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Research article

Integrative biomarker response - Threshold (IBR-T): Refinement of IBRv2 to consider the reference and threshold values of biomarkers

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ABSTRACT

The Integrated Biomarker Response (IBR) is one of the most used index in biomonitoring, especially the IBRv2 integrating a reference condition. However, some limitations remain for its routine and large-scale use. The IBRv2 is proportional to the total number of biomarkers, is dependent on the nature of biomarkers and considers all biomarkers modulations, even small and biologically non-significant. In addition, IBRv2 relies on reference values but the references are often different between each study, making it difficult to compare results between studies and/or campaigns. To overcome these limitations, the present work proposed a new index called IBR-T ("Integrated Biomarker Response – Threshold") which considers the threshold values of biomarkers by limiting the calculation of the IBR value to biomarkers with significant modulations. The IBRv2 and the IBR-T were calculated and compared on four datasets from active biomonitoring campaigns using *Dreissena polymorpha,* a bivalve widely used in freshwater biomonitoring studies. The comparison between indices has demonstrated that the IBR-T presents a better correlation ($0.907 < r^2 < 0.998$) with the percentage of biomarkers significantly modulated than the IBRv2 ($0.002 < r^2 < 0.759$). The IBRv2 could not be equal to 0 ($0.915 <$ intercept < 1.694) because the value was dependent on the total number of biomarkers, whereas the IBR-T reached 0 when no biomarker was significantly modulated, which appears more biologically relevant. The final ranking of sites was different between the two index and the IBR-T ranking tends to be more ecologically relevant that the IBRv2 ranking. This IBR-T have shown an undeniable interest for biomonitoring and could be used by environmental managers to simplify the interpretation of large datasets, directly interpret the contamination status of the site, use it to decision-making, and finally to easily communicate the results of biomonitoring studies to the general public.

1. Introduction

Many tools have been developed to assess the effects of contamination on the health of organisms. Among them, biomarkers provide an early warning of a potential alteration of the ecosystem health status (Milinkovitch et al., 2019). A multibiomarker approach is needed to ensure a good understanding of this pressure, leading to the production of large datasets. One of the challenges for environmental monitoring is to integrate all these data within an index to easily interpret them and compare individuals, populations, sites, seasons, and even generations at large geographical and temporal scales (Broeg and Lehtonen, 2006). In short, the index should be as independent as possible from biotic (*i.e.*,

Abbreviations: IBR, Integrated Biomarker Response; IBR-T, Integrated Biomarker Response - Threshold.

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age, size, sex of organisms) and abiotic (*i.e.*, physicochemical parameters) variations.

Among all the biomarker indexes developed in the last twenty years, the most used index is the "Integrated Biomarker Response" (IBR) index developed on the blue mussel (*Mytilus edulis*) by Beliaeff and Burgeot (2002) and on the European flounder (*Platichthys flesus*) by the BIOMAR program. Briefly, the biomarker responses are normalized and represented on a radar chart (one biomarker per branch). The area of the resulting geometric shape constitutes the final IBR index of the site. The greater the IBR index, the more modulated the biomarkers, the more impacted the site. This graphical representation easily shows biomarker and environmental quality modulations among sites, and makes it easier to interpret the results. Devin et al. (2014) improved IBR calculation considering all possible permutations of the biomarkers in the radar chart and then among sites by comparing the distribution of these multiple IBR values (depending on the number of biomarkers) thanks to statistical analyses. At the same time, Sanchez et al. (2013) proposed to no longer consider a geographical area but the distance from a reference condition for each biomarker, which can be positive or negative following induction and inhibition, respectively. The sum of these absolute distances constitutes the final IBRv2 index. IBRv2 was successfully used in the field (Cao et al., 2018; Catteau et al., 2020, 2022) and in laboratory studies (Beghin et al., 2021). Marigómez et al. (2013) and Pires et al. (2021) compared the efficiency of different indices. They highlighted the interest of the IBR index notably because it is only calculated using mathematical transformations whereas the other indexes require significant mechanistic knowledge, especially to establish the classes of importance.

Nevertheless, some points remain to be improved, such as characterizing the natural variability of biomarkers and including it in IBR calculations (Marigómez et al., 2013). Biomarker levels can vary independently from chemical contamination, and can have a strong impact on the reference value and in turn on the final score of the index. The use of independent reference values based on the range of natural variability of biomarkers offers the possibility to distinguish natural modulation from significant modulation linked with a toxic impact (Chaumot et al., 2015). Moreover, it currently appears difficult to find reference sites free from any anthropogenic pressure for field studies. This supports the trend towards defining generic reference values. These values can be defined empirically, based on annual and spatial variability within stations (Besse et al., 2013; Leprêtre et al., 2022), or with modeling tools when environmental influences are large (Geffard et al., 2010; Coulaud et al., 2011; Marchand et al., 2019). They consider the variations of the biomarkers linked to the physicochemical parameters and overcome the influence of the abiotic confounding factors in the final interpretation. Threshold values of real induction and/or inhibition can be extrapolated, represented by the higher and/or lower reference range bounds. The use of these reference and threshold values would make it possible to calculate indices that are more representative of the real toxic impact of the environment on organisms. Associated with these reference ranges, the active biomonitoring approach using transplanted organisms coming from a reference site limits the variability linked with certain biotic factors (*i.e.*, the age, size and sex of individuals exposed) and exposure conditions (*i.e.*, exposure duration, localization) by controlling these parameters. In addition, the active approach bypasses the mechanisms of acclimatization or adaptation to chronic contamination that may appear in native populations and lead to modulated biomarker levels. These active approaches have been deployed with several aquatic model species, such as fish, amphipods or bivalves (Besse et al., 2013; Catteau et al., 2022; Hani et al., 2021; Lacaze et al., 2011; Le Guernic et al., 2016) and appear to be the most promising strategy for processing biomonitoring studies. One other limitation of the IBR index is directly related to the calculation process. Whatever the IBR index, the final score is obtained by summing up the parameters derived from the biomarker values. This makes the final score closely dependent on the number of biomarkers considered. Some authors have proposed to replace the sum by the mean value (Broeg and Lehtonen, 2006). With this slight modification, these authors compared sites or conditions even if one biomarker was missing, making IBRv2 less dependent on the total number of measured biomarkers.

