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1 *Integrative Biomarker Response - Threshold (IBR-T): refinement of IBRv2*
2 *to consider the reference and threshold values of biomarkers.*

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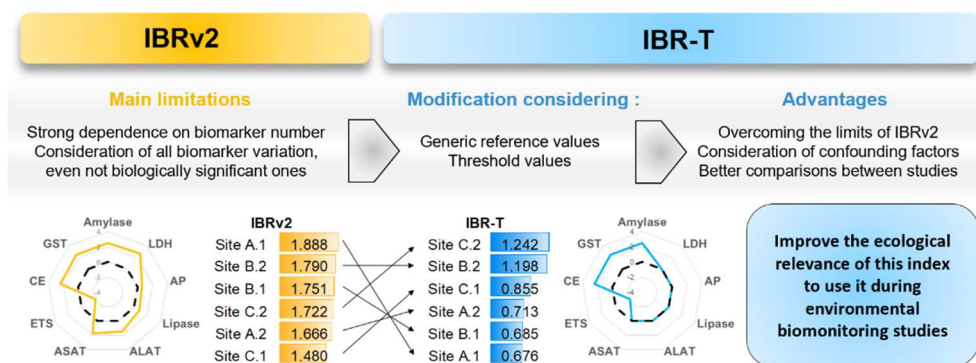
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17 Abstract

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

39

40 Graphical Abstract



41

42

43

44 **Keywords:** *Dreissena polymorpha*; Biomonitoring; Integration; Biomarkers; References; Caging.

45 **Highlights:**

- 46 • A new Integrative biomarker response index – IBR-T – is proposed to improve IBRV2
- 47 • IBR-T integrates reference and threshold values
- 48 • IBR-T improves site classification and includes biological variation of biomarkers
- 49 • IBR-T appears more ecologically relevant and allows comparing studies and years

50 1. Introduction

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

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

77 Nevertheless, some points remain to be improved, such as characterizing the natural variability of
78 biomarkers and including it in IBR calculations (Marigómez et al., 2013). Biomarker levels can vary
79 independently from chemical contamination, and can have a strong impact on the reference value
80 and in turn on the final score of the index. The use of independent reference values based on the
81 range of natural variability of biomarkers offers the possibility to distinguish natural modulation from
82 significant modulation linked with a toxic impact (Chaumot et al., 2015). Moreover, it currently
83 appears difficult to find reference sites free from any anthropogenic pressure for field studies. This

84 supports the trend towards defining generic reference values. These values can be defined
85 empirically, based on annual and spatial variability within stations (Besse et al., 2013; Leprêtre et al.,
86 2022), or with modeling tools when environmental influences are large (Geffard et al., 2010; Coulaud
87 et al., 2011; Marchand et al., 2019). They consider the variations of the biomarkers linked to the
88 physicochemical parameters and overcome the influence of the abiotic confounding factors in the
89 final interpretation. Threshold values of real induction and/or inhibition can be extrapolated,
90 represented by the higher and/or lower reference range bounds. The use of these reference and
91 threshold values would make it possible to calculate indices that are more representative of the real
92 toxic impact of the environment on organisms. Associated with these reference ranges, the active
93 biomonitoring approach using transplanted organisms coming from a reference site limits the
94 variability linked with certain biotic factors (*i.e.*, the age, size and sex of individuals exposed) and
95 exposure conditions (*i.e.*, exposure duration, localization) by controlling these parameters. In addition,
96 the active approach bypasses the mechanisms of acclimatization or adaptation to chronic
97 contamination that may appear in native populations and lead to modulated biomarker levels. These
98 active approaches have been deployed with several aquatic model species, such as fish, amphipods or
99 bivalves (Besse et al., 2013; Catteau et al., 2022; Hani et al., 2021; Lacaze et al., 2011; Le Guernic et
100 al., 2016) and appear to be the most promising strategy for processing biomonitoring studies. One
101 other limitation of the IBR index is directly related to the calculation process. Whatever the IBR index,
102 the final score is obtained by summing up the parameters derived from the biomarker values. This
103 makes the final score closely dependent on the number of biomarkers considered. Some authors have
104 proposed to replace the sum by the mean value (Broeg and Lehtonen, 2006). With this slight
105 modification, these authors compared sites or conditions even if one biomarker was missing, making
106 IBRV2 less dependent on the total number of measured biomarkers.

