradation using the biomarker of genotoxicity were highlighted between 2020 and 2021 for all concerned species at SE12. The same observation can be made at SE9 except for *D. polymorpha* for which a "strong effect" level was determined in 2020 and 2021. This difference can be attributed to the specific characteristics of *D. polymorpha* (sessile and filter-feeding organisms) compared to *G. fossarum*, *P. longirostris*, *G. aculeatus*, and *P. flesus*.

As previously mentioned in Section 3.2.1, results from mollusk and crustacean species corroborate the improvement in environmental quality in the water column at downstream and littoral sites. Interestingly, this observation is contrasted by results obtained with *P. flesus*, potentially providing valuable insights to refine the assessment of environmental quality. This difference can be explained by the distinct ecological traits of *P. flesus*, which need to be caged in another environmental compartment—directly in contact with the sediment (Borcier et al., 2019; Diop et al., 2022), unlike *M. edulis* and *P. serratus*, which need to be caged in the water column (Erraud et al., 2018a; Hagger et al., 2008). Moreover, these species do not occupy the same trophic level. In

consequence, they may not be exposed to the same bioavailable fraction of contaminants.

In continental and transitional water sites (SE1-9, OR1-4, VI1-2), a degradation of the environmental quality, as determined by the immunotoxic biomarker, was generally observed across all concerned species between 2020 and 2021 (*M. edulis*, *P. serratus*, and *P. flesus* not considered as they were deployed in only one transitional water site each, Table 6). It is particularly noteworthy that for *D. polymorpha*, all sites with an "no effect" level in 2020 were downgraded in 2021. In the other sites, we did not determine a modification in the environmental quality between 2020 and 2021, except at SE7, which experienced an improvement. However, the opposite was determined for SE7 using *G. aculeatus*. The same observation can be made for *M. leucophaeata*. With that species, 60% of sites had a worse quality in 2021 compared to 2020, and 40% remained at the same level ("effect" or "strong effect"). Interestingly, this degradation trend assessed by the immunotoxic biomarker can be mitigated by the evaluation using *G. fossarum*. In fact, even though 42.9% of sites were downgraded between 2020 and 2021, we observed an improvement in environmental quality also in 42.9% of sites (including SE1 and OR1 for which a degradation was determined using both mollusk species). It confirms the usefulness of deploying different species, which may not respond to the contamination exposure in the same manner.

3.2.4. Comparison with WFD assessment

The biomarker analysis outcomes were compared to the chemical and ecological assessments determined under the Water Framework Directive (WFD). This comparison aimed to determine whether our findings aligned with regulatory environmental assessments or if they provided complementary information.

As shown in Table 7, the overall analysis in 2020 of the biomarker results indicated a "no effect" level at CO sites and an "effect" level at OR4 and VI2. In 2021, a "no effect" level was observed at CO1 and CO3-5, while an "effect" level was determined at OR4 and CO2. For all other sites, a "strong effect" level was consistently observed in both 2020 and 2021, accounting for 70.8% of sites in 2020 and 75% in 2021.

First, a strong coherence between the WFD statuses and the biomarker outcomes was observed. Among the sites which was aligned fort both the WFD chemical and ecological status, the biomarker analyses performed in 2020 and 2021 confirmed the status in 78.6% of cases (i.e., 11 sites for a total of 14: SE3, SE6, SE7, SE9, SE11, OR1, OR2, VI1, VI2, VI3, and CO5, Table 7). These sites did not meet the criteria for good status, with the exception of CO5. Regarding SE8, both chemical and ecological assessments indicated good environmental status, which contrast with the results of the biomarker analysis in 2020 and 2021. This site was located within the WFD surface water body FRHR261 (Eure River at its confluence with the Seine River), the latter was selected as the reference station. However, it is worth noting that the exact location of the WFD sampling was notably upstream of SE8 (data not shown), which may also be subject to tidal influences. In consequence, SE8 could be integrated to the WFD surface water body FRHT01 (upper Seine estuary as SE9). In this case, the results of the biomarker evaluation were more consistent with the WFD outcomes (fail to achieve a good status). At CO1, the biomarker-based methodology indicated a "no effect" level, which was in line with the majority of CO sites (2020 and 2021). However, these assessments did not align with WFD approaches. In terms of the chemical aspect, CO1 did not achieve a good status due to the presence of compounds classified as ubiquitous, persistent, bio-accumulative, and toxic (uPBT), as indicated in Table 1: PCB 118. The exposure to this compound among present contaminants or their bioavailable fraction was not associated with the genotoxic and immunotoxic responses under studied, which were not specific to this particular contaminant. It is possible that the substances did not affect the biological responses investigated in this work or that they were not bioavailable for the concerned species. The use of additional biomarkers to encompass a wide range of biological functions could be

Table 7

Quality status defined according to the biomarker analyses applying the “one out-all out” principle and WFD chemical and ecological status (2022) associated to each study site.