In this context, the present work aimed to improve the original IBRv2 calculation method by proposing a new index called "Integrative Biomarker Response - Threshold" (IBR-T). We first formalized the use of the mean of the absolute distances instead of the sum by comparing IBRv2 calculated with each strategy. Then, we introduced reference values to avoid the need for a reference site, and finally threshold values were included in IBRv2 calculation to propose this new IBR-T. We compared the results and the final rankings obtained with each index. This work was conducted using datasets from several active biomonitoring programs of the zebra mussel (*Dreissena polymorpha*).

2. Materials and methods

2.1. Datasets

The datasets of three different biomonitoring programs using the zebra mussel (*D. polymorpha*) were used to meet the aims of this study. This mussel is a bivalve with bioecological traits that make it particularly relevant for biomonitoring. Its high filtration rate and high bioaccumulation potential make it an extensively used indicator for field biomonitoring studies and laboratory experiments (Evariste et al., 2018; Kerambrun et al., 2016a, 2016b; Le Guernic et al., 2022). The mussels used for the caging experiments came from the Lac du Der Chantecoq (51,290 Giffaumont-Champaubert, France, N 48°33′35″; E 4°45′11″) and were kept several weeks in aerated tanks in darkness with a controlled temperature. Two weeks before experiments, adult mussels (>20 mm) were randomly

Table 1
Description of the biomonitoring of *Dreissena polymorpha* in fall (from 2018 to 2020). The distribution map in available in Supp. Data 1.

Experiment	Date of caging		Localization	
	From	To		
DIADeM	3/10/2018	29/11/2018	Upstream/Downstream of WWTPs in the Meuse Watershed	
			Namur upstream:	Namur Downstream:
			NAM_Up	NAM_Down
			Charleroi upstream:	Charleroi Downstream:
			CHR_Up	CHR_Down
			Charleville-Mézière upstream: CHV_Up	Charleville-Mézière downstream: CHV_Down
MAQUEREAU			Caging in 4 rivers in northern France	
C1	7/11/2019	28/11/2019	The Deule at Courrière: COU	The Scarpe at Brebières: BRE
C2	8/9/2020	29/9/2020	The Sensée at Bouchain: BOU	The Yser at Bmabecque: BAM
BIOSURVEILLANCE	4/10/2020	28/10/2020	Caging in the Seine and two tributaries (The Vire and the Orne)	
			La Touques: TOU	Soissons: SOI
			Martot-Les-Damps: MLD	Triel: TRI
			Fontaine-Le-Port: FLP	La Meauffe: MEA
			Pont-Sur-Yonne: PSY	Melz-sur-Seine: MSS
			Verberie: VER	Petit-Couronne: PCO

placed in 2-mm-mesh polyethylene cages (7 × 7 × 14 cm) (from 180 to 200 mussels per cage according to the campaign) and the cage were maintained in the aerated tanks until the beginning of *in situ* exposures. During this acclimation time, mussels were fed *as libitum* twice a week with a mix (50/50) of two commercial solutions of microorganisms adapted for shellfish diet, including Nannochloropsis, *Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana* (Nanno 3600 and Shellfish Diet 1800; Planktovie, France). For each experiment, the caging was carried out in autumn during the resting period of *Dreissena polymorpha* and mussels were caged for 21 days (one cage per site studied). The different datasets are described in Table 1 and the distribution map of sites are presented in Supp. Data 1.

The caging experiment led within the framework of the Interreg DIADeM program aimed to assess the impact of several wastewater treatment plants (WWTPs) on the water quality in the Meuse watershed (across the Franco-Belgian border). Mussels were caged upstream and downstream of three WWTPs in 2018 (Namur, Charleroi and Charleville-Mézières) (Hani et al., 2021). The MAQUEREAU program (funded by the French Water Agency of Artois-Picardie) (Cant et al., 2022) aimed to assess the quality of several rivers in northern France, particularly impacted by agricultural contamination and metal trace elements. Two campaigns were carried out during the program (campaign 1 = C1 in 2019; campaign 2 = C2 in 2020). Finally, the BIOSURVEILLANCE program (funded by the French Water Agency of Seine-Normandie) aimed to assess the quality of the water in the Seine River and its tributaries. This monitoring campaign was led in autumn 2020.

For each caging experiment, a large set of biomarkers were measured in caged mussels to characterize their energy metabolism (n = 6 b y studied site for each biomarker), immunity capacity (n = 10), detoxification capacity (n = 9) and DNA damage (n = 10). These biomarkers were chosen to be representative to the most important physiological functions in living organisms. The modulations and the responses of these biomarkers are well known on *Dreissena polymorpha*, since they have been used for several years in many studies (Evariste et al., 2017; Joachim et al., 2021; Palais et al., 2011, 2012). The detailed procedures for biomarkers measurements can be found in previous recent works (Catteau et al., 2022; Hani et al., 2021). The biomarkers measured, their abbreviations and units, organs in which the measurements were made and the original publications for the protocols are summarized in Supp. Data 2.

2.2. Index calculation

2.2.1. Determination of reference and threshold values

The determination of biomarker thresholds for zebra mussel is described in details in Leprêtre et al. (2022), following the method initially developed by Besse et al. (2013) for bioaccumulation data. This methodology assumes that the natural variability of a biomarker for organisms living in a "healthy" unpolluted environment would be characterized by a Gaussian distribution. Biomarker threshold and reference values were determined using mussel biomarker datasets obtained from the EQUAL project.