107 In this context, the present work aimed to improve the original IBRV2 calculation method by proposing
108 a new index called “Integrative Biomarker Response - Threshold” (IBR-T). We first formalized the use
109 of the mean of the absolute distances instead of the sum by comparing IBRV2 calculated with each
110 strategy. Then, we introduced reference values to avoid the need for a reference site, and finally
111 threshold values were included in IBRV2 calculation to propose this new IBR-T. We compared the
112 results and the final rankings obtained with each index. This work was conducted using datasets from
113 several active biomonitoring programs of the zebra mussel (*Dreissena polymorpha*).

114 2. Materials and Methods

115 2.1. Datasets

116 The datasets of three different biomonitoring programs using the zebra mussel (*D. polymorpha*) were
117 used to meet the aims of this study. This mussel is a bivalve with bioecological traits that make it
118 particularly relevant for biomonitoring. Its high filtration rate and high bioaccumulation potential
119 make it an extensively used indicator for field biomonitoring studies and laboratory experiments
120 (Evariste et al., 2018; Kerambrun et al., 2016a, 2016b; Le Guernic et al., 2022). The mussels used for
121 the caging experiments came from the Lac du Der Chantecoq (51290 Giffaumont-Champaubert,
122 France, N 48°33'35"; E 4°45'11") and were kept several weeks in aerated tanks in darkness with a
123 controlled temperature. Two weeks before experiments, adult mussels (> 20 mm) were randomly
124 placed in 2-mm-mesh polyethylene cages (7 × 7 × 14 cm) (from 180 to 200 mussels per cage according
125 to the campaign) and the cage were maintained in the aerated tanks until the beginning of *in situ*
126 exposures. During this acclimation time, mussels were fed *as libitum* twice a week with a mix (50/50)
127 of two commercial solutions of microorganisms adapted for shellfish diet, including *Nannochloropsis*,
128 *Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira weissflogii* and *Thalassiosira pseudonana* (Nanno 3600
129 and Shellfish Diet 1800, Planktovie, France). For each experiment, the caging was carried out in
130 autumn during the resting period of *Dreissena polymorpha* and mussels were caged for 21 days (one
131 cage per site studied). The different datasets are described in Table 1 and the distribution map of sites
132 are presented in Supp. data 1.

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

143 For each caging experiment, a large set of biomarkers were measured in caged mussels to
144 characterize their energy metabolism (n = 6 by studied site for each biomarker), immunity capacity (n
145 = 10), detoxification capacity (n = 9) and DNA damage (n = 10). These biomarkers were chosen to be
146 representative to the most important physiological functions in living organisms. The modulations and

147 the responses of these biomarkers are well known on *Dreissena polymorpha*, since they have been
 148 used for several years in many studies (Evariste et al., 2017; Joachim et al., 2021; Palais et al., 2012,
 149 2011). The detailed procedures for biomarkers measurements can be found in previous recent works
 150 (Catteau et al., 2022; Hani et al., 2021). The biomarkers measured, their abbreviations and units,
 151 organs in which the measurements were made and the original publications for the protocols are
 152 summarized in Supp. data 2.

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

Experiment	Date of caging		Localisation	
	From	To		
Upstream/Downstream of WWTPs in the Meuse Watershed				
DIADeM	3/10/2018	29/11/2018	Namur upstream :	Namur Downstream :
			NAM_Up	NAM_Down
			Charleroi upstream :	Charleroi Downstream :
			CHR_Up	CHR_Down
Charleville-Mézière upstream :	Charleville-Mézière downstream :			
CHV_Up	CHV_Down			
Caging in 4 rivers in northern France				
MAQUEREAU				
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
Caging in the Seine and two tributaries (The Vire and the Orne)				
BIOSURVEILLANCE	4/10/2020	28/10/2020	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

155 2.2. Index calculation

156 2.2.1. Determination of reference and threshold values

157 The determination of biomarker thresholds for zebra mussel is described in details in Leprêtre et al.
 158 (2022), following the method initially developed by Besse et al. (2013) for bioaccumulation data. This
 159 methodology assumes that the natural variability of a biomarker for organisms living in a “healthy”
 160 unpolluted environment would be characterized by a Gaussian distribution. Biomarker threshold and
 161 reference values were determined using mussel biomarker datasets obtained from the EQUAL project.

