This is the **accepted manuscript version** of the article:
Gil-Solsona, R., Álvarez-Muñoz, D., Serra-Compte, A., Rodríguez-Moza, S. (2021). "(Xeno)metabolomics for the evaluation of aquatic organism's exposure to field contaminated water" Trends in Environmental Analytical Chemistry 31 (2021) e00132 available online at https://doi.org/10.1016/j.teac.2021.e00132
© 2021. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http:// creativecommons.org/licenses/by-nc-nd/4.0/

1

2 **(Xeno)Metabolomics for the evaluation of aquatic organism's exposure to field**

3 **contaminated water.**

4

5 Ruben Gil-Solsona[1,a], Diana Álvarez-Muñoz[1,2,a,*], Albert Serra-Compte[1], Sara Rodríguez-

6 Mozaz [1,*]

7

8 [1]Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat

9 de Girona, C/ Emili Grahit, 101 Edifici H2O, E-17003 Girona, Spain.

10 [2] Water and Soil Quality Research Group, Department of Environmental Chemistry, IDAEA-

11 CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.

12

13 [a] Ruben Gil-Solsona and Diana Álvarez-Muñoz contributed equally to this work

14 *Corresponding author. Tel.: +34934006100; Fax +34932045904; E-mail address:

15 dalvarez@icra.cat

16 srodriguez@icra.cat

17 **Abstract**

18 Environmental (xeno)metabolomics offers a major advantage compared to other approaches

19 for the evaluation of aquatic organism's exposure to contaminated water because its allows

20 the simultaneous profiling of the xenometabolome (chemical xenobiotics and their

21 metabolites accumulated in an organism exposed to environmental contaminants) and the

22 metabolome (endogenous metabolites whose levels are altered due to an external stressor).

23 This approach has been widely explored in lab exposure experiments, however in field

24 studies environmental (xeno)metabolomics has only started in the last years. In this review,

the papers published so far that have performed different (xeno)metabolomics approaches for the evaluation of aquatic organisms exposed to contaminated water are presented, together with their main achievements, current limitations, and future perspectives. The different analytical methods applied including sample pre-treatment (considering matrix type), platforms used (Nuclear Magnetic Resonance (NMR) and low- or high-resolution Mass Spectrometry (MS or HRMS)), and the analytical strategy (target vs non-target analysis) are discussed. The application of (xeno)metabolomics to provide information of xenobiotics mixtures accumulated in exposed organisms, either in lab or field studies, as well as biomarkers of exposure and biomarkers of effect are debated, and finally, the most commonly metabolic pathways disrupted by chemical contamination are highlighted.

Keywords: mixtures, contaminants, risk, toxicity, metabolome, xenometabolome, exposome, biomarkers

1. Introduction

Human activities release large quantities of xenobiotics to the aquatic environment causing dramatic effects not only in the closest area to the discharge, but also in remote locations (e.g. marine environment in Antarctica [1]) by means of trasnsport of pollutants in water and pollution shifting . These xenobiotics can include both inorganic and organic contaminants such as metals, polycyclic aromatic hydrocarbons, surfactants, polychlorinated biphenyls, pesticides, dioxins, polyfluorinated alkyl substances, flame retardants, pharmaceuticals and personal care products, nanomaterials, siloxanes, plastics, etc. [2]. Besides, transformation products (TPs) can also be generated after biological or chemical degradation of other contaminants [3], as well as natural products like marine algal toxins [4]. All these

compounds potentially present in the aquatic environment may pose a risk for resident organisms due to their toxic effects. Therefore, it is crucial to develop appropriate strategies for assessing the environmental risk of these chemical mixtures and identifying the contaminants of potential concern [5].

Traditionally, the approach used to characterize chemical contamination in environmental matrices has been to apply several analytical methods for identifying and quantifying different chemical groups. In this sense, multi-residue methods focused on the separate analysis of different chemical families (e.g. pesticides, personal care products, perfluorinated compounds, etc) have been used [6–8]. In these methods, only levels of previously selected xenobiotics are studied, and when applied to biological samples they are considered biomonitoring studies. However, in order to cope with the analysis of the broad spectrum of contaminants present in aquatic organisms in a faster and cheaper manner, multi-residue methods are being developed devoted to the analysis of relevant contaminants mixtures [9–11]. These methods consider selected contaminants from different chemical families or perform a suspect screening of large lists of contaminants (e.g. using NORMAN suspect lists [12]). This is especially interesting when a high-resolution mass analyser is used because it gives the opportunity of digging in the complexity of the contaminant's mixture accumulated in an organism by using a non-target approach. Non-target approaches give the opportunity of obtaining information without pre-selecting compounds, so any kind of possible contaminant (as well as their transformation products) can be observed. Despite the huge opportunity it gives, the main drawbacks of non-target approaches are that HRMS instruments has lower sensitivity compared with the ones used for target analysis (usually QqQ or QTrap) and might hinder the annotation of features observed in this non-target analysis. Notwithstanding, the application of non-target analysis seems to be the way forward

73 to fill this knowledge gap. Particularly environmental (xeno)metabolomics offers a major
74 advantage compared to other approaches because, by comparing a control group with an
75 exposed group, both the xenometabolome or exposome (chemical compounds and their
76 metabolites present in an organism as a result of environmental exposure and that are not
77 naturally expected in the studied organism) and the metabolome (endogenous metabolites
78 whose levels are altered due to an external stressor) can be simultaneously profiled [2]. This
79 approach has been widely explored in lab exposure experiments, where exposure conditions
80 can be closely controlled, while in field studies environmental (xeno)metabolomics has only
81 been started in the last few years, despite being a powerful tool for the assessment of chemical
82 and biological health status of an ecosystem. Its popularity for the analysis of biological
83 samples directly from the field is rapidly increasing and it is anticipated to escalate as
84 metabolomics becomes a more routine tool for environmental monitoring [13]. In fact, the
85 application of this approach in the environmental field started 10 years ago, but it has been
86 in the last 5 years when it has gained a lot of attention from the scientific community.
87 Both, xenometabolomics and metabolomics in environmental science shares the goal of
88 obtaining biomarkers of (chemical) exposure and/or effect. In line with these definitions, a
89 biomarker in experimental biology has been "a defined characteristic that is measured as an
90 indicator of normal biological processes, pathogenic processes or responses to an exposure
91 or intervention" [14,15]. This definition only refers to endogenous compounds altered by an
92 external cause. However, xenobiotic' metabolites or transformation products generated by
93 its metabolism could also be named biomarkers, as they can be considered "endogenous
94 metabolites" though derived from a xenobiotic source [16]. Thus, in this review we will
95 define biomarkers derived from xenobiotic sources (and not normally present in the studied

96 organism) as “biomarkers of exposure”, while endogenous biomarkers (normally present in
97 the studied organism) will be named “biomarkers of effect”.
98 This paper presents studies addressing different (xeno)metabolomics approaches for the
99 evaluation of aquatic organisms exposed to contaminated water, where bioaccumulated
100 xenobiotics, biomarkers of exposure and biomarkers of effect have been studied, together
101 with their current limitations and future perspectives.
102

103 ## 2. *Analytical methods applied*

104 ### *2.1. Sample pre-treatment*

105 A total of 22 studies have been published in the last decade (from 2011 to 2020), where
106 (xeno)metabolomics approaches have been applied for testing the effects of exposure to
107 contaminated water in aquatic organisms (**Table 1**). In these scientific articles, liver has been
108 the tissue of preference for analysis (9 articles) followed by the whole organism (5 papers).
109 Other tissues such as gonads or digestive gland have also been analysed (3 articles) as well
110 as biofluids like plasma or serum (4 papers) or skin mucus (1 article). An in-vivo extraction
111 with Solid Phase MicroExtraction (SPME) from muscle has also been performed in one
112 occasion to conduct a metabolomics study. Depending on the matrix type, different sample
113 pre-treatments have been used.

114 #### *2.1.1. Liver*

115 Liver samples (or hepatocyte cells) have been studied in 9 out of the 22 papers published,
116 while other 3 have studied liver samples in combination with plasma or gonads, pointing out
117 the importance of liver as a natural detoxification organ. The main drawback of using this
118 tissue is that it requires the sacrifice of the animals.

119 Several works [17–22] have applied in their studies with liver samples the extraction method
120 proposed by Wu et. al. [23] followed by a clean-up procedure for lipids extraction reported
121 by Bligh & Dyer [24]. This method consists on the extraction of homogenized tissue with
122 methanol (MeOH) (4 mL/g) and water (H_2O) (0.85 mL/g) in an orbital shaker (or enhanced
123 with a tissuelyzer), followed by the addition of chloroform ($CHCl_3$) (2 mL/g) and H_2O (2
124 mL/g) forming two phases (with a final proportion of MeOH:H_2O:$CHCl_3$, 2:1.425:1). After
125 this step, dryness followed by reconstitution with deuterated water (D_2O) buffered with
126 sodium phosphate (pH 7.0-7.4) is usually performed for the analysis. Polar compounds
127 remain in the aqueous phase, while lipids are found in the chloroform, therefore obtaining
128 less interferences in the analysis of the polar analytes.

129 Other studies have followed a similar procedure to this biphasic extraction with little
130 modifications. The biphasic system was obtained by analysing separately polar compounds
131 via Nuclear Magnetic Resonance (NMR) and non-polar via High Resolution Mass
132 Spectrometry (HRMS). In both Glazer [25] and Park [26] studies, they performed the
133 extraction by vortex agitation with MeOH:H_2O, followed by addition of $CHCl_3$ and H_2O.
134 Then, samples were incubated in an ice-bath and centrifuged (with a final proportion of
135 MeOH:H_2O:$CHCl_3$ of 2:1.425:1 (Glazer) and 2:1.75:1 (Park)). In the case of Glazer and Park,
136 only the polar fraction was analysed.