Briefly, all the data measured in mussels exposed at different sites were sorted from the lowest to the highest value for a given substance. A Shapiro-Wilk test was applied on the overall dataset to test if the distribution was Gaussian. If not, the highest value was removed from the dataset and normality was tested again. This iterative process was stopped when the dataset was distributed according to a Gaussian law. The reference and threshold values were defined from the resulting Gaussian distribution as the 50th and the 95th percentiles, respectively. The approach was improved by adding bootstrapping (Leprêtre et al., 2022), and adapted to biomarkers possibly inhibited by a pollutant (for which an inhibition threshold should be defined) and biomarkers possibly induced and inhibited (for which both induction and inhibition thresholds should be defined) (Fig. 1).

For biomarkers that are only induced, the methodology used to define an induction threshold was as follows: (i) a random sample of n = 30 values was taken from the complete initial dataset, sorted from the lowest to the highest value, and the above-described approach (Besse et al., 2013) was applied to obtain an induction threshold value; (ii) this procedure was repeated z = 1000 times to deduce z threshold values from the z data samples; (iii) *in fine*, the estimated reference and induction threshold values corresponded to the means of the z reference and threshold values, respectively.

For biomarkers that are only inhibited, the methodology used to define an inhibition threshold was as follows: (i) a random sample of n = 30 values was taken from the complete initial dataset, and sorted from the highest to the lowest value. Then, a Shapiro-Wilk test was performed, and the lowest value was removed from the data if the Gaussian distribution was not verified. Normality was tested again, and the iterative process was run until a Gaussian distribution was obtained, from which an inhibition threshold was defined as the 95th percentile; (ii) as before, this procedure was repeated z = 1000 times to deduce z inhibition threshold values from the z data samples; (iii) *in fine*, the retained estimated reference and inhibition threshold values corresponded to the means of the z threshold values.

For biomarkers possibly induced and inhibited, we used the two previously described methods to separately estimate the inhibition and induction thresholds. The issue here was to define a methodology to separate the "inhibition dataset" from the "induction" dataset. For that, we sorted the whole dataset from the highest to the lowest value (as represented in Fig. 1), and divided it into two sub-datasets: (i) the induction dataset, from which the induction threshold was estimated, was defined by the q = 65% highest values (*i.e.*, q = 65 first percentiles); and (ii) the inhibition dataset, from which the inhibition threshold was estimated, was defined by the q = 65% lowest values (*i.e.*, q = 65 last percentiles). In this way, the data representing natural variability (*i.e.*,

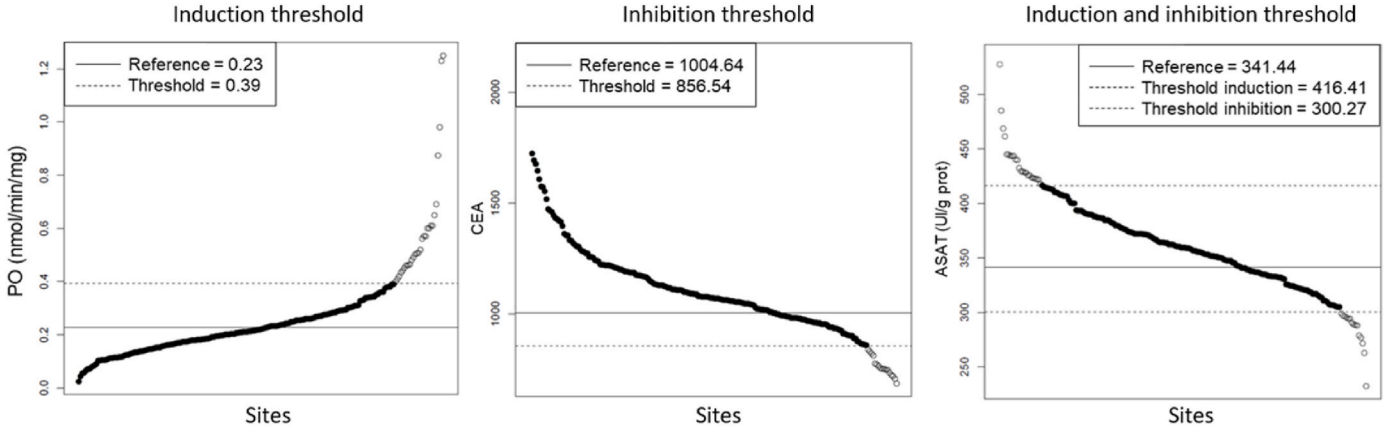


Fig. 1. Determination of reference and threshold values for biomarkers that can only be induced (e.g., PO), inhibited (e.g., CEA) or both induced or inhibited (e.g., ASAT).

basal modulation) were in both datasets. Then, the above-described methodology for determining an induction threshold was applied to the induction dataset, and the one used to determine an inhibition threshold was applied to the inhibition dataset.

We tested different values for n, z and q, and those retained for all datasets ensured the robustness of the results. The q value more particularly guaranteed that the reference value estimated from the induction dataset was the same as the one estimated from the inhibition dataset. All the calculations were performed with R software version 3.3.2 (R Development Core Team, 2014). The reference and threshold values were defined for all the biomarkers of this study and are presented in Supp. Data 4.

2.2.2. IBRv2 and IBR-T calculation

The procedure for calculating IBRv2 and IBR-T is described in Fig. 2. It is based on the method developed by Sanchez et al. (2013). Indexes were calculated independently for each experiment. Based on the value of a biomarker j ($j = 1 \ldots J$) for an individual i ($i = 1 \ldots n$) at site k ($k = 1. m$; $m = 4$ for the MAQUEREAU dataset; $m = 6$ for the DIADeM dataset, $m = 10$ for the BIOSURVEILLANCE dataset) called $X_{j,i,k}$, the IBRv2 index was calculated as follows.