162 Briefly, all the data measured in mussels exposed at different sites were sorted from the lowest to the
 163 highest value for a given substance. A Shapiro-Wilk test was applied on the overall dataset to test if
 164 the distribution was Gaussian. If not, the highest value was removed from the dataset and normality
 165 was tested again. This iterative process was stopped when the dataset was distributed according to a
 166 Gaussian law. The reference and threshold values were defined from the resulting Gaussian
 167 distribution as the 50th and the 95th percentiles, respectively. The approach was improved by adding

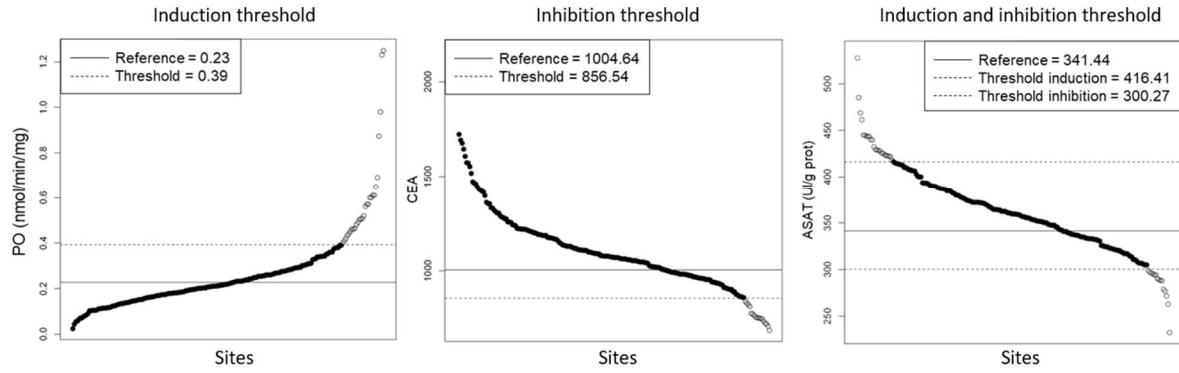
168 bootstrapping (Leprêtre et al., 2022), and adapted to biomarkers possibly inhibited by a pollutant (for
169 which an inhibition threshold should be defined) and biomarkers possibly induced and inhibited (for
170 which both induction and inhibition thresholds should be defined) (Fig. 1).

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

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

186 For biomarkers possibly induced and inhibited, we used the two previously described methods to
187 separately estimate the inhibition and induction thresholds. The issue here was to define a
188 methodology to separate the “inhibition dataset” from the “induction” dataset. For that, we sorted
189 the whole dataset from the highest to the lowest value (as represented in Fig. 1), and divided it into
190 two sub-datasets: (i) the induction dataset, from which the induction threshold was estimated, was
191 defined by the $q=65\%$ highest values (*i.e.*, $q= 65$ first percentiles); and (ii) the inhibition dataset, from
192 which the inhibition threshold was estimated, was defined by the $q=65\%$ lowest values (*i.e.*, $q= 65$ last
193 percentiles). In this way, the data representing natural variability (*i.e.*, basal modulation) were in both
194 datasets. Then, the above-described methodology for determining an induction threshold was applied
195 to the induction dataset, and the one used to determine an inhibition threshold was applied to the
196 inhibition dataset.