137 ***2.1.2. Whole body/other tissues***

138 Although the whole body of a single organism or a pool of individuals are the type of samples
139 most analysed, other tissues like digestive gland, kidney, gonads, or gills are also used for
140 the (xeno)metabolomics approach.

141 For some analysis, the same strategy proposed for liver (biphasic system) have been followed
142 with little modifications. Cappello et.al. [27], for gill tissue, used ultraturrax homogenization

143 with MeOH, followed by the previously exposed bi-phasic separation (with a final proportion
144 of MeOH:H_2O:$CHCl_3$ of 2:1:4 for Melvin [28] et.al. and 2:1.425:1 for Cappello et.al.).
145 For the rest of studies, the authors have preferred bead beating extraction for the whole body,
146 incubation [7] or simply vortex agitation [29].
147 In the case of bead beating, different bead materials have been employed, such as zirconium
148 (Huang et.al. [30], both 100mg) or ceramic beads (Jeppe et.al. [31]). These studies used
149 solvents with different polarities and the biphasic extraction. Huang et.al. [30] applied an
150 additional clean-up procedure with Nunc 96-well plates while Jeppe et.al. [31] only analysed
151 the polar fraction.
152 Studies analysing digestive gland carried out an easier extraction protocol. Campillo et.al.
153 [7] incubated digestive gland with a mixture of acetonitrile (ACN):10mM KH_2PO_4, (3:1, v/v)
154 and Dumas et.al. [29] preferred to vortex samples following the biphasic extraction
155 (MeOH:H_2O:$CHCl_3$ of 1:0.8:1).
156 Previšić et.al. [32] sonicated aquatic invertebrate samples with a sonication probe, cleaning
157 the extract with a SPE procedure. The use of SPE can help to reduce the number of
158 interferences, enhancing the possibilities of detecting xenobiotics in the sample. However,
159 there is a potential loss of other compounds that may be not retained in the cartridge.
160 Whole body or tissues sampling is an invasive technique as they require scarifying the
161 animals. For this reason, other studies have proposed different non-lethal and less invasive
162 options to study the (xeno)metabolome such as the analysis of external parts of the organisms
163 performing an in-vivo solid phase extraction. In this sense, Roszkowska et.al. [33] inserted a
164 C18-coated blade in field fish muscles, which was desorbed with ACN:H_2O (20:80) and
165 directly analysed by MS. This strategy shows an interesting non-slaughtering sampling
166 alternative, which avoids sacrifice and allows applying (xeno)metabolomics in tissues.

167 ***2.1.3. Biofluids***

168 An alternative less-invasive approach is the extraction of biofluids such as plasma or skin
169 mucus. This type of sample is easy to obtain and the organism can be safely returned to the
170 environment after taking it, avoiding animals' sacrifice. Plasma (or serum) has been pointed
171 out as an interesting biofluid in the metabolomics analysis because it offers information
172 regarding the health status of the organism as it contains many endogenous metabolites.
173 Moreover, xenobiotics, as for instance polar contaminants, are also found there and they can
174 be distributed to other tissues through it. Finally, it makes possible to study contaminants
175 mixtures accumulated in wild individuals as well as their effects in vulnerable populations,
176 as sacrifice is not necessary.

177 The first step of the treatment of serum/plasma before analysis is normally a deproteinization.
178 Acetonitrile and ice-cold methanol have been mostly used with this purpose but also other
179 less polar solvents, like a mixture of methanol:ethanol, which can extract less-polar
180 compounds from samples [34].

181 Acetonitrile was added to plasma samples by Heffernan et. al. [35], followed by
182 centrifugation and lipid precipitation at -20°C. A C18-endcapped lipid cartridge was used as
183 final clean-up procedure to eliminate possible interferences. In the case of Al-Salhi et. al.
184 [36], MeOH was selected for deproteinization but no further treatment was applied. Simmons
185 et.al. [37] also employed MeOH as protein removal solvent with phenyl isothiocyanate and
186 ammonium acetate. David et. al. [38] eliminates protein and phospholipids by using *Phree*
187 phospholipid removal plates of samples diluted with MeOH 1% formic acid followed by
188 Strata-X-C (cation exchange cartridges). They also analysed fish gonads, liver and kidney
189 with the same treatment, performing samples extraction by sonication with MeOH, applying
190 then the same clean-up procedure.

191 Mosley et.al. [39] studied skin mucus of fathead minnows with a simple blotting with glass-
192 fiber filter strips, which were eluted with ice-cold MeOH, vacuum dried and reconstituted
193 with ACN:H_2O (1:19).
194 As a take home message of this section, full body extractions with bead beater have been
195 preferred by most of the authors for analysing whole body samples using a polar and non-
196 polar biphasic system (water:methanol:chloroform) also described for liver tissues. This
197 approach helps reducing matrix effect of these complex matrices and therefore its use is
198 recommended. Finally, a deproteinization step in biofluids analysis is mandatory when
199 analysing them directly. This is usually achieved by using organic solvents such as
200 acetonitrile (ACN) or ice-cold methanol, which allows to precipitate and separate the proteins
201 from the rest of the fluids. Moreover, the use of ACN as solvent avoids the requirement of
202 working with freeze-dried solvents, avoiding incomplete precipitation and further problems
203 as column damage.
204
205 ***2.2. Analysis by HRMS vs NMR***
206 Two analytical platforms are mainly used to carry out metabolomics studies: Nuclear
207 Magnetic Resonance (NMR) employed in 9 studies [17–22,27,28,40], low- or high-
208 resolution Mass Spectrometry (MS or HRMS) in 16 [7,21,35–40,22,25,26,29–33], whereas
209 both instrumental set-ups were applied in 3 out of 22 papers.
210 The pros and cons of NMR and HRMS for metabolomic applications have been widely
211 discussed in the literature [41]. On the one hand, NMR are non-invasive and non-destructive
212 procedures, and they have high reproducibility and greater elucidation power for unknown
213 compounds than HRMS. On the other hand, MS and HRMS have higher sensitivity,
214 achieving the detection of lower concentrations for target and non-target compounds.

215 Consequently, in a non-target analysis more compounds can be detected using MS than
216 NMR. Concretely in the articles reported in table 1 up to 208 compounds were detected using
217 MS techniques, while between 3 and 31 compounds were observed when NMR was selected
218 as analysis technique. In reference to xenometabolomics applications, none of the articles
219 reviewed used NMR to monitor xenobiotics, most likely due to their lower sensitivity.
220 An important drawback of MS is that the sample treatment required is usually more
221 challenging than in NMR and some compounds could be lost during those extraction
222 procedures. Solid samples (e.g. liver or other tissues) must be extracted with solvent in order
223 to be in a liquid phase for their analysis by liquid chromatography, and further converted in
224 gas phase (usually by Electrospray) for their ionization and MS detection. In the case of liquid
225 samples (e.g. plasma or serum), their deproteinization is mandatory before liquid-
226 chromatography analysis for avoiding any clogging and damage of the chromatographic
227 column due to protein precipitation. By applying this step, the poor ionization efficiency, the
228 potential detector saturation or any matrix effects caused by proteins or phospholipid species
229 in MS detectors [42] are reduced, as well as the potential loss of compounds of interest.
230 As we have previously stated, a critical comparison of NMR and MS is out of the scope of
231 this manuscript, and can be checked more deeply in the literature [41]. However, considering
232 NMR and MS (MS/MS or HRMS) benefits and drawbacks, the use of HRMS is encouraged,
233 as it allows the analysis of low concentrated compounds, both endogenous and xenobiotic
234 compounds, which cannot be studied with the less sensitive NMR instruments.
235
236 ***2.3. Target / Non-target strategy***
237 In the previous sections, sample treatment and instrument selection have been exposed. In
238 this subsection, the data acquisition strategy followed for the analysis of metabolites and

239 contaminants is discussed. There are two main approaches regarding compound preselection,
240 target and non-target strategies. Target analysis is based on the identification and
241 quantification of a previously selected set of compounds, depending on their expected
242 relevance in the experiment. For instance, in the case of MS/MS based target analysis, both
243 parent and daughter ions are selected before acquisition experiments. Normally, a validated
244 method based on the comparison with corresponding analytical standards is applied in order
245 to quantify these compounds. However, information about any other ion from the matrix not
246 included in the acquisition list will not be obtained a *posteriori*. This strategy has been
247 followed in 7 of the papers published [7,17,25,27,28,30,31] (**Table 1**).
248 Non-target strategies are based on the full-scan data acquisition (in the case of MS based
249 approaches) and statistical data treatment to mark features that better explain the differences
250 between groups of samples. In this strategy, the lack of information before acquisition makes
251 more challenging the identification of the compounds that vary the most between the groups
252 (filters are usually applied to decrease the dataset size), but offers the possibility to move
253 from the classical hypothesis-driven research to a data generating hypothesis-driven
254 approach, more interesting in metabolomics experiments [43]. This powerful tool allows
255 performing post-target analysis of the acquired data, clarifying the highlighted results, or
256 even revealing new hypothesis previously not observed, which is very useful in
257 xenometabolomics studies in order to search and identify chemicals. This strategy has been
258 used in most of the studies reviewed in this paper, in 16 scientific articles (**Table 1**). It is
259 based on the comparison of areas between a control group of a certain organism and a group
260 of organisms exposed to a particular stressor. It is mainly a qualitative technique, but
261 quantification of compounds can be performed after identification if chemical standards are
262 available.