1. Calculation of the mean of each biomarker for each site (X

$$\overline{X}_{j,k} = \frac{1}{n}\sum_{i=1}^{n} X_{j,i,k} \quad (1)$$

2. Transformation into an induction/inhibition index (Y) relative to the reference value of the given biomarker ($X0_j$):

$$Y_{j,k} = log\left(\frac{\overline{X}_{j,k}}{X0_j}\right) \quad (2)$$

3. Calculation of an index of deviation from the reference:

$$A_{j,k} = \frac{Y_{j,k}}{\sigma_{Y_j}} \quad (3)$$

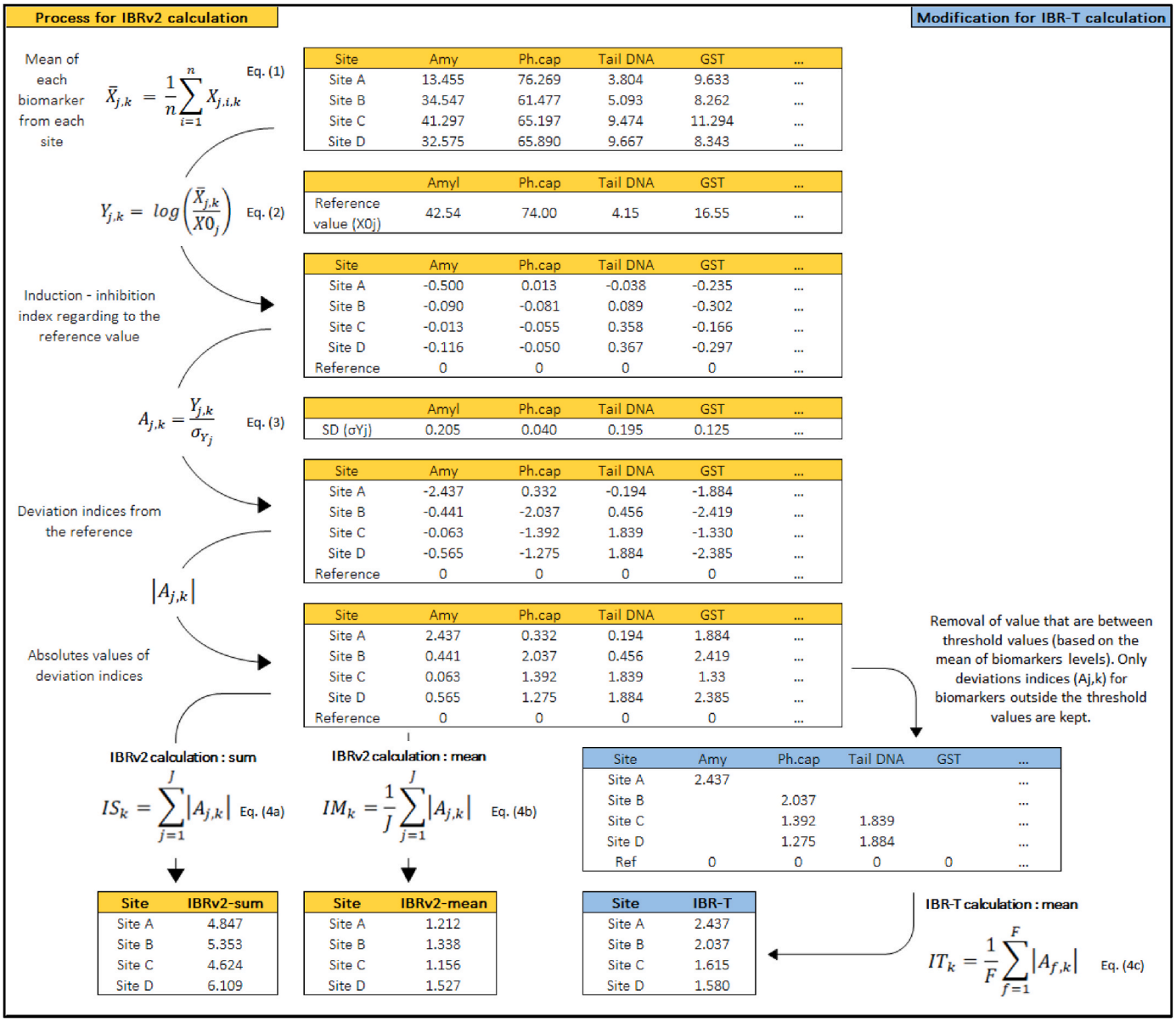


Fig. 2. Diagram of the procedure for IBRv2-mean, IBRv2-sum and IBR-T calculations.

With σ_{Yj} the standard deviation of the $Y_{j,k}$ values among the m sites. This equation is a simplification of the calculation presented in Sanchez et al. (2013). The mathematical demonstration of this simplification is available in Supp. Data 3.

4. Calculation of the IBRv2 value at site k. According to Sanchez et al. (2013), the final IBRv2 corresponds to the sum of the absolute values of the deviation indices of all biomarkers.

$$IS_k = \sum_{j=1}^{J} |A_{j,k}| \tag{4a}$$

Thus, we obtained an IBRv2-sum for each site and each biomonitoring experiment. We also calculated an IBRv2-mean by averaging the absolute values of the deviation indices:

$$IM_k = \frac{1}{J} \sum_{j=1}^{J} |A_{j,k}| \tag{4b}$$

To obtain IBR-T, the same first three steps were applied to estimate the deviation indices ($A_{j,k}$). Once deviation indices were calculated, we referred to the mean of each biomarker response in each site ($X_{j,k}$) to keep only deviation indices for the sites that presented a mean value higher or lower than the threshold previously defined for each biomarker (Supp. Data 4). Only these deviation indices were used to calculate IBR-T. In addition, the mean of the deviation indices was preferred to the sum in order to normalize the values according to the number of biomarkers considered and be able to compare indices among sites even if the numbers of biomarkers measured in each site were different. The final formula for IBR-T was:

$$IT_k = \frac{1}{F} \sum_{f=1}^{F} |A_{f,k}| \tag{4c}$$

With f the number of biomarkers out of their respective threshold values, and $A_{f,k}$ the deviation indices for biomarkers lower or higher than their threshold values. The calculation of IBRv2, IBR-T and the starplots (Fig. 3) were realised with Microsoft Excel.