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



202

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

205 2.2.2. IBRv2 and IBR-T calculation

206 The procedure for calculating IBRv2 and IBR-T is described in Fig. 2. It is based on the method
 207 developed by Sanchez et al. (2013). Indexes were calculated independently for each experiment.
 208 Based on the value of a biomarker j ($j=1\dots J$) for an individual i ($i=1\dots n$) at site k ($k=1\dots m$; $m=4$ for the
 209 MAQUEREAU dataset; $m=6$ for the DIADeM dataset, $m=10$ for the BIOSURVEILLANCE dataset) called
 210 $X_{j,i,k}$, the IBRv2 index was calculated as follows:

- 211 1. Calculation of the mean of each biomarker for each site (\bar{X}):

212
$$\bar{X}_{j,k} = \frac{1}{n} \sum_{i=1}^n X_{j,i,k} \quad \text{Eq. (1)}$$

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

215
$$Y_{j,k} = \log \left(\frac{\bar{X}_{j,k}}{X0_j} \right) \quad \text{Eq. (2)}$$

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

218
$$A_{j,k} = \frac{Y_{j,k}}{\sigma_{Y_j}} \quad \text{Eq. (3)}$$

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

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

224
$$IS_k = \sum_{j=1}^J |A_{j,k}| \quad \text{Eq. (4a)}$$

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

227
$$IM_k = \frac{1}{J} \sum_{j=1}^J |A_{j,k}| \quad \text{Eq. (4b)}$$

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

236