263 The main bottleneck of non-target strategies, as stated before, is the elucidation process for
264 highlighted compounds. On-line spectral databases (e.g. METLIN[44], Massbank[45] or
265 MZCloud, among others) are very useful for the identification of compounds and are
266 constantly being expanded. However, not all the compounds detected are registered and
267 available to check in these databases (i.e. TPs of some contaminants).
268 Some in-silico software (e.g. SIRIUS 4 [46]) have also appeared for fulfilling this gap,
269 allowing to search possible candidate identities for compounds included in databases (e.g.
270 PubChem [47]) without spectral information. Comparing experimental tandem mass spectra
271 information with in-silico spectra prediction, a candidate list can be shortened, reducing
272 elucidation time for molecules not included in databases.
273 Other studies have employed both target and non-target strategies with more than a single
274 analytical platform (combining NMR with MS, or LC-MS with GC-MS) to obtain widened
275 information [17,21,28,31]. However, with the continuous increase in sensitivity of HRMS
276 instruments combined with their versatility, the use of a single instrument in full-scan
277 acquisition (in the so-called data independent or data dependent acquisition modes) have
278 made possible to perform both kind of analysis in the same run. As previously pointed out
279 and shown in the literature, full scan acquisition offers information about both contaminants
280 (which can be impacting the metabolism) and endogenous metabolites present in the sample
281 that are up or down regulated because of the chemical stress. Therefore, HRMS allows the
282 possibility of performing *a-posteriori* suspect analysis of compounds, which had not been
283 targeted before acquisition.
284 As an example, Gago-Ferrero et.al. [48] performed suspect analysis of more than 2000
285 compounds by means of UPLC- HRMS in a single run and in full-scan acquisition mode.
286 This wide-scope screening or suspect analysis is based on a previously defined set of

287 compounds (2316 different substances) and, only those detected were further quantified in
288 wastewater using a calibration curve (target analysis). The same data also allows to perform
289 non-target analysis, opening the possibility to widen the number of identified compounds.
290 The same approach has been applied to organisms in the XENOMETABOLOMIC project
291 (CTM2015-73179-JIN, AEI/FEDER/UE). A target method using HRMS was developed for
292 the analysis of a mixture of relevant contaminants in mussels [11]. The compounds included
293 in the method were quantified in mussels from Ebro Delta (Spain) [49]. Later on, a non-target
294 approach was applied allowing the identification of other significant contaminants previously
295 not included in the method [50]

296 Target and non-target approaches can be considered complementary approaches and their
297 combined used is recommended, as well as the wide-scope suspect screening strategies, for
298 (xeno)metabolomics applications.

299

300 **3. (Xeno)metabolomics approaches in environmental studies**

301 Among the 22 papers (**Table 2**) published in the last decade, only 8 have applied
302 (xeno)metabolomics to evaluate the presence of xenobiotics in aquatic organisms as well as
303 their effects (metabolomics), therefore studying both biomarkers of exposure and effect. The
304 rest of publications have been devoted to the study of biomarkers of effects solely.
305 Consequently, this section is divided in two subsections, one dedicated to the study of
306 contaminants (profiling of the xenometabolome and biomarkers of exposure), and another
307 one to detect early stage metabolic dysregulations provoked by these xenobiotics in the
308 organisms (profiling of the metabolome and biomarkers of effect).

309 **3.1. Xenometabolomics and biomarkers of exposure**

310 Xenobiotics profiling in an organism, also known as xenometabolome or exposome, is of
311 high importance for connecting contaminant levels and toxic effects. The analysis of the
312 compounds bioaccumulated in an organism allows to evaluate chemical contamination, and
313 to correlate their presence and levels with metabolic alterations or even with diseases. Despite
314 the encouraging possibilities of xenometabolomics, only 8 out of the 22 publications included
315 in this review have studied the presence of xenobiotics or their TPs in organisms (**Table 2**)
316 besides the endogenous metabolites. Among them only 5 applied xenometabolomics using a
317 non target methodology ([33], [35], [36], [38] and [39]) whereas 3 studies performed a target
318 screening of preselected substances (biomonitoring) ([32], [37] and [40]). However,
319 xenometabolomics popularity for analysing biological samples directly from the field is
320 being boosted and it is foreseen to grow even faster as metabolomics becomes a more routine
321 tool for environmental monitoring [13].
322

323 ***3.1.1. Lab exposure to real contaminated waters***

324 In three out of the 5 studies where xenometabolomics was undertaken, exposure experiments
325 were carried out at lab-scale [36,38,39]. Xenometabolomics was employed for the first time
326 by Al-Salhi et al. [36] in 2012. From a total amount of 242 compounds that significantly
327 contributed to the separation of control and exposed fish (to WWTP effluent) only 8 were
328 endogenous metabolites. The remaining 236 were xenobiotics, mainly surfactants, but also
329 chlorinated phenols, xylenols or phenoxyphenols. They also found TPs such as glucuronide
330 metabolites for pharmaceuticals and sunscreen products in bile and plasma samples. David
331 et.al. [38] identified 54 exogenous compounds and TPs in plasma and tissues of fish exposed
332 to WWTP effluent, including pharmaceuticals, endocrine disruptors, personal care products
333 or pesticides, among others. Mosley et al. [39] also found 4 xenobiotics and TPs in skin

334 mucus of fish exposed to WWTP effluent (BPA, 1,7-dimethylxanthine, cotinine and triclosan
335 transformation products).

336 ***3.1.2. Field exposure***

337 In addition to the studies carried out under lab-controlled conditions with real contaminated
338 waters, other 2 articles applied a xenometabolomics approach using wild animals directly
339 collected from the field [33,35]. Heffernan et.al. [35] sampled plasma of green sea turtles
340 from the Great Barrier Reef (Australia) finding 13 xenobiotics and TPs (including pesticides
341 or additives). Roszkowska et.al. [33] applied an SPME extraction in fish muscle exposed to
342 pulp and paper mill discharge in a contaminated area, and observed 42 different xenobiotics,
343 including pesticides, aromatic hydrocarbons, phthalates, mycotoxins or organometallic
344 compounds. These studies show the strength of the xenometabolomics strategy to cover both
345 xenobiotic compounds and their possible transformation products. At lab exposure
346 conditions, parent compounds and TPs are more readily detected when a depuration phase is
347 not undertaken before sampling, as the organisms have limited time to eliminate the
348 toxicants. However, this clearance is continuously done in wild animals, which may difficult
349 the task.

350 Compounds found by the different authors comprehend classical micropollutants usually
351 included in target and suspect screening lists (e.g. NORMAN suspect lists) but also many
352 glucuronide metabolites of chemical contaminants (e.g. chlorinated phenols,
353 pharmaceuticals, UV filters, etc.) showing the potential of xenometabolomics approaches for
354 obtaining a good overview of relevant biomarkers of exposure generally not included in
355 target methodologies.

356 **3.2. Metabolomics and biomarkers of effect**

357 A metabolomics approach was applied to study the impact of chemical contamination in
358 organisms exposed to real contaminated waters either at the lab (under control conditions) or
359 in the field (natural conditions).

360 ***3.2.1. Lab exposure to real contaminated waters***

361 A total of 8 studies have exposed aquatic organism (fish [20,22,30,36,38,39], crustacean [40]
362 or mussels [29]) to real contaminated samples (water from WWTP [22,30,36,38–40] or
363 sediments [20]) in lab controlled conditions. This type of experiments helps to understand
364 the effects that real mixtures of contaminants (mainly wastewater samples) have on aquatic
365 organisms in a controlled scenario.

366 Wagner et.al. [40] exposed crustacean to two stages of wastewater, the pre-chlorinated
367 wastewater and the final effluent (in addition to the final effluent spiked with
368 perfluorooctanesulfonic acid (PFOS)). They found significant alterations in amino acid
369 metabolism and depletion of sugar and energy metabolites, pointing out a probable
370 gluconeogenesis activation to provide energy into the organism. The authors also observed
371 an increased toxicity of the chlorinated effluent, which could be attributed to the presence of
372 disinfection by-products (DBPs). This study indicates that applying metabolomics can
373 greatly assist to understand how DBPs alter the metabolome of aquatic organisms and to
374 determine their mode of action (MOA).