3. Results and discussion

3.1. Mean values allow comparing studies

The final IBRv2 score calculated with the sums (IBRv2-sum) and the means (IBRv2-mean) of the absolute values of the index deviations from the reference were compared (Table 2). The IBRv2-sum indices ranged between 16.70 and 30.68 for all studies. In comparison, the IBRv2-mean indices ranged between 1.193 and 2.192 (Table 2). The use of the mean instead of the sum did not change the ranking of the sites within each study or the ratio between the most and the least impacted sites (ratio = 1.837 for both strategies). However, IBRv2-mean smoothed out the final result. Because IBRv2-sum was closely linked with the number of biomarkers included in the calculation, the higher the number of biomarkers, the higher IBRv2-sum, until it could reach a very high value. In contrast, with IBRv2-mean the sum of absolute distances was divided by the total number of biomarkers, and this decreased their importance in the final result. Thus, IBRv2-mean allows comparing indices among studies and years. When using the sum of biomarkers, it is essential to have strictly identical biomarkers among studies in terms of number and nature, biomonitoring campaigns, and years. Adding or deleting one or

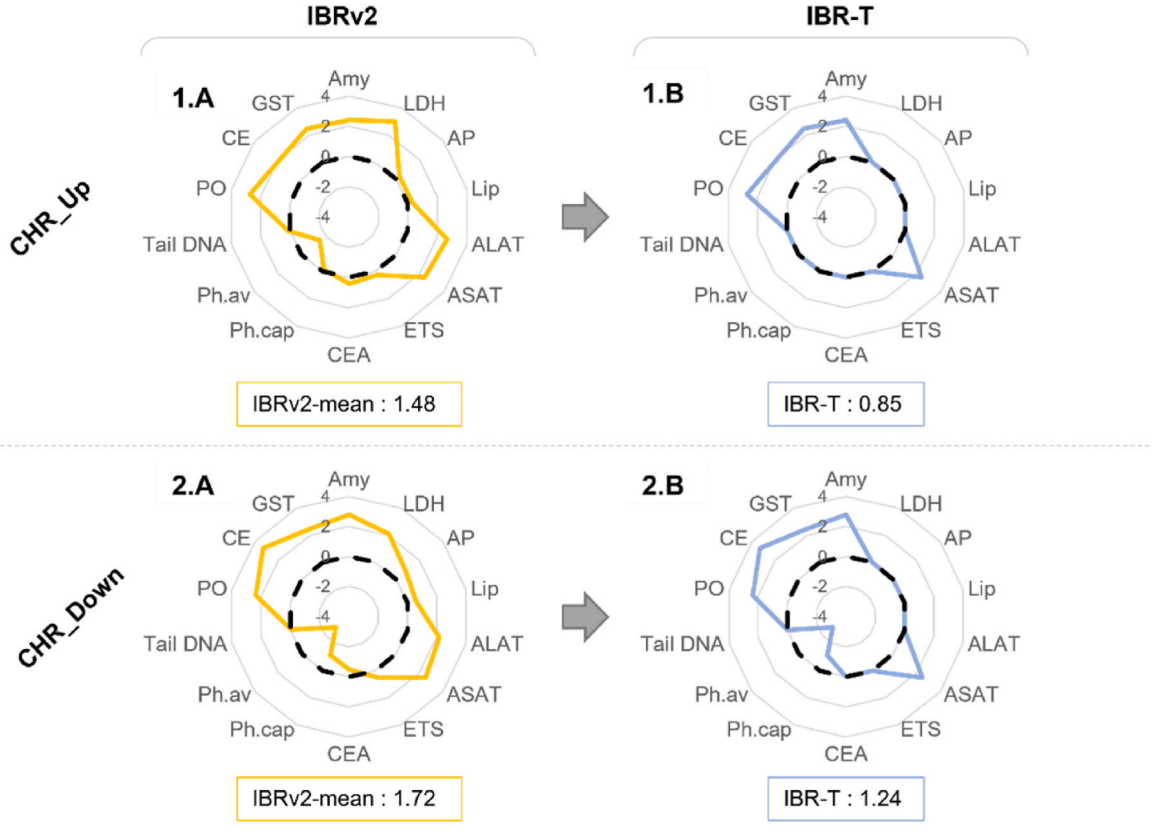
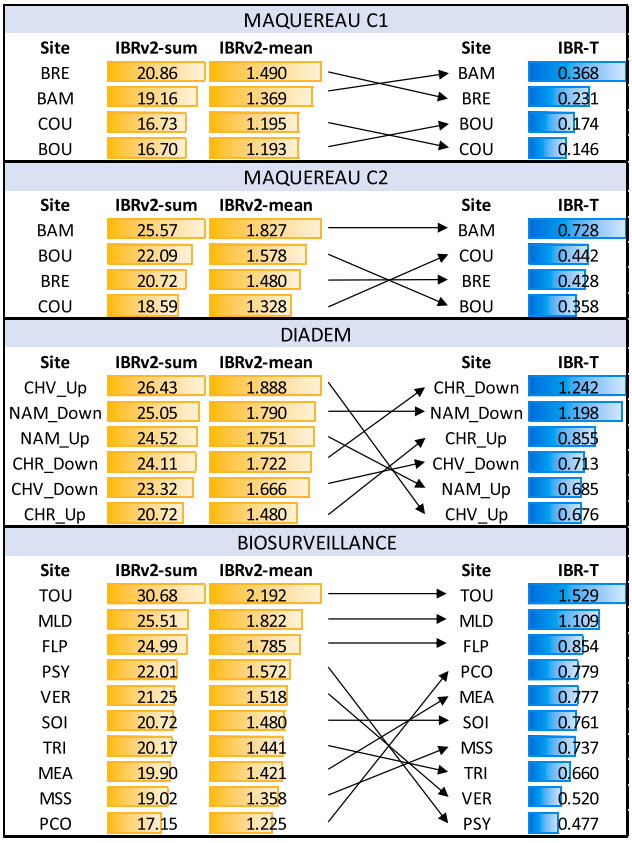