$$IT_k = \frac{1}{F} \sum_{f=1}^F |A_{f,k}| \quad \text{Eq. (4c)}$$

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

240

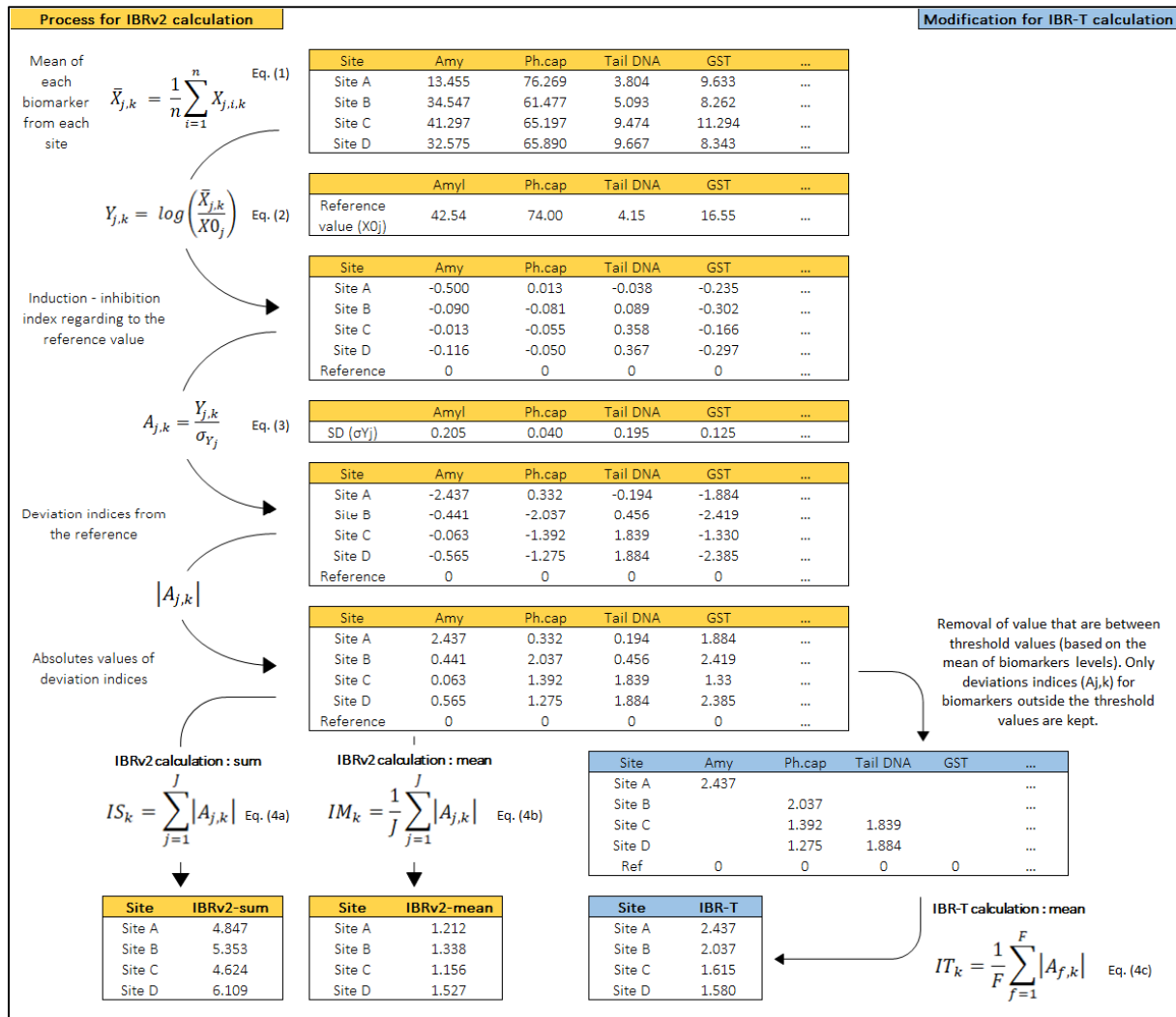


Fig. 2: Diagram of the procedure for IBRv2-mean, IBRv2-sum and IBR-T calculations.

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

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

270
 271

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

MAQUEREAU C1					
Site	IBRv2-sum	IBRv2-mean		Site	IBR-T
BRE	20.86	1.490		BAM	0.368
BAM	19.16	1.369		BRE	0.231
COU	16.73	1.195		BOU	0.174
BOU	16.70	1.193		COU	0.146
MAQUEREAU C2					
Site	IBRv2-sum	IBRv2-mean		Site	IBR-T
BAM	25.57	1.827		BAM	0.728
BOU	22.09	1.578		COU	0.442
BRE	20.72	1.480		BRE	0.428
COU	18.59	1.328		BOU	0.358
DIADEM					
Site	IBRv2-sum	IBRv2-mean		Site	IBR-T
CHV_Up	26.43	1.888		CHR_Down	1.242
NAM_Down	25.05	1.790		NAM_Down	1.198
NAM_Up	24.52	1.751		CHR_Up	0.855
CHR_Down	24.11	1.722		CHV_Down	0.713
CHV_Down	23.32	1.666		NAM_Up	0.685
CHR_Up	20.72	1.480		CHV_Up	0.676
BIOSURVEILLANCE					
Site	IBRv2-sum	IBRv2-mean		Site	IBR-T
TOU	30.68	2.192		TOU	1.529
MLD	25.51	1.822		MLD	1.109
FLP	24.99	1.785		FLP	0.854
PSY	22.01	1.572		PCO	0.779
VER	21.25	1.518		MEA	0.777
SOI	20.72	1.480		SOI	0.761
TRI	20.17	1.441		MSS	0.737
MEA	19.90	1.421		TRI	0.660
MSS	19.02	1.358		VER	0.520
PCO	17.15	1.225		PSY	0.477

272

273

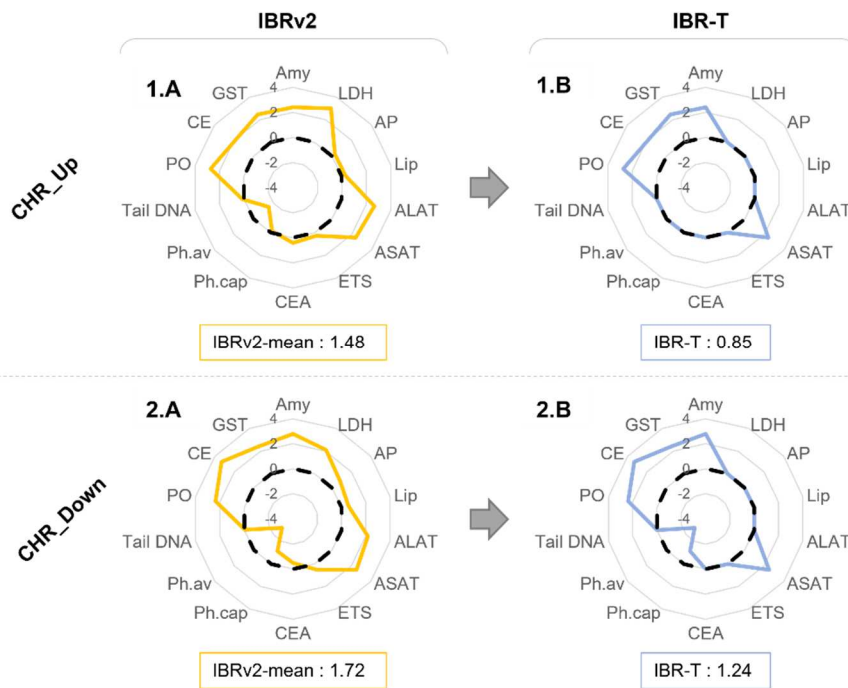
3.2. Reference values allow to dispense with reference sites