375 Huang et.al. [30] applied a wide target analysis, including 21 amino acids (AA), 21 biogenic
376 amines (BAs), 4 bile acids, ∑hexose, 17 fatty acids (FAs), 40 acylcarnitines (ACs), 89
377 phosphatidylcholines (PCs), and 15 sphingomyelins (SMs). The authors exposed zebrafish
378 to different lab-prepared contaminant mixtures, a real wastewater effluent (WWE) and a
379 spiked WWE. They found that the matrix effluent can have different effects on the metabolic
380 responses induced by some contaminants. For example, in the case of fluoxetine, strong

381 effects on the metabolome profile were observed in combination with the effluent, while for
382 PFOS the same effect was not observed probably due to possible interactions affecting
383 bioavailability.
384 Al-Salhi et.al. [36] exposed rainbow trout to WWE during 10 days, when some of the fish
385 were sampled at the end of the exposure period and two subgroups were transferred to clean
386 water for depuration during 4 and 7 days respectively. They observed a significant increase
387 in the plasma concentration of the bile acids cyprinol sulfate, taurocholic acid and
388 lysophospholipids, and a decrease in the lipid sphingosine. These findings showed the
389 disruption of bile acid and lipid homeostasis with probable consequences for cellular
390 signalling and maintenance of cell membrane integrity. However, after 11 days of depuration,
391 with around 90-100% of xenobiotics elimination, all markers returned to normal levels and
392 the initial health status of organisms was nearly recovered.
393 David et.al. [38] exposed *Rutilus rutilus* to 100% wastewater effluent observing mortality of
394 8 out of 60 fish. The plasma of survivors was collected and analysed by nanoflow Ultra High
395 Performance Liquid chromatography– High Resolution Mass Spectrometry (nUHPLC-
396 HRMS). The authors observed alterations in tryptophan metabolism, bile acid metabolism as
397 well as serotonin metabolism and sphinganine signalling disruption. Mosley et.al. [39] also
398 exposed sexually mature fish to different WWE dilutions (5, 20 and 100%), observing 52
399 altered pathways related with energy, amino acids, oxidative stress, nitrogen, vitamins and
400 phospholipid metabolism among others, and including biotransformed xenobiotics.
401 Zhen et.al. [22], exposed liver cells to 75% concentration extract of wastewater effluent and
402 some other river waters up- and downstream. They also found alterations in energy
403 metabolism, oxidative degradation and amino acid metabolism. This study demonstrated the

404 utility of cell-based metabolomics for assessing the biological effects of contaminant
405 mixtures.
406 Water has been deeply explored as the main source of contamination of aquatic
407 environments. However, lipophilic and less polar compounds tend to be present in sediments
408 at higher levels than in water. In the study by Williams [20] European flounders were exposed
409 to contaminated estuarine sediments in a mesocosms study. They observed alterations in liver
410 immune response by means of transcriptomic analysis of the fish samples. They also found
411 eighteen altered tentative metabolites using a NMR-based metabolomics although their
412 identity was not further confirmed. Dumas et.al. [29] performed a SPE extraction from a
413 WWTP effluent to trap lipophilic compounds, and exposed mussels to the extracts. They
414 found alterations in some amino acid metabolisms, but also in purine, pyrimidine, pyruvate
415 and glutathione metabolites.
416 ***3.2.2. Field Exposure***
417 In order to study the effects of contaminants exposure in the field (an uncontrolled exposure
418 scenario) two different strategies have been followed. Wild animals can be sampled in
419 specific sites in the environment so that their metabolome reflect the stressors present in their
420 natural habitat, including different contaminants. Alternatively, the organisms can be placed
421 in a natural site inside cages for a specific period of time in order to reduce specimen mobility,
422 which is especially critical in the case of fish for instance. That way, organism metabolome
423 profile reflects more accurately the contamination impact of a particular area under study.
424 Most works published so far have dealt mainly with fish captured from aquatic environments
425 but also midge has been studied, an insect that lives in wetlands. Field exposure has been
426 studied in 13 out of the 22 papers considered in this review (**Table 2),** 7 of them used caged
427 organisms to perform their study and 6 sampled wild organisms.

428

429 ***Caged animals***

430 The exposure to contaminated waters inside a cage provides a snapshot of a certain period of
431 time. This kind of experiments have been applied in 6 studies, reflected in 7 papers by
432 Campillo et.al. [7], Ekman et.al. [17], Davis et.al. in 2013 [18] and 2016 [19], Skelton et.al.
433 [21], Cappello et.al. [27] and Previšić et.al. [32].

434 Campillo et.al. [7] used target analysis for quantifying more than 70 metabolites. The authors
435 collected clams from a clean area, after 10 days of depuration in lab conditions they were
436 transferred to 3 different zones in *Mar Menor* lagoon. Two of them were used as reference
437 sites, and the other one was located near the most important contamination source named El
438 Albujón, a watercourse that discharges pollutants from the surrounding area. They found
439 alterations in amino acid metabolism, oxidative response system or taurine metabolism,
440 closely related with contamination stress.

441 Cappello et.al. [27] caged mussels at a highly polluted petrochemical area mainly
442 contaminated with Hg and Polycyclic Aromatic Hydrocarbons (PAHs), and they were
443 compared with mussels exposed in a non-contaminated site. They carried out target NMR to
444 study serotoninergic, cholinergic as well as dopaminergic systems by means of
445 neurotransmitters quantification, pointing out that all these systems are affected by Hg
446 pollution in this area.

447 Ekman et.al. [17] deployed cages containing fish at different sites in the Platte River near
448 two WWTPs, and they applied target in combination with non-target NMR. Target NMR was
449 employed to assess estrogenic effects, based on glutamate, alanine and vitellogenin that have
450 been previously observed as biomarkers of estrogenicity by themselves. Statistical analysis

451 was applied to highlight non-estrogenic biomarkers of effect, pointing out alterations in
452 oxidative stress and phosphocholine metabolism.
453 Skelton et.al. [21] applied non-target NMR to fish caged in three different zones of a river
454 impacted by WWTPs. One of them was taken as a control group and the other two as exposed
455 from contaminated areas near the effluents, one highly urbanized and the other with huge
456 farm and agricultural impact. The fish were caged upstream, in the effluent mouth and
457 downstream. They pointed out differences in amino acid metabolism, phospholipid,
458 cholesterol, and energy metabolism affected by the WWTPs.
459 Davis et.al. [18] exposed fish to different distances from a pulp and paper mill outflow as
460 well as a control site. The authors observed differences in amino acid, creatine, and taurine
461 metabolism, as well as energy metabolism alterations and liver toxicity biomarkers.
462 From all these manuscripts, we consider important to enhance the manuscript that Davis et.al.
463 published in 2016 [19]. The authors caged different fish in 18 sites from 5 lakes (Great Lakes
464 basin) and performed PCA analysis of endogenous compounds. Then, with these variations,
465 the authors carried out a Partial Least Squares (PLS) regression with contaminants levels
466 analysed in a target way, pointing out those who better explain differences observed in
467 endogenous metabolites. This model highlights xenobiotic compounds affecting the
468 metabolism and helps to discriminate contaminants without effects observed in the dataset.
469 The authors concluded that up to 52% of detected contaminants were not correlated with
470 changes in endogenous metabolites. This interesting approach can help xenometabolomics
471 field to observe the impact of xenobiotics in the metabolome, helping to prioritize
472 anthropogenic contaminants with real relevance in metabolome alterations.

473 Previšić et. al. [32] in a non-target analysis of the whole body of *Hydropsyche sp.* larvae
474 observed alterations in 32 compounds, with disruptions in fatty acid metabolism (energy
475 metabolism), phospholipid metabolism and oxidative stress.

476 ***Wild animals***

477 Wild animals captured in situ provide a more realistic picture of long-term exposure
478 conditions than individuals caged in the environment, where a shorter exposure time is
479 considered. However, despite in caged experiments contaminant levels can be better
480 controlled or known (by means of target analysis in the exposure site), wild organisms could
481 have been exposed to a more complex mixture of environmental xenobiotics due to their free
482 mobility through the environment. For this reason, the use of wild animals can yield to
483 conclusions about the level of contamination in a wider zone at long-term exposure. All the
484 papers that performed this kind of experiments (Glazer et al [25], Park el.al. [26], Jeppe et.al.
485 [31], Roszkowska et.al. [33], Heffernan et.al. [35] and Simmons et.al. [37]) used mass
486 spectrometry for these analyses. Five publications used fish as a model organism but also
487 turtles were studied following this strategy.

488 In the case of Simmons et.al. [37], they combined taking wild animals with caged ones, as
489 they exposed caged goldfish to a possible WWTP contaminated area at different distances
490 downriver and took wild fish from the Jordan Harbour (near the point of the caged ones). All
491 the plasma samples were analysed by means of a multi-targeted set of metabolites. They
492 observed that some contaminants were detected in both caged and wild animals at similar
493 levels. The authors also discussed that the magnitude of fold change was higher in wild
494 organisms due to their long-term and constant exposure (gemfibrozil as an example). For the
495 ones caged near the WWTP, they highlighted 47 altered biological functions compared to
496 fish caged in a reference site. They found liver necrosis and metal ion transport functions

497 activated, as well as depletion of the synthesis of cyclic adenosine monophosphates (AMPs).
498 Additionally, in the other sampling points, an increase in amino acids, accumulation of lipids
499 and glyceride, inhibition of steroid synthesis or increasing in glutathione concentration were
500 some of the effects observed. Overall, the expression of plasma metabolites and proteins in
501 caged goldfish agreed well with those in the wild goldfish, suggesting that the combined use
502 of omics approaches and caged surrogates is a useful way to predict the molecular effects of
503 contaminants in wild fish [37].
504 Park et.al. [26] exposed *Danio rerio* to water from different zones (reference sites and other
505 contaminated with different xenobiotics) in order to study its liver, as well as wild fish
506 captured in the study sites. The highlighted compounds observed in the environmental fish
507 also shown differences in lab-exposure conditions, but with smaller ratios. The authors
508 observed that lab exposure experiments have lower impact in the metabolism of exposed
509 organisms than others captured directly from the environment.
510 Glazer et al [25] also performed a target analysis of liver samples from adult fish collected
511 from a PCB-contaminated area (Acushnet River Estuary) and from pristine site (Scorton
512 Creek). They found alterations of one-carbon metabolic pathway and amino acid imbalance.
513 Roszkowska et.al. [33] also captured wild individuals and performed in-vivo SPME
514 sampling in their tissues with a PAN-C18-coated blade. Different metabolites related with
515 lipid metabolism were observed by means of this technique designed to study less polar
516 contaminants spectrum.
517 Heffernan et.al. [35] took blood samples from turtles from different zones of Australia, an
518 offshore control site and two coastal areas exposed to urban/industrial and agricultural
519 activities and performed a non-target strategy. They pointed out alterations in some
520 nucleotides, fatty acids and vitamin related compounds.