Fig. 3. Starplots of IBRv2 (orange, 1. A and 2. A) and IBR-T (blue, 1. B and 2. B) for the two Charleroi upstream (1. A and 1. B) and Charleroi downstream (2. A and 2. B) sites in the DIADeM caging experiment. The reference condition is represented by the black dotted curve. Amy: amylase; LDH: lactate deshydrogenase; AP: acid phosphatase; Lip: lipase; ALAT: alanine aminotransferase; ASAT: aspartame aminotransferase; ETS: electron transport system activity; CEA: cellular energy allocation; Ph. cap: phagocytic capacity; Ph. av: phagocytic avidity; Tail DNA: DNA damages; PO: phenoloxidase; CE: carboxylesterase; GST: glutathione-S-transferase.

Table 2
IBRv2-sum, IBRv2-mean and IBR-T scores in the different caging experiments. Sites are listed in ascending order of IBR.



more biomarkers is not possible, although it is one of the main purposes of this index. It would be ideal to have a constant number of biomarkers representing several biological functions (Devin et al., 2014), but reality is different because of experimental hazards (loss of samples, mortality) and certain limitations (lack of data, equipment, etc.). When using the mean instead of the sum, the importance of the number of biomarkers is lower, and the comparison of IBR from different studies, campaigns and years seems easier. IBR can be calculated even if one or more biomarkers are missing. However, although the total number of biomarkers is less important in IBRv2-mean than in IBRv2-sum, the number of biomarkers assessed for each physiological function remains essential. If a physiological function characterized by a high number of biomarkers is strongly impacted on a site, the final IBRv2 will increase proportionally (based on the mean as well as the sum). On the contrary, if this function is characterized by a small number of biomarkers, the impact on the final IBRv2 index may be negligible. This underlines the importance of a balanced number of biomarkers to assess each physiological function.

3.2. *Reference values allow to dispense with reference sites*

The reference values as well as the inhibition and induction thresholds were calculated on a large number of data recovered during various *in situ* caging experiments using the same population of zebra mussels from the Lac du Der Chantecoq (Leprêtre et al., 2022). Working with individuals coming from the same mussel population with the same exposure history supports the estimated reference and threshold values, even if individuals come from different generations. The reference values allow to dispense having a "reference" site, with the lowest possible anthropogenic impact. This has several advantages. First, finding a "reference" site free of any contamination is an illusion nowadays (Tlili et al., 2013). Most "reference" sites are chosen because they are far from a source of contamination. Sites can be also chosen according to an upstream-downstream approach around a source of contamination. However, these "reference" sites may also be impacted by the same source of contamination or another one and represent the downstream point of a new contaminant. Broeg and Lehtonen (2006) observed an increase of the IBR calculated on a "reference" site between two campaigns. Moreover, Tsangaris et al. (2011) measured higher metal contents at one or more of their "reference" sites compared to other sites. In addition, depending on the criteria retained to define a reference site, the same site may be selected as a contaminated site during one study and as a reference one for another. Knowing that geographical variability influences biological responses (Pain-Devin et al., 2014; González-Fernández et al., 2015), the use of a different reference site could overestimate the IBR score when comparing data from separate sites. The use of threshold and reference values calculated considering numerous studies and various sites, contaminated or not, will allow to reduce this overestimation of the IBR-T score considering a greater response variability, and decreasing the importance of geographic variability. Calculating IBR-T by considering reference values avoids having to find "reference" sites for each experiment (Devin et al., 2014). Another advantage is that these reference values include a greater number of values than when a "reference" site is used for each experiment. Therefore, the dataset used for calculating reference and threshold values can include individual seasonal variation due to confounding (abiotic and biotic) factors, which is closer to environmental reality (Beliaeff and Burgeot, 2002). By determining reference and threshold values using a mathematical statistical method based on a large dataset, one removes the historical scientific obstacle in biomonitoring represented by the reference site. Although building up the dataset used to calculate these reference and threshold values can prove tedious, this method subsequently saves time and environmental increases the reliability of the diagnosis.

3.3. *Differences in ranking sites between IBRv2 and IBR-T in biomonitoring studies*

Table 2 specifies and organizes IBRv2 and IBR-T scores according to the fourth caging experiment. Arrows highlight classification differences between IBRv2 and IBR-T at each site. Only seven sites out of 24 retained their ranking whatever the calculated IBR; therefore, the ranking changed for most of them (Table 2). In the MAQUEREAU C2 and BIOSURVEILLANCE campaigns, the sites with the highest IBR scores remained the same regardless of the IBR calculation method, but the other sites did not, and the sites with the highest IBR scores differed in the MAQUEREAU C1 or DIADeM campaigns.

These classification differences were particularly exacerbated in the DIADeM experiment, when mussels were caged upstream and downstream of different WWTPs (Table 2). Logically and according to the known chemical contamination (Catteau et al., 2022), the IBR score of the downstream sites should have been higher than those of the upstream sites. This theoretical classification was found with IBR-T, not with IBRv2. More precisely, the difference between the upstream and downstream sites highly increased according to IBR-T in comparison with IBRv2 for NAM and CHR (Fig. 3). For CHV, the ranking of the upstream and downstream sites was even inverted with IBR-T compared with IBRv2. This new ranking highlights stronger modulations in the downstream site than in the upstream site, which seems more ecologically relevant than the initial ranking with IBRv2. In addition, Charleroi was the biggest of the three WWTPs studied during this campaign, and the CHR_Down site was the one with the highest concentrations in pharmaceutical and domestic activity tracers (Catteau et al., 2022). The IBR-T score ranked this site as the most disturbed one, which IBRv2 did not. Therefore, site classification from an ecotoxicological point of view in the DIADeM caging experiment seemed more relevant according to IBR-T scores than according to IBRv2 scores.