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

304 3.3. Differences in ranking sites between IBRv2 and IBR-T in biomonitoring 305 studies

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

312 These classification differences were particularly exacerbated in the DIADeM experiment, when
313 mussels were caged upstream and downstream of different WWTPs (Table 2). Logically and according
314 to the known chemical contamination (Catteau et al., 2022), the IBR score of the downstream sites
315 should have been higher than those of the upstream sites. This theoretical classification was found
316 with IBR-T, not with IBRv2. More precisely, the difference between the upstream and downstream
317 sites highly increased according to IBR-T in comparison with IBRv2 for NAM and CHR (Fig. 3). For CHV,
318 the ranking of the upstream and downstream sites was even inverted with IBR-T compared with
319 IBRv2. This new ranking highlights stronger modulations in the downstream site than in the upstream
320 site, which seems more ecologically relevant than the initial ranking with IBRv2. In addition, Charleroi
321 was the biggest of the three WWTPs studied during this campaign, and the CHR_Down site was the
322 one with the highest concentrations in pharmaceutical and domestic activity tracers (Catteau et al.,
323 2022). The IBR-T score ranked this site as the most disturbed one, which IBRv2 did not. Therefore, site
324 classification from an ecotoxicological point of view in the DIADeM caging experiment seemed more
325 relevant according to IBR-T scores than according to IBRv2 scores.



326

327 *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*
 328 *Charleroi downstream (2.A and 2.B) sites in the DIADeM caging experiment. The reference condition is represented by the*
 329 *black dotted curve. Amy: amylase; LDH: lactate dehydrogenase; AP: acid phosphatase; Lip: lipase; ALAT: alanine*
 330 *aminotransferase; ASAT: aspartame aminotransferase; ETS: electron transport system activity; CEA: cellular energy*
 331 *allocation; Ph.cap: phagocytic capacity; Ph.av: phagocytic avidity; Tail DNA: DNA damages; PO: phenoloxidase; CE:*
 332 *carboxylesterase; GST: glutathione-S-transferase.*

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

349 In addition, IBR-T better discriminated sites than IBRv2 did: a mean ratio of 2.40 was found between
 350 the highest and lowest IBR scores for IBR-T, versus 1.42 for IBRv2 (calculated from Table 2). This

351 discriminated or grouped several sites according to their IBR-T score. In the MAQUEREAU campaigns
 352 and as mentioned above, BAM site had a higher IBR-T than the other sites every year, not found with
 353 IBRv2 scores. In the DIADeM campaign, CHR_Down and NAM_Down sites were grouped according to
 354 their close IBR-T scores. In the BIOSURVEILLANCE campaign, four groups were visually formed based
 355 on IBR-T values: TOU, MLD, FLP-PCO-MEA-SOI-MSS-TRI, and VER-PSY (Table 2).