521 Jeppe et.al. [31] studied midge captured in different wetlands where sediments shown diverse
522 contamination. They pointed out alterations in methionine metabolism, glycolipid
523 metabolism and sugar metabolism by metals and total petroleum hydrocarbon contaminated
524 zones. Mitochondrial electron transport and urea cycle metabolism were individually related
525 to bifenthrin contamination, as well as sugar metabolism related to all contaminant inputs.
526
527 **3.3. Metabolic alterations**
528 After profiling the metabolome, further data analysis is usually undertaken to find out the
529 most altered metabolic pathways and draw biological conclusions. In this review, despite the
530 experiments correspond to different exposure conditions, organisms, and analytical
531 platforms, some metabolic pathways can be pointed out as the most commonly disrupted by
532 chemical contamination, showing important alterations in organism that may be related to
533 pathologies. Figure 1 presents these metabolic pathways reported as disrupted due to the
534 exposure to contaminants (according to table 2). The sixteen pathways reported can be ranked
535 based on the number of times that a certain pathway has been presented in a paper as
536 disrupted. The top 3 are hold by energy metabolism, followed by aminoacid and phospholipid
537 metabolism (Figure 1 in red colour). Considering the total number of papers included in table
538 2, energy metabolism has been reported in 57% of the papers published and aminoacid
539 metabolism in 50%. Therefore, these two metabolic routes are highlighted as the most
540 commonly altered (nearly half of the experiments). Similar pathways were previously
541 reported as the most affected ones when aquatic organisms are exposed to a single
542 contaminant [1]. Phospholipid metabolism has been mentioned in 29% of the papers. After
543 them, other altered pathways were oxidative degradation and liver toxicity in 21% and 18%
544 of the papers respectively (Fig. 1 in orange), nitrogen and taurine metabolism in 11% of the

545 papers (Fig. 1 in yellow), bile acid metabolism, carnitine metabolism, cholesterol
546 metabolism, sphinganine metabolites, steroid biosynthesis and vitamin metabolism in 7% of
547 the papers (Fig. 1 in blue), creatine metabolism, serotonin metabolism and repair capacity in
548 4% of the papers (Fig. 1 in green).

549 Some of these dysregulations have important implications in animals' metabolism and
550 development. The exposure to highly contaminated waters has shown to produce an
551 important impact in their energy sources, such as aminoacids or lipids, which may affect their
552 growing, reproduction and movement. Besides, phospholipids are structural components in
553 cells and membrane stability may be compromised. Liver toxicity has been detected in some
554 cases too, showing the important implications of contaminants in their metabolism, as well
555 as the increase in oxidative stress, conditioning their health status and survival.

556

557 **4. Conclusions and future research**

558 **Conclusions:**

559 1. The analysis of a single tissue or fluid at lab-scale and field experiments with a single
560 platform (e.g. GC-MS, LC-MS, NMR) might give incomplete information and thus
561 bias the conclusion of the study. Therefore, the study of several matrices with
562 different analytical techniques is preferred as it provides wider information.
563 2. The analysis of blood derived samples (serum, plasma) or/and in-field extraction
564 procedures from different tissues is preferable because they are non-invasive
565 techniques and avoid animals' sacrifice.
566 3. Despite the higher elucidation power of NMR, MS is preferred as low concentrated
567 compounds can be highlighted, allowing the detection of chemical pollutants and
568 therefore, expanding metabolomics to xenometabolomics approaches.

569 4. Xenometabolomics studies have risen with the application of untargeted strategies.

570 Even though the huge potential and applicability of the methodology have been demonstrated
571 some **limitations** remain and should be considered:

572 1. Exposure time at lab-scale experiments are usually shorter than real exposure of wild
573 animals in their natural environment. Moreover, lab studies represent constant
574 exposure conditions, while in a field study exposure conditions varies along the time.
575 Also, synergic effect of varying stressors can be different. Therefore, slight
576 differences can be found in the altered pathways. Finally, water is not the only
577 contamination source in the aquatic environment and exposure to more realistic
578 conditions (e.g. water in combination with sediments) is highly recommended.
579 Hence, the study of wild individuals is encouraged although this approach is more
580 challenging.
581 2. Despite exposure to contaminants mixtures remains as the most realistic scenario, it
582 does not allow differentiating which component of the mixture produces a specific
583 effect and therefore, lab experiments of exposure to single compounds are still
584 needed. To this respect, multivariate analysis can greatly assist by linking endogenous
585 compounds' modifications with different contaminant levels in the organisms,
586 helping to better understand the dose-effects relationships at low doses.
587 3. There is a lack of standardization for metabolomics approaches in the studies about
588 exposure to contaminant mixtures. Due to the possibilities of (xeno)metabolomics,
589 its implementation in laboratories should be carried out ensuring the comparability
590 between studies. For this purpose, a working group for inter-lab (xeno)metabolomics
591 studies is highly recommended.

592 **Acknowledgments**

593 This study was supported by the Spanish Ministry of Economy and Competitiveness, State
594 Research Agency, and by the European Union through the European Regional Development
595 Fund through the projects XENOMETABOLOMIC (CTM2015-73179-JIN)
596 (AEI/FEDER/UE) and PLAS-MED (CTM2017-89701-C3-2-R). Authors acknowledge the
597 support from the Economy and Knowledge Department of the Catalan Government through
598 Consolidated Research Groups ICRA-ENV 2017 SGR 1124 and 2017 SGR 01404. Sara
599 Rodriguez-Mozaz acknowledges the Ramon y Cajal program (RYC-2014-16707).

600

601 **References**

602 [1] J.T. Kim, Y.J. Choi, M. Barghi, J.H. Kim, J.W. Jung, K. Kim, J.H. Kang, G. Lammel,
603 Y.S. Chang, Occurrence, distribution, and bioaccumulation of new and legacy
604 persistent organic pollutants in an ecosystem on King George Island, maritime
605 Antarctica, J. Hazard. Mater. 405 (2021) 124141.
606 https://doi.org/10.1016/j.jhazmat.2020.124141.

607 [2] M. Alvarez-Muñoz, Diana; Farré, ENVIRONMENTAL METABOLOMICS
608 APPLICATIONS IN FIELD AND LABORATORY STUDIES., ELSEVIER, 2020.

609 [3] Y. Picó, D. Barceló, Transformation products of emerging contaminants in the
610 environment and high-resolution mass spectrometry: A new horizon, Anal. Bioanal.
611 Chem. 407 (2015) 6257–6273. https://doi.org/10.1007/s00216-015-8739-6.

612 [4] L. Soliño, P.R. Costa, Differential toxin profiles of ciguatoxins in marine organisms:
613 Chemistry, fate and global distribution, Toxicon. 150 (2018) 124–143.
614 https://doi.org/10.1016/j.toxicon.2018.05.005.

615 [5] European Commission, The combination effects of chemicals. Chemical mixtures,
616 Communication from the Commission to the Council, Brussels, (2012).

617 [6] N.G. Dodder, K.A. Maruya, P. Lee Ferguson, R. Grace, S. Klosterhaus, M.J. La
618 Guardia, G.G. Lauenstein, J. Ramirez, Occurrence of contaminants of emerging
619 concern in mussels (Mytilus spp.) along the California coast and the influence of land
620 use, storm water discharge, and treated wastewater effluent, Mar. Pollut. Bull. 81
621 (2014) 340–346. https://doi.org/10.1016/j.marpolbul.2013.06.041.

622 [7] J.A. Campillo, A. Sevilla, M. Albentosa, C. Bernal, A.B. Lozano, M. Cánovas, V.M.
623 León, Metabolomic responses in caged clams, Ruditapes decussatus, exposed to
624 agricultural and urban inputs in a Mediterranean coastal lagoon (Mar Menor, SE

625 Spain), Sci. Total Environ. 524–525 (2015) 136–147.
626 https://doi.org/10.1016/J.SCITOTENV.2015.03.136.
627 [8] D. Álvarez-Muñoz, S. Rodríguez-Mozaz, A.L. Maulvault, A. Tediosi, M. Fernández-
628 Tejedor, F. Van den Heuvel, M. Kotterman, A. Marques, D. Barceló, Occurrence of
629 pharmaceuticals and endocrine disrupting compounds in macroalgaes, bivalves, and
630 fish from coastal areas in Europe, Environ. Res. 143 (2015) 56–64.
631 https://doi.org/10.1016/j.envres.2015.09.018.
632 [9] S. Pizzini, R. Piazza, G. Cozzi, C. Barbante, Simultaneous determination of
633 halogenated contaminants and polycyclic aromatic hydrocarbons: a multi-analyte
634 method applied to filter-feeding edible organisms, Anal. Bioanal. Chem. 408 (2016)
635 7991–7999. https://doi.org/10.1007/s00216-016-9897-x.
636 [10] L. Mijangos, H. Ziarrusta, I. Zabaleta, A. Usobiaga, M. Olivares, O. Zuloaga, N.
637 Etxebarria, A. Prieto, Multiresidue analytical method for the determination of 41
638 multiclass organic pollutants in mussel and fish tissues and biofluids by liquid
639 chromatography coupled to tandem mass spectrometry, Anal. Bioanal. Chem. 411
640 (2019) 493–506. https://doi.org/10.1007/s00216-018-1474-z.
641 [11] D. Álvarez-Muñoz, M. Rambla-Alegre, N. Carrasco, M. Lopez de Alda, D. Barceló,
642 Fast analysis of relevant contaminants mixture in commercial shellfish, Talanta. 205
643 (2019) 119884. https://doi.org/10.1016/j.talanta.2019.04.085.
644 [12] N.A. Alygizakis, P. Oswald, N.S. Thomaidis, E.L. Schymanski, R. Aalizadeh, T.
645 Schulze, M. Oswaldova, J. Slobodnik, NORMAN digital sample freezing platform: A
646 European virtual platform to exchange liquid chromatography high resolution-mass
647 spectrometry data and screen suspects in “digitally frozen” environmental samples,
648 TrAC - Trends Anal. Chem. 115 (2019) 129–137.