Another example was revealed by the MAQUEREAU campaigns. While the IBRv2 classification completely differed across years, a common trend emerged using IBR-T scores. Whatever the year, IBR-T from BAM site was higher and separated from the other sites (Table 2). Similarly, strong disparities of site classification were reported within the BIOSURVEILLANCE experiment (Table 2). The site with the fourth highest IBR-T score (PCO) ranked last according to IBRv2, and conversely for the PSY site. These differences can lead to major divergences in the interpretation of results during biomonitoring campaigns. Although IBR-T better differentiated the sites and seemed to classify them in an ecotoxicologically relevant way, it is necessary to keep analyzing the differences within each of the biomarkers used to construct this index. The final IBR-T score can indeed provide information on the intensity of the pressure suffered by the organisms and classifies the sites, but it should always be completed by detailed biomarker analyses to improve the understanding of the measured physiological impacts. This tool can be constantly remodeled by adding, modifying and, to a lesser extent, deleting markers within the battery used for its calculation. It could therefore be personalized according to the laboratory and the contamination analyzed - to be studied - as long as various biological functions are included in its calculation. Thus, IBR-T is a synthetic tool complementary to the multibiomarker approach used in environmental biomonitoring.

In addition, IBR-T better discriminated sites than IBRv2 did: a mean ratio of 2.40 was found between the highest and lowest IBR scores for IBR-T, versus 1.42 for IBRv2 (calculated from Table 2). This discriminated or grouped several sites according to their IBR-T score. In the MAQUEREAU campaigns and as mentioned above, BAM site had a higher IBR-T than the other sites every year, not found with IBRv2 scores. In the DIADeM campaign, CHR_Down and NAM_Down sites were grouped according to their close IBR-T scores. In the BIOSURVEILLANCE campaign, four groups were visually formed based on IBR-T values: TOU, MLD, FLP-PCO-MEA–SOI–MSS-TRI, and VER-PSY (Table 2).

In addition to inducing differences in site classification, the nature of IBR calculation modulated the consistency of the percentage of modulated biomarkers (induced and/or inhibited) and the IBR scores (IBRv2 or IBR-T) (Table 3; Supp. Data 6). As expected, a strong correlation was highlighted between the percentage of modulated biomarkers and the IBR-T scores (from 0.907 to 0.998 in the individual caging experiments; 0.882 when these experiments were combined). This correlation was much weaker for IBRv2 (from 0.002 to 0.759; 0.545 when the experiments were combined) (Table 3; Supp. Data 6). This low correlation between the percentage of modulated biomarkers and the IBRv2 values supports the importance given to small variations of the biomarkers that may have little or no biological significance. This was underlined in the DIADeM campaign where the correlation between the modulated biomarkers and IBR-T was much higher than that obtained with IBRv2 (0.915 with IBR-T versus 0.002 with IBRv2) (Table 3; Supp. Data 6). It is also important to note that the y-ordinate tended towards zero for IBR-T (from 0.132 to 0.035), in particular when the number of sites increased (0.013 for all experiments), whereas those of IBRv2 were higher (from 0.915 to 1.694) (Table 3; Supp. Data 6).

Table 3
Summary of the correlation coefficients and intercepts of the trend curves of the correlations between the IBR scores (IBRv2 or IBR-T) and the percentages of modulated biomarkers. The "all" condition groups all caging experiments. The correlation coefficients and intercepts were extracted from Supp. Data 6.

Experiment	Number of sites	IBRv2		IBR-T	
		R^2	Intercept	R^2	Intercept
MAQUEREAU C1	4	0.496	1.125	0.959	0.053
MAQUEREAU C2	4	0.759	0.915	0.998	0.132
DIADeM	6	0.002	1.694	0.915	0.077
BIOSURVEILLANCE	10	0.431	1.038	0.907	0.035
ALL	24	0.545	0.750	0.882	0.013

The main difference in the calculation of IBRv2 and IBR-T is that the distances to the reference value are not considered when the biomarker level does not exceed the threshold(s). Including these induction or inhibition thresholds in IBR construction presents some benefits.