356 In addition to inducing differences in site classification, the nature of IBR calculation modulated the
 357 consistency of the percentage of modulated biomarkers (induced and/or inhibited) and the IBR scores
 358 (IBRv2 or IBR-T) (Table 3; Supp. data 6). As expected, a strong correlation was highlighted between
 359 the percentage of modulated biomarkers and the IBR-T scores (from 0.907 to 0.998 in the individual
 360 caging experiments; 0.882 when these experiments were combined). This correlation was much
 361 weaker for IBRv2 (from 0.002 to 0.759; 0.545 when the experiments were combined) (Table 3; Supp.
 362 data 6). This low correlation between the percentage of modulated biomarkers and the IBRv2 values
 363 supports the importance given to small variations of the biomarkers that may have little or no
 364 biological significance. This was underlined in the DIADeM campaign where the correlation between
 365 the modulated biomarkers and IBR-T was much higher than that obtained with IBRv2 (0.915 with IBR-
 366 T versus 0.002 with IBRv2) (Table 3; Supp. data 6). It is also important to note that the y-ordinate
 367 tended towards zero for IBR-T (from 0.132 to 0.035), in particular when the number of sites increased
 368 (0.013 for all experiments), whereas those of IBRv2 were higher (from 0.915 to 1.694) (Table 3; Supp.

Table 1: 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 Error! Reference source not

369 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

370

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

374 First, the induction and inhibition thresholds reflect the biological reality of the biomarker variations.
 375 The former forms of IBR endeavoured to consider each variation of each biomarker compared to the
 376 reference condition, whatever the value or the orientation of this variation, whereas IBR-T only
 377 considers variations whose level and orientation highlight a real impact of a pressure on the organism

378 under study. The orientation may be unilateral (an induction or an inhibition threshold) or bilateral (an
379 induction threshold and an inhibition threshold) (Supp. data 4). This orientation depends on the
380 biomarker nature as well as the possible impacts of environmental and anthropogenic pressures
381 exerted on them. DNA damage assessment (genotoxicity or tail DNA in this study) is a good example
382 of a biomarker for which only the induction threshold is calculated. DNA damage increases following
383 exposure to various contaminants (Binelli et al., 2009; Juhel et al., 2007; Vincent-Hubert et al., 2011).
384 Similarly, the decrease in phagocytosis parameters seems more interesting in order to evidence the
385 impact of a contaminant rather than its increase. This is supported by various studies including
386 different types of exposure (Couleau et al., 2012; Evariste et al., 2017; Le Guernic et al., 2020).
387 Induction as well as inhibition of other biomarkers could highlight exposure to xenobiotics. For
388 example, amylase activity can be stimulated or inhibited by contaminants (Amiard-Triquet et al., 2015;
389 Catteau et al., 2022; Kerambrun et al., 2016a; Yan et al., 1996). Thus, the fact that IBR-T takes the
390 biological meaning of modulations into account makes it more representative of environmental
391 reality. Nonetheless, the biological meaning of modulations and the resulting threshold values should
392 be revised for each biomarker and confirmed for each species. In this study, transplanted mussels
393 came from the same population from the Lac du Der Chantecoq. Threshold and reference values
394 should be verified if another population were used. Indeed, biological variations can vary according to
395 geographical variability of sentinel species. Thus, different autochthonous populations can respond
396 differently to a same environmental stress (González-Fernández et al., 2015). This may be due to
397 different genetic characteristics and environmental conditions between populations (Pain-Devin et al.,
398 2014). Nonetheless, Pain-Devin et al. (2014) estimated that IBR calculated on different populations of
399 zebra mussels varied mainly according to environmental conditions (including contamination) than
400 according to their genetic characteristics. This was also shown in *Mytilus sp.* where the geographical
401 location of individuals had much less effect on several biomarkers than seasonal variations (Benito et
402 al., 2019; Storhaug et al., 2019). These small differences could induce slight modulations of the IBR-T
403 values if the reference and threshold values are not obtained on the same population as that used for
404 biomonitoring. It is therefore necessary to limit these potential biases by knowing the population(s)
405 used and by respecting a common acclimation step before deployment to dissipate their ecological
406 memory (González-Fernández et al., 2015). However, these studies showed the strong importance of
407 the season in the responses of biomarkers. In the zebra mussel, the weight of the season has already
408 been assessed on various biomarkers, whether acetylcholinesterase, glutathione-s-transferase, energy
409 reserves, digestive enzymes, gene expression, etc. (Kerambrun et al., 2016b; Palais et al., 2012). The
410 importance of the season in biological responses is notably linked to many biotic and abiotic factors
411 (reproductive status, temperature, food availability, etc.). The season could then induce strong
412 disparities in the values of the IBR. To limit this, data used to calculate the reference and threshold
413 values, as well as those from the caging experiments described in this study, were all obtained *via*

414 caging carried out in the fall. With the aim to apply the active biomonitoring approach throughout the
415 year, the generic references and the threshold values should thus be established for each season.