649 https://doi.org/10.1016/j.trac.2019.04.008.

650 [13] A.D. Southam, A. Lange, R. Al-Salhi, E.M. Hill, C.R. Tyler, M.R. Viant,
651 Distinguishing between the metabolome and xenobiotic exposome in environmental
652 field samples analysed by direct-infusion mass spectrometry based metabolomics and
653 lipidomics, Metabolomics. 10 (2014) 1050–1058. https://doi.org/10.1007/s11306-
654 014-0693-3.

655 [14] A.J. Atkinson, W.A. Colburn, V.G. DeGruttola, D.L. DeMets, G.J. Downing, D.F.
656 Hoth, J.A. Oates, C.C. Peck, R.T. Schooley, B.A. Spilker, J. Woodcock, S.L. Zeger,
657 Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework,
658 Clin. Pharmacol. Ther. 69 (2001) 89–95. https://doi.org/10.1067/mcp.2001.113989.

659 [15] R.M. Califf, Biomarker definitions and their applications, Exp. Biol. Med. 243 (2018)
660 213–221. https://doi.org/10.1177/1535370217750088.

661 [16] I. Senta, S. Rodríguez-Mozaz, L. Corominas, M. Petrovic, Wastewater-based
662 epidemiology to assess human exposure to personal care and household products – A
663 review of biomarkers, analytical methods, and applications, Trends Environ. Anal.
664 Chem. 28 (2020) e00103. https://doi.org/10.1016/j.teac.2020.e00103.

665 [17] D.R. Ekman, K. Keteles, J. Beihoffer, J.E. Cavallin, K. Dahlin, J.M. Davis, A. Jastrow,
666 J.M. Lazorchak, M.A. Mills, M. Murphy, D. Nguyen, A.M. Vajda, D.L. Villeneuve,
667 D.L. Winkelman, T.W. Collette, Evaluation of targeted and untargeted effects-based
668 monitoring tools to assess impacts of contaminants of emerging concern on fish in the
669 South Platte River, CO, Environ. Pollut. 239 (2018) 706–713.
670 https://doi.org/10.1016/J.ENVPOL.2018.04.054.

671 [18] J.M. Davis, T.W. Collette, D.L. Villeneuve, J.E. Cavallin, Q. Teng, K.M. Jensen, M.D.
672 Kahl, J.M. Mayasich, G.T. Ankley, D.R. Ekman, Field-Based Approach for Assessing

673 the Impact of Treated Pulp and Paper Mill Effluent on Endogenous Metabolites of
674 Fathead Minnows (Pimephales promelas), Environ. Sci. Technol. (2013)
675 130830150520002. https://doi.org/10.1021/es401961j.

676 [19] J.M. Davis, D.R. Ekman, Q. Teng, G.T. Ankley, J.P. Berninger, J.E. Cavallin, K.M.
677 Jensen, M.D. Kahl, A.L. Schroeder, D.L. Villeneuve, Z.G. Jorgenson, K.E. Lee, T.W.
678 Collette, Linking field-based metabolomics and chemical analyses to prioritize
679 contaminants of emerging concern in the Great Lakes basin, Environ. Toxicol. Chem.
680 35 (2016) 2493–2502. https://doi.org/10.1002/etc.3409.

681 [20] T.D. Williams, I.M. Davies, H. Wu, A.M. Diab, L. Webster, M.R. Viant, J.K.
682 Chipman, M.J. Leaver, S.G. George, C.F. Moffat, C.D. Robinson, Molecular
683 responses of European flounder (Platichthys flesus) chronically exposed to
684 contaminated estuarine sediments, Chemosphere. 108 (2014) 152–158.
685 https://doi.org/10.1016/J.CHEMOSPHERE.2014.01.028.

686 [21] D.M. Skelton, D.R. Ekman, D. Martinović-Weigelt, G.T. Ankley, D.L. Villeneuve, Q.
687 Teng, T.W. Collette, Metabolomics for in Situ Environmental Monitoring of Surface
688 Waters Impacted by Contaminants from Both Point and Nonpoint Sources, Environ.
689 Sci. Technol. 48 (2014) 140115140646006. https://doi.org/10.1021/es404021f.

690 [22] H. Zhen, D.R. Ekman, T.W. Collette, S.T. Glassmeyer, M.A. Mills, E.T. Furlong,
691 D.W. Kolpin, Q. Teng, Assessing the impact of wastewater treatment plant effluent
692 on downstream drinking water-source quality using a zebrafish (Danio Rerio) liver
693 cell-based metabolomics approach, Water Res. 145 (2018) 198–209.
694 https://doi.org/10.1016/J.WATRES.2018.08.028.

695 [23] H. Wu, A.D. Southam, A. Hines, M.R. Viant, High-throughput tissue extraction
696 protocol for NMR- and MS-based metabolomics, Anal. Biochem. 372 (2008) 204–

697 212. https://doi.org/10.1016/j.ab.2007.10.002.
698 [24] E.G. BLIGH, W.J. DYER, A rapid method of total lipid extraction and purification.,
699 Can. J. Biochem. Physiol. 37 (1959) 911–917. https://doi.org/10.1139/o59-099.
700 [25] L. Glazer, M.C. Kido Soule, K. Longnecker, E.B. Kujawinski, N. Aluru, Hepatic
701 metabolite profiling of polychlorinated biphenyl (PCB)-resistant and sensitive
702 populations of Atlantic killifish (Fundulus heteroclitus), Aquat. Toxicol. 205 (2018)
703 114–122. https://doi.org/10.1016/J.AQUATOX.2018.10.007.
704 [26] M. Park, Y. Lee, A. Khan, P. Aleta, Y. Cho, H. Park, Y.H. Park, S. Kim, Metabolite
705 tracking to elucidate the effects of environmental pollutants, J. Hazard. Mater. (2019)
706 112–124. https://doi.org/10.1016/j.jhazmat.2019.05.024.
707 [27] T. Cappello, M. Maisano, A. Giannetto, V. Parrino, A. Mauceri, S. Fasulo,
708 Neurotoxicological effects on marine mussel Mytilus galloprovincialis caged at
709 petrochemical contaminated areas (eastern Sicily, Italy): 1H NMR and
710 immunohistochemical assays, Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.
711 169 (2015) 7–15. https://doi.org/10.1016/J.CBPC.2014.12.006.
712 [28] S.D. Melvin, O.A.H. Jones, A.R. Carroll, F.D.L. Leusch, 1H NMR-based
713 metabolomics reveals interactive effects between the carrier solvent methanol and a
714 pharmaceutical mixture in an amphibian developmental bioassay with Limnodynastes
715 peronii, Chemosphere. 199 (2018) 372–381.
716 https://doi.org/10.1016/J.CHEMOSPHERE.2018.02.063.
717 [29] T. Dumas, B. Bonnefille, E. Gomez, J. Boccard, N.A. Castro, H. Fenet, F. Courant,
718 Metabolomics approach reveals disruption of metabolic pathways in the marine
719 bivalve Mytilus galloprovincialis exposed to a WWTP effluent extract, Sci. Total
720 Environ. 712 (2020) 136551. https://doi.org/10.1016/j.scitotenv.2020.136551.

721 [30] S.S.Y. Huang, J.P. Benskin, B. Chandramouli, H. Butler, C.C. Helbing, J.R. Cosgrove,
722 Xenobiotics Produce Distinct Metabolomic Responses in Zebrafish Larvae (Danio
723 rerio), Environ. Sci. Technol. 50 (2016) 6526–6535.
724 https://doi.org/10.1021/acs.est.6b01128.
725 [31] K.J. Jeppe, K.A. Kouremenos, K.R. Townsend, D.F. Macmahon, D. Sharley, D.L.
726 Tull, A.A. Hoffmann, V. Pettigrove, S.M. Long, Metabolomic profiles of a midge
727 (Procladius villosimanus, kieffer) are associated with sediment contamination in urban
728 wetlands, Metabolites. 7 (2017). https://doi.org/10.3390/metabo7040064.
729 [32] A. Previšić, M. Rožman, J.R. Mor, V. Acuña, A. Serra-Compte, M. Petrović, S.
730 Sabater, Aquatic macroinvertebrates under stress: Bioaccumulation of emerging
731 contaminants and metabolomics implications, Sci. Total Environ. 704 (2019) 135333.
732 https://doi.org/10.1016/j.scitotenv.2019.135333.
733 [33] A. Roszkowska, M. Yu, V. Bessonneau, J. Ings, M. McMaster, R. Smith, L. Bragg,
734 M. Servos, J. Pawliszyn, In vivo solid-phase microextraction sampling combined with
735 metabolomics and toxicological studies for the non-lethal monitoring of the exposome
736 in fish tissue, Environ. Pollut. (2019) 109–115.
737 https://doi.org/10.1016/j.envpol.2019.03.024.
738 [34] Y. Yang, C. Cruickshank, M. Armstrong, S. Mahaffey, R. Reisdorph, N. Reisdorph,
739 New sample preparation approach for mass spectrometry-based profiling of plasma
740 results in improved coverage of metabolome, J. Chromatogr. A. 1300 (2013) 217–226.
741 https://doi.org/10.1016/j.chroma.2013.04.030.
742 [35] A.L. Heffernan, M.M. Gómez-Ramos, C. Gaus, S. Vijayasarathy, I. Bell, C. Hof, J.F.
743 Mueller, M.J. Gómez-Ramos, Non-targeted, high resolution mass spectrometry
744 strategy for simultaneous monitoring of xenobiotics and endogenous compounds in