First, the induction and inhibition thresholds reflect the biological reality of the biomarker variations. The former forms of IBR endeavoured to consider each variation of each biomarker compared to the reference condition, whatever the value or the orientation of this variation, whereas IBR-T only considers variations whose level and orientation highlight a real impact of a pressure on the organism under study. The orientation may be unilateral (an induction or an inhibition threshold) or bilateral (an induction threshold and an inhibition threshold) (Supp. Data 4). This orientation depends on the biomarker nature as well as the possible impacts of environmental and anthropogenic pressures exerted on them. DNA damage assessment (genotoxicity or tail DNA in this study) is a good example of a biomarker for which only the induction threshold is calculated. DNA damage increases following exposure to various contaminants (Binelli et al., 2009; Juhel et al., 2007; Vincent-Hubert et al., 2011). Similarly, the decrease in phagocytosis parameters seems more interesting in order to evidence the impact of a contaminant rather than its increase. This is supported by various studies including different types of exposure (Couleau et al., 2012; Evariste et al., 2017; Le Guernic et al., 2020). Induction as well as inhibition of other biomarkers could highlight exposure to xenobiotics. For example, amylase activity can be stimulated or inhibited by contaminants (Amiard-Triquet et al., 2015; Catteau et al., 2022; Kerambrun et al., 2016a; Yan et al., 1996). Thus, the fact that IBR-T takes the biological meaning of modulations into account makes it more representative of environmental reality. Nonetheless, the biological meaning of modulations and the resulting threshold values should be revised for each biomarker and confirmed for each species. In this study, transplanted mussels came from the same population from the Lac du Der Chantecoq. Threshold and reference values should be verified if another population were used. Indeed, biological variations can vary according to geographical variability of sentinel species. Thus, different autochthonous populations can respond differently to a same environmental stress (González-Fernández et al., 2015). This may be due to different genetic characteristics and environmental conditions between populations (Pain-Devin et al., 2014). Nonetheless, Pain-Devin et al. (2014) estimated that IBR calculated on different populations of zebra mussels varied mainly according to environmental conditions (including contamination) than according to their genetic characteristics. This was also shown in *Mytilus* sp. Where the geographical location of individuals had much less effect on several biomarkers than seasonal variations (Benito et al., 2019; Storhaug et al., 2019). These small differences could induce slight modulations of the IBR-T values if the reference and threshold values are not obtained on the same population as that used for biomonitoring. It is therefore necessary to limit these potential biases by knowing the population(s) used and by respecting a common acclimation step before deployment to dissipate their ecological memory (González-Fernández et al., 2015). However, these studies showed the strong importance of the season in the responses of biomarkers. In the zebra mussel, the weight of the season has already been assessed on various biomarkers, whether acetylcholinesterase, glutathione-s-transferase, energy reserves, digestive enzymes, gene expression, etc. (Kerambrun et al., 2016b; Palais et al., 2012). The importance of the season in biological responses is notably linked to many biotic and abiotic factors (reproductive status, temperature, food availability, etc.). The season could then induce strong disparities in the values of the IBR. To limit this, data used to calculate the reference and threshold values, as well as those from the caging experiments described in this study, were all obtained *via* caging carried out in the fall. With the aim to apply the active biomonitoring approach throughout the year, the generic references and the threshold values should thus be established

Table 4
List of the benefits and limitations of the modifications of the IBR index proposed in this study.

Parameter	Advantages	Limitations
Mean instead of sum	Decrease the importance of the biomarker number	
	Possible loss of data without strongly impact the score	
	Allow comparisons between seasons, years and biomonitoring campaigns.	
Reference values	Avoid to have a reference site	Time-consuming method for obtaining data
	Consider individual variability	
	Integrate confounding factors	
	Potential large amount of data	
	Can be refined	
Thresholds	Consider the biological reality of the variations of the biomarkers	Time-consuming method for obtaining data
	No consider small biomarkers variations	Depending on the biomarker nature and species studied
	Potential large amount of data	
	Can be refined	
	Start with an IBR-T score of 0 (when no biomarker are modulated)	
Calculation and results	Great concordance between the percentage of modulated biomarkers and the IBR-T score	
	Y-ordinate tends towards zero	
	Better classification of sites	

for each season.

Secondly, the definition of threshold values for each biomarker as proposed by Leprêtre et al. (2022) allows considering only significantly modulated biomarker responses. The small variations in biomarkers, not exceeding the 95th percentiles obtained from a database respecting the same conditions as those of the three different biomonitoring programs described in this study, were therefore removed from the calculation of the IBR-T. This allowed to consider only biologically significant variations and not to add a weight to this index. Consequently, a site with no impact on the tested biomarkers (no mean value exceeding the induction and/or inhibition threshold(s)) will have an IBR-T score equal to zero. In IBRv2, all biomarker modulations from the reference are considered – even non-significant ones – and increase the final index score (Sanchez et al., 2013). Therefore, IBRv2 can never be equal to zero despite the absence of significant effects. To illustrate this advantage with the present dataset, the biomarkers that did not exceed the threshold values contributed 32.2%–57.3% of the IBRv2 scores of the MAQUEREAU campaigns, 25.5%–50.2% of the IBRv2 scores of the DIADeM campaign, and 6.3%–35.5% of the IBRv2 scores of the BIOSURVEILLANCE campaign (Supp. Data 5). These results again emphasize that small modulations largely contribute to IBRv2 scores.

The final advantage of the use of thresholds in IBR-T calculation is the strong score disparities compared to IBRv2 which can result in a better or at least modified interpretation of biomonitoring program results. Various studies using the first form of IBR (Beliaeff and Burgeot, 2002) or the one revised by Sanchez et al. (2013) have showed a correlation between environmental contamination or bioaccumulation and the score of these indices (Damiens et al., 2007; Lu et al., 2010; Perussolo, 2019; Pytharopoulou et al., 2008). Nonetheless, other studies do not report this correlation, suggesting a possible impact of individual variation, confounding factors, or the number and nature of selected biomarkers (Broeg and Lehtonen, 2006; Catteau et al., 2022; Marigómez et al., 2013; Tsangaris et al., 2011). In this study, the scores obtained in the four caging experiments differed according to IBRv2 or IBR-T calculations. These strong disparities are linked to the biomarkers that did not exceed the threshold values, included in IBRv2 but not in IBR-T. As discussed above, excluding the biomarkers with small variations from the reference range cancels their importance in the calculation of IBR and has a certain influence on the final classification of sites and may have a greater ecotoxicological significance.

4. Conclusion

Present-day new technologies and methods allow acquiring a large amount of data within a short time, but interpreting these datasets remains tricky. Therefore, integrating and summarizing data within a simple index with an easy and transferable calculation method appears as a major challenge in ecotoxicology. The modifications proposed in the present study – (i) using mean distances to the reference value instead of sums of the distances to the reference value, (ii) adding reference values instead of reference sites – and during the calculation of IBR – (iii) deleting biomarkers not exceeding the thresholds – overcome some limitations of the previous forms of IBR. These benefits are summarized in Table 4. This improved index could be used in other environmental biomonitoring programs and on various biological species to prove its effectiveness in synthesizing and integrating large datasets and its correlation with environmental pressures.

Credit author statement

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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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