Site ID	2020			2021			WFD status	
	PE	DD	Global	PE	DD	Global	Chem	Eco
SE1								
SE2								
SE3								
SE4								
SE5								
SE6								
SE7								
SE8								
SE9								
SE10								
SE11								
SE12								
OR1								
OR2								
OR3								
OR4								
OR5								
VI1								
VI2								
VI3								
CO1		NA						
CO2								
CO3								
CO4								
CO5								

No effect

Effect

Strong effect

Cage loss

Good

Moderate

Bad

PE: Phagocytosis efficiency, DD: DNA damages, NA: Not analyzed.

recommended, especially since a moderate ecological status was determined for CO1. Worldwide, the inclusion of uPBT compounds importantly contributes to the failure to achieve a good status (Zacharias et al., 2020). In our study, they were detected in 55% of the WFD surface water bodies. The use of biomarkers in this context offers an alternative to evaluations using contamination thresholds and addresses their drawbacks by assessing the toxicity of bioavailable compounds present in the environment. For CO2, the WFD assessments determined good status in contrary to the biomarker results only in 2021. The shift from a “no effect” (2020) to an “effect” level (2021) using our methodology can be attributed to the fact that biomarkers act as early warning systems compared to the assessment carried at larger biological scales, such as communities or populations (Wernersson et al., 2015). It was also worth considering the possibility of an adaptation by local populations. However, a more insightful study would be required to draw such conclusions.

Then, it appeared that the chemical and ecological statuses provided contradictory quality assessments in 8 sites (i.e., SE1, SE2, SE4, SE5, SE12, OR5, CO3, and CO4, Table 7). In such cases, biomarker analyses

provided new information, offering potential explanations for the discrepancies. At SE1, while the chemical status was good, the opposite result was obtained with the ecological and biomarker analyses. This can be explained by the fact that biological tools provide a more integrative analysis, considering factors such as cocktail effects and contaminant bioavailability, compared to the conventional chemical assessment based on a priority list of compounds. At CO4, a similar disparity between the chemical and ecological statuses was observed, however, biomarker results indicated a “no effect” level suggesting a potential influence of other stresses besides chemical contaminants on the environment. Concerning SE2, SE4, SE5, SE12, and OR5, the ecological status was good, contrary to the results of chemical and biomarker analyses. As previously mentioned for CO2, biomarkers could act as early warning systems or maybe there is adaptation of local populations. At last, CO3 exhibited good status with the biomarker and ecological analyses but a degraded one with the chemical evaluation. Similarly to CO1, a hypothesis that needs verification is that the compounds known to be present (uPBT: PCB 118 and tributyltin, Table 1) might not be bioavailable for the caged species or may not induce effects on the

analyzed parameters.

4. What improvement levers for the use of biomarkers in the study of the continuum?

This study has demonstrated the feasibility of defining environmental quality using biomarkers. It has proved that this methodology can be applied on a large geographic scale, suggesting its potential incorporation into regulatory monitoring programs. One critical factor for the success of this continuum study project, based on biomarker analysis, was the establishment of reference values and thresholds. This paper does not aim to propose a universal method to define them, as it was based on existing statistical methodologies designed by experts of the various model species. Here we showed the strong interest to obtain these values and to use it in a biomonitoring context. Reference values and thresholds permitted us to consider the natural variability of biomarkers and the influence of confounding factors. However, their accuracy depends on the quality and quantity available data. In this study, certain species, such as *G. fossarum*, *D. polymorpha* or *G. aculeatus*, benefited from a strong hindsight that directly influenced the accuracy of the site grading. We strongly encourage the study of biomarker functioning under realistic conditions (i.e., *in situ*) to generate data that can enhance or establish rigorous interpretation grids.

One limitation of this work was to study only two biomarkers. There is a real interest to include more biomarkers into the program to encompass a broader range of contaminant modes of action. However, one of the difficulties remains the availability of consensual approaches that would enable interspecific comparisons and thus to cover an entire continuum. In this regard, high-throughput omics-based technologies appear to offer good candidates offering the possibility to study different modes of action related to physiological functions through a unique analysis and using a common method across different species (Bahamonde et al., 2016; Espeyte et al., 2023; Gouveia et al., 2019; Leprêtre et al., 2023). The development of novel biomarkers using these technologies is a topical focus. For instance, analyzing the screening of *G. fossarum* proteomes, coupled with data from field exposure, enabled the identification of protein modulations associated with environmental pressures (Leprêtre et al., 2022b). In order to propose tools for use in biomonitoring, new methodologies are being developed and proposed with the aim to reduce the number of samplings, as well as the quantity and cost of analyses. In a recent article, Faugere et al. (2023) introduced a multi-omics assessment that allows the screening of proteins, metabolites, and lipids and successfully applied it to a unique *G. fossarum* sample. This kind of approach is of significant interest and provide valuable perspectives in the context of biomarker studies involving multiple species.