745 green sea turtles on the Great Barrier Reef, Sci. Total Environ. 599–600 (2017) 1251–
746 1262. https://doi.org/10.1016/J.SCITOTENV.2017.05.016.
747 [36] R. Al-Salhi, A. Abdul-Sada, A. Lange, C.R. Tyler, E.M. Hill, The xenometabolome
748 and novel contaminant markers in fish exposed to a wastewater treatment works
749 effluent, Environ. Sci. Technol. 46 (2012) 9080–9088.
750 https://doi.org/10.1021/es3014453.
751 [37] D.B.D. Simmons, J. Miller, S. Clarence, E.S. McCallum, S. Balshine, B.
752 Chandramouli, J. Cosgrove, J.P. Sherry, Altered expression of metabolites and
753 proteins in wild and caged fish exposed to wastewater effluents in situ, Sci. Rep. 7
754 (2017). https://doi.org/10.1038/s41598-017-12473-6.
755 [38] A. David, A. Lange, A. Abdul-Sada, C.R. Tyler, E.M. Hill, Disruption of the
756 Prostaglandin Metabolome and Characterization of the Pharmaceutical Exposome in
757 Fish Exposed to Wastewater Treatment Works Effluent As Revealed by Nanoflow-
758 Nanospray Mass Spectrometry-Based Metabolomics, Environ. Sci. Technol. 51
759 (2017) 616–624. https://doi.org/10.1021/acs.est.6b04365.
760 [39] J.D.. Mosley, D.R.. Ekman, J.E.. Cavallin, D.L.. Villeneuve, G.T.. Ankley, T.W.
761 Collette, High-resolution mass spectrometry of skin mucus for monitoring
762 physiological impacts and contaminant biotransformation products in fathead
763 minnows exposed to wastewater effluent, Environ. Toxicol. Chem. 37 (2018) 788–
764 796.
765 [40] N.D. Wagner, P.A. Helm, A.J. Simpson, M.J. Simpson, Metabolomic responses to
766 pre-chlorinated and final effluent wastewater with the addition of a sub-lethal
767 persistent contaminant in Daphnia magna, Environ. Sci. Pollut. Res. (2019) 1–13.
768 https://doi.org/10.1007/s11356-019-04318-8.

769 [41] A.H.M. Emwas, The strengths and weaknesses of NMR spectroscopy and mass
770 spectrometry with particular focus on metabolomics research, Methods Mol. Biol.
771 1277 (2015) 161–193. https://doi.org/10.1007/978-1-4939-2377-9_13.
772 [42] A. David, A. Abdul-Sada, A. Lange, C.R. Tyler, E.M. Hill, A new approach for plasma
773 (xeno)metabolomics based on solid-phase extraction and nanoflow liquid
774 chromatography-nanoelectrospray ionisation mass spectrometry, J. Chromatogr. A.
775 1365 (2014) 72–85. https://doi.org/10.1016/j.chroma.2014.09.001.
776 [43] M. Pourchet, L. Debrauwer, J. Klanova, E.J. Price, A. Covaci, N. Caballero-Casero,
777 H. Oberacher, M. Lamoree, A. Damont, F. Fenaille, J. Vlaanderen, J. Meijer, M.
778 Krauss, D. Sarigiannis, R. Barouki, B. Le Bizec, J.P. Antignac, Suspect and non-
779 targeted screening of chemicals of emerging concern for human biomonitoring,
780 environmental health studies and support to risk assessment: From promises to
781 challenges and harmonisation issues, Environ. Int. 139 (2020) 105545.
782 https://doi.org/10.1016/j.envint.2020.105545.
783 [44] C. Guijas, J.R. Montenegro-Burke, X. Domingo-Almenara, A. Palermo, B. Warth, G.
784 Hermann, G. Koellensperger, T. Huan, W. Uritboonthai, A.E. Aisporna, D.W. Wolan,
785 M.E. Spilker, H.P. Benton, G. Siuzdak, METLIN: A Technology Platform for
786 Identifying Knowns and Unknowns, Anal. Chem. 90 (2018) 3156–3164.
787 https://doi.org/10.1021/acs.analchem.7b04424.
788 [45] MassBank | European MassBank (NORMAN MassBank) Mass Spectral DataBase,
789 (n.d.). http://massbank.eu/MassBank/Index (accessed February 18, 2019).
790 [46] K. Dührkop, M. Fleischauer, M. Ludwig, A.A. Aksenov, A. V. Melnik, M. Meusel,
791 P.C. Dorrestein, J. Rousu, S. Böcker, SIRIUS 4: a rapid tool for turning tandem mass
792 spectra into metabolite structure information, Nat. Methods. 16 (2019) 299–302.

793 https://doi.org/10.1038/s41592-019-0344-8.
794 [47] PubChem, (n.d.) https://pubchem.ncbi.nlm.nih.gov/.
795 [48] P. Gago-Ferrero, A.A. Bletsou, D.E. Damalas, R. Aalizadeh, N.A. Alygizakis, H.P.
796 Singer, J. Hollender, N.S. Thomaidis, Wide-scope target screening of >2000 emerging
797 contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS and smart
798 evaluation of its performance through the validation of 195 selected representative
799 analytes, J. Hazard. Mater. 387 (2020) 121712.
800 https://doi.org/10.1016/j.jhazmat.2019.121712.
801 [49] L. Terrado-Rouera, M. Martínez-López, D. Alvarez-Muñoz, Identification of organic
802 contaminants accumulated in mollusc by means of High Resolution Mass
803 Spectrometry (http://hdl.handle.net/2445/125789), 2018.
804 [50] L. Molina-Millán, M. Martínez-López, D. Álvarez-Muñoz, Treball Final de Grau
805 Non-target analysis of organic contaminants mixtures in mussel from Ebro Delta
806 Anàlisi non-target de mescles de contaminants orgànics en musclos del Delta de l'Ebre
807 (http://diposit.ub.edu/dspace/handle/2445/137961), 2019.
808
809

Table 1: Metabolomics studies conducted to evaluate the effects of exposure to real contaminated waters in biota from aquatic environments. Details about the stressor used, organism, tissue, instrument, strategy followed (target/non-target) and short sample treatment summary.

Article	Stressor	Organism	Tissue	Analysis	Target / Non-target	Sample treatment summary
[7] Campillo 2015	Lagoon (Mar menor) contamination	**Clams**	Digestive gland	MS	Target MS	Addition to sample of 2 mL ACN:10mM KH2PO4, 3:1, v/v at pH 7.4, **incubation**, centrifugation (15000g, 20 min, 4°C),**iquid-liquid extraction** of non-polar compounds with $CHCl_3$ for MS analysis.
[17] Ekman 2018	River (South Platte River) contamination	**Fish**	Liver	NMR	Target / Non-target 1H-NMR	Homogenization of grilled tissue with MeOH (4ml/g) and H2O (0.85 mL/g) by **orbital shake**r, addition of **$CHCl_3$** (2 mL/g) and H2O (2 mL/g), vortex (60 s), centrifugation (1000g, 4 °C,15 min), **polar phase** dried and reconstituted (600 µL D2O buffered 100mM sodium phosphate pH 7.4)
[18] Davis 2013	Lake impacted by Pulp and Paper Mill Effluent (Lake Superior)	**Fish**	Liver	NMR	Non-target H-NMR	Homogenization of grilled tissue with MeOH (4ml/g) and H2O (0.85 mL/g) by **orbital shake**r, addition of **CHCl3** (2 mL/g) and H2O (2 mL/g), vortex (60 s), centrifugation (1000g, 4 °C,15 min), **polar phase** dried and reconstituted (600 µL D2O buffered 100mM sodium phosphate pH 7.4)
[19] Davis 2016	Lakes (5) impacted by different contaminants	**Fish**	Liver	NMR	Non-target H-NMR	Homogenization of grilled tissue with MeOH (4ml/g) and H2O (0.85 mL/g) by **mechanical tissuelyzer**, addition of **$CHCl_3$** (2 mL/g) , tissuelyzed (20 min), addition of H_2O (2 mL/g), centrifugation (3200g, 4 °C,20 min), **polar phase** dried and reconstituted (600 µL D2O buffered 100mM sodium phosphate pH 7.4) and filtered (0.45 µm)
[20] Williams 2014	Contaminated sediments	**Fish**	Liver,muscle, bile and plasma	NMR	Non-target H-NMR	Homogenization of grilled tissue with MeOH (4ml/g) and H2O (0.85 mL/g) by **orbital shaker**, addition of **CHCl3** (2 mL/g) and H2O (2 mL/g), vortex (60 s), centrifugation (1000g, 4 °C,15 min), **polar phase** dried and reconstituted (600 µL D2O buffered 100mM sodium phosphate pH 7.4)
[21] Skelton 2014	Rivers impacted by WWTPs	**Fish**	Liver	NMR & MS	Non-target 1H-NMR & GC-MS	Homogenization of grilled tissue with MeOH (4ml/g) and H2O (0.85 mL/g) by **orbital shaker**, addition of **$CHCl_3$** (2 mL/g) and H2O (2 mL/g), vortex (60 s), centrifugation (1000g, 4 °C,15 min), **polar phase** dried and reconstituted (600 µL D2O buffered 100mM sodium phosphate pH 7.4)
[22] Zhen 2018	River + WWTP effluents	**Fish**	Liver cells	NMR & MS	Non-target H-NMR & GC-MS	Homogeneization of cells in MeOH (15 min), **tissuelyzed**, addition of **$CHCl_3$**(0.24 mL), homogeneized (20 min), addition of H2O (0.22 mL), homogeneized (15 min), centrifuged (3000g, 15 min), **polar phase** led to dryness and reconstituted (0.1 M sodium phosphate buffered D2O containing 20µM TSP), **lipidic part** led to dryness and reconstituted (CDCl3:CD3OD (2:1) containing 1mM TMS)
[25] Glazer 2018	Estuary PCB-contaminated area	**Fish**	Liver	MS	Target LC-MS	Snap-frozen sample extracted with MeOH:H2O (1mL:212.5µL), **vortexed** 60s,addition of **$CHCl_3$** (0.5mL), vortexed 60s, **incubated** in ice 10 min (shaking every 60s), addition of 0.5mL CHCl3 and 0.5mL H2O ,

						vortexed 60 s, centrifuged (1000g, 15 min, 4°C), **polar fraction** vacuum dried and reconstituted in ACN:H_2O (5:95).
[26] Park 2019	Contamination of Nakdong river by Zinc industry	**Fish**	Liver (For Zebrafish of lab, whole organism)	MS	Non-target HPLC-HRMS	Liophilized sample (1g) extracted with MeOH (1.6 mL) and H_2O (0.6 mL), **vortexed** (5 min), addition of H_2O (0.8 mL) and **$CHCl_3$** (0.8 mL), **vortexed** (5 min), incubated in ice bath (15 min), **centrifuged** (1000g, 4°C, 15 min), **polar fraction** freeze dried and reconstituted with mobile phases.
[27] Capello 2015	Petrochemical contaminated area	**Mussels**	Tissue	NMR	Target H-NMR	Homogenization of grilled tissue with MeOH (4ml/g) and H2O (0.85 mL/g) by **Ultraturrax**, addition of **CHCl3** (2 mL/g) and H2O (2 mL/g), vortex, centrifugation (2000g, 4 °C, 5 min), **polar phase** dried and reconstituted (100 µL D2O buffered 240mM sodium phosphate pH 7.0)
[28] Melvin 2018	Contaminated area with metalloids	**Fish**	whole body	NMR	Non-target H-NMR	Homogeneization with ice-cold MeOH (400 µg/L) with **Ultraturrax**, sonication, incubation (1h, -20 °C), addition of $CHCl_3$ (800 µL), H_2O (200 µL), vortexed, centrifuged (16000g, 4°C, 10 min) and stored at -80 °C.
				NMR		**Sonication** (15 min) of liophilized sample (1 mg) with 0.2M phosphate buffer in D2O containing 0.1 w/v sodium azide (45 µL), centrifuged (15000g, 4°C, 20 min) and stored
[29] Dumas 2020	WWTP eluted extracts from effluent	**Mussels**	Digestive gland	MS	Non-target MS	Addition of 0.25mL MeOH and 75 µL H_2O to 30mg sample, **vortex** 60s, addition of 0.24mL CH_2Cl_2 and 0.12 mL H_2O, **vortex** 60s, centrifuged (2000g, 15 min, 4°C) and 50 µL supernatant led to dryness and reconstituted witn 0.2 mL ACN:H_2O (5:95, v/v) and filtered with 0.2µm PTFE sytnge filter
[30] Huang 2016	Exposition to Exogenous endocrine compound, Performance chemicals, PhACs and PCPs, Petroleum derivative, heavy metals and EWW	**Fish**	Whole organism	MS	Target HPLC-MS/MS	**Bead beating** (2 min) with **ZrO_2** (100 mg), MeOH (200 µL), centrifugation (10000rpm, 30s), supernatant collection, **CHCl3** addition (200 µL), centrifugation (10000rpm, 30s), **mix of both extracts**, One portion derivatized with phenylisothiocyanate, Other Nunc 96-deep well plate extraction with 5 mM CH_3COONH_4 in MeOH, divided in 2 aliquots, one diluted with MeOH (lipid analysis) and other with H_2O (Bile acids, FAs and hexoses).
[31] Jeppe 2017	Contamination of animals exposed to different sediments	**Mosquito**	Whole organism	MS	Target GC-MS and target LC-MS	**Bead beating** with ceramic lysisng beads (6800rpm, -10 °C), **ice-cold MeOH** (330 µL) and H2O (110 µL), addition of ice-cold **$CHCl_3$** (110 µL), shaken (15 min, 2°C),addition of H_2O (220 µL), centrifuged (14000g, 0°C, 5min) and upper phase was stored.
[32] Previšić 2020	WWTP effluent impact in river	**Insect larvae**	Whole body	MS	Non-target HRMS	**Freeze-dried** samples were **sonicated** (3 cycles of 120s at 30% in ultrasonic probe) with MeOH. Evaporated to dryness, redosilved in H_2O with EDTA 1%. Extraction with **Oasis HLB**, elution with MeOH, dryness and reconstitution with MeOH:H_2O (10:90)
[33] Roszkowska 2019	Contamination of Athabasca river by pulp and paper mill	**Fish**	SPME from muscle	MS	Non-target LC-HRMS	**PAN-C18** coated blade inserted in dorsal-epaxial muscle (20 min), rinsed with nanopure H_2O (10 s), frozen, desorpted in vortex agitation (90min, 1000rpm) with ACN:H_2O (80:20)

[35] Heffernan 2017	Contaminated bays	**Turtles**	Plasma	MS	Non-target HRMS	Plasma samples (1 g) mixed with **ACN** (3 mL) and H_2O (1 mL), 0.2g NaCl, 1g anhidrous $MgSO_4$ and c**eramic homogeneizer, manually shaken**, centrifuged (3700rpm, 10 min, 4°C), **stored at -20°C** for lipid precipitation and supernatant acidified with 0.1% Formic acid, filtered through a **lipid cartridge**, evaporated to near dryness, reconstituted with MeOH:H_2O (20:80)
[36] Al-Salhi 2012	WWTP effluents	**Fish**	Plasma and bile	MS	Non-target HRMS and GC-MS	Plasma was **deproteinized with ice-cold MeOH** (sample 20%). Bile diluted 50-fold with MeOH:H_2O (1:1, v/v)
[37] Simmons 2017	Contamination of Hamilton Harbour by WWTP	**Fish**	Plasma	MS	Target HPLC-MS/MS	Plasma sample (10 µL) added to a **96-well filter plate**, Phenylisothiocyanate addition, dried, addition of 5mM CH_3COONH_4 in MeOH(250 µL), shaken (30 min), eluted to a Nunc 96-deep well plate by centrifugation (100g, 2 min), diluted with MeOH.
[38] David 2017	WWTP effluents	**Fish**	Plasma, gonads, gill, liver and kidney tissues	MS	Non-target HRMS	Tissues were mixed with **MeOH**, sonicated (30s), centrifuged and supernatant diluted with 20% H2O. Tissues extract and **plasma** passed through **Phree plates (Phospholipid and protein removal**), MeOH 1% formic acid addition (100 µL), extraction with **Strata-X-C**, elution with 5% NH_4OH MeOH, Ethyl acetate, dryness and reconstitution with MeOH:H_2O (20:80).
[39] Mosley 2018	WWTP infl + effl	**Fish**	Skin mucus	MS	Non-target HRMS	**Glass-fiber filter strip** blotted in fish skin mucus, extraction with ice-cold MeOH (400 µL), centrifuged (10 min, 4°C), vacuum dried and reconstitution with ACN:H_2O (1:19, v/v, 150 µL).
[40] Wagner 2019	Influent (post-secondary clarification) and Effluent Wastewater (EWW) with PFOSs addition in EWW	**Crustacean**	Whole organism	MS	Target H-NMR and target LC-MS/MS	**Sonication** (15 min) with MeOH:H_2O (80:20, 200 µL), incubation (1h, 4°C), centrifugation (13000g at 4°C for 20 min), N_2 dryness, resuspension with Mobile phases

Table 2: Metabolomics studies conducted to evaluate the effects of exposure to real contaminated waters in biota from aquatic environments. Details about the stressor used, organism, kind of exposure (lab or field) and number of detected xenobiotics (Xenometabolomics column) and endogenous metabolites (Metabolomics column).

Article	Stressor	Organism	Exposure	Xeno metabolomics	Metabolomics
[7] Campillo 2015	Lagoon (Mar menor) contamination	**Clams**	Field (Caged)	NO	**74 compounds**
[17] Ekman 2018	River (South Platte River) contamination	**Fish**	Field (Caged)	NO	**4 compounds**
[18] Davis 2013	Lake impacted by Pulp and Paper Mill Effluent (Lake Superior)	**Fish**	Field (Caged)	NO	**18 compounds**
[19] Davis 2016	Lakes (5) impacted by different contaminants	**Fish**	Field (Caged)	NO	**21 compounds**
[20] Williams 2014	Contaminated sediments	**Fish**	Lab	NO	**18 compounds**
[21] Skelton 2014	Rivers impacted by WWTPs	**Fish**	Field (Caged)	NO	**12 compounds**
[22] Zhen 2018	River + WWTP effluents	**Fish**	Lab	NO	**31 compounds**
[25] Glazer 2018	Estuary PCB-contaminated area	**Fish**	Field (Wild animals)	NO	**72 compounds**
[26] Park 2019	Contamination of Nakdong river by Zinc industry	**Fish**	Field (Wild animals)	NO	**6 compounds**
[27] Capello 2015	Petrochemical contaminated area	**Mussels**	Field (Caged)	NO	**3 compounds**
[28] Melvin 2018	Contaminated area with metalloids	**Fish**	Field (Caged)	YES (13 metals)	