416 Secondly, the definition of threshold values for each biomarker as proposed by Leprêtre et al. (2022)
417 allows considering only significantly modulated biomarker responses. The small variations in
418 biomarkers, not exceeding the 95th percentiles obtained from a database respecting the same
419 conditions as those of the three different biomonitoring programs described in this study, were
420 therefore removed from the calculation of the IBR-T. This allowed to consider only biologically
421 significant variations and not to add a weight to this index. Consequently, a site with no impact on the
422 tested biomarkers (no mean value exceeding the induction and/or inhibition threshold(s)) will have an
423 IBR-T score equal to zero. In IBRv2, all biomarker modulations from the reference are considered –
424 even non-significant ones – and increase the final index score (Sanchez et al., 2013). Therefore, IBRv2
425 can never be equal to zero despite the absence of significant effects. To illustrate this advantage with
426 the present dataset, the biomarkers that did not exceed the threshold values contributed 32.2% to
427 57.3% of the IBRv2 scores of the MAQUEREAU campaigns, 25.5% to 50.2% of the IBRv2 scores of the
428 DIADeM campaign, and 6.3% to 35.5% of the IBRv2 scores of the BIOSURVEILLANCE campaign (Supp.
429 data 5). These results again emphasize that small modulations largely contribute to IBRv2 scores.

430 The final advantage of the use of thresholds in IBR-T calculation is the strong score disparities
431 compared to IBRv2 which can result in a better or at least modified interpretation of biomonitoring
432 program results. Various studies using the first form of IBR (Beliaeff and Burgeot, 2002) or the one
433 revised by Sanchez et al. (2013) have showed a correlation between environmental contamination or
434 bioaccumulation and the score of these indices (Damiens et al., 2007; Lu et al., 2010; Perussolo, 2019;
435 Pytharopoulou et al., 2008). Nonetheless, other studies do not report this correlation, suggesting a
436 possible impact of individual variation, confounding factors, or the number and nature of selected
437 biomarkers (Broeg and Lehtonen, 2006; Catteau et al., 2022; Marigómez et al., 2013; Tsangaris et al.,
438 2011). In this study, the scores obtained in the four caging experiments differed according to IBRv2 or
439 IBR-T calculations. These strong disparities are linked to the biomarkers that did not exceed the
440 threshold values, included in IBRv2 but not in IBR-T. As discussed above, excluding the biomarkers
441 with small variations from the reference range cancels their importance in the calculation of IBR and
442 has a certain influence on the final classification of sites and may have a greater ecotoxicological
443 significance.

444 **4. Conclusion**

445 Present-day new technologies and methods allow acquiring a large amount of data within a short
446 time, but interpreting these datasets remains tricky. Therefore, integrating and summarizing data

447 within a simple index with an easy and transferable calculation method appears as a major challenge
 448 in ecotoxicology. The modifications proposed in the present study – (i) using mean distances to the
 449 reference value instead of sums of the distances to the reference value, (ii) adding reference values
 450 instead of reference sites – and during the calculation of IBR – (iii) deleting biomarkers not exceeding
 451 the thresholds – overcome some limitations of the previous forms of IBR. These benefits are
 452 summarized in Table 4. This improved index could be used in other environmental biomonitoring
 453 programs and on various biological species to prove its effectiveness in synthesizing and integrating
 454 large datasets and its correlation with environmental pressures.

455 *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	
Reference values	Allow comparisons between seasons, years and biomonitoring campaigns.	
	Avoid to have a reference site Consider individual variability Integrate confounding factors Potential large amount of data Can be refined	Time-consuming method for obtaining data
Thresholds	Consider the biological reality of the variations of the biomarkers	Time-consuming method for obtaining data
	No consider small biomarkers variations Potential large amount of data Can be refined Start with an IBR-T score of 0 (when no biomarker are modulated)	Depending on the biomarker nature and species studied
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	

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