The inclusion of several biomarkers in the analyses raises the question of how integrate their responses. The “one out-all out” strategy was implemented in the WFD to ensure a conservative and standardized environmental assessment. This precautionary principle has faced criticism for potentially underestimating the number of sites with a good status (Moe et al., 2015; Prato et al., 2014). In the present study, we have chosen to apply it, and, even with only two biomarkers, it also tended to downgrade sites. When the phagocytosis efficiency and the DNA damage responses were interpreted together, all sites failed to achieve good status, with the exception of CO1-5 sites in 2020 and CO1 and CO3-5 in 2021 (Table 7). The development and validation of the methodology will inevitably involve an increase in the number of biomarkers analyzed to cover a wider range of effects, to provide a more accurate assessment, and potentially a comprehensive health index for the exposed organisms. In this context, the issue caused by the “one out-all out” strategy becomes more critical, as the likelihood of downgrading an environment would be positively correlated with the number of parameters studied. With the growing number of biomarkers in the methodology, the question of integration of results should also be considered. In this context, the Integrated Biomarker Responses version

2 (IBRv2) was developed (Sanchez et al., 2013) and recently modified in order to determine a score from the biomarker responses using reference and threshold values instead of a reference site (IBR-T, Catteau et al., 2023; Leprêtre et al., 2022a). This approach considers significant variations in several biomarker responses and is intended to be a robust method in the biomonitoring field. Future challenges will involve the ability to assign different weights to biomarkers according to their respective physiological and ecological relevance (i.e., their ability to predict effects for populations or communities).

5. Conclusion

The use of biomarkers in water quality assessment has been shown to be relevant for defining a quality status, prioritizing sites for remediation actions, and evaluating the improvements linked to regulatory decisions. However, employing biomarkers for studying various types of environment along continua poses challenges. The present study proposed and, above all, implemented a consistent methodology across species and sites including the characterization of the technical specificities, analysis, and interpretation of results. The methodology was successfully applied during two campaign periods, along different continua. It allowed us to characterize an environmental status based on biological effects for each site, that can be easily useable by stakeholders and other water protection actors. The results confirmed the trends of the chemical and ecological assessment of WFD, when the two statuses were similar. Additionally, they provided additional insights when the WFD statuses contradicted each other.

However, this work remains a pilot test which should serve as a basis for future studies. The challenge now is to repeat this type of deployment in order to improve hindsight. Indeed, there is a need to generate large datasets in order to (1) establish robust reference values and thresholds (whatever the species or biomarker considered), and (2) better define the links and complementarity between biomarker outputs and chemical and ecological indices. In this context, collaboration with stakeholders and water protection actors is necessary to establish scopes and strategies (e.g., regulatory framework or otherwise, large-scale surveys, or targeted impact studies) for the optimal use of biomarkers approaches in monitoring programs.

CRedit authorship contribution statement

Sylvain Slaby: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Alain Geffard:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Cédric Fisson:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Matthieu Bonnevalle-Normand:** Visualization, Formal analysis, Data curation. **Anne-Sophie Allonier-Fernandes:** Project administration. **Rachid Amara:** Resources, Methodology, Investigation. **Anne Bado-Nilles:** Writing – review & editing, Resources, Methodology, Investigation. **Isabelle Bonnard:** Investigation. **Marc Bonnard:** Writing – review & editing, Resources, Methodology, Investigation. **Mayélé Burlion-Giorgi:** Investigation. **Amélie Cant:** Writing – review & editing, Investigation, Formal analysis. **Audrey Catteau:** Writing – review & editing, Investigation, Formal analysis. **Arnaud Chaumot:** Writing – review & editing, Resources, Methodology. **Katherine Costil:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Romain Coulaud:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Laurence Delahaut:** Investigation. **Mamadou Diop:** Investigation, Formal analysis. **Aurélié Duflot:** Investigation. **Olivier Geffard:** Writing – review & editing, Visualization, Resources, Methodology. **Emmanuel Jestin:** Project administration. **Frank Le Foll:** Resources, Methodology. **Antoine Le Guernic:** Investigation,

Formal analysis. **Christelle Lopes:** Writing – review & editing, Resources, Methodology. **Mélissa Palos-Ladeiro:** Writing – review & editing, Resources, Methodology, Investigation. **Quentin Peignot:** Investigation. **Agnès Poret:** Investigation. **Antoine Serpentin:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Gauthier Tremolet:** Investigation. **Cyril Turiès:** Investigation. **Benoît Xuereb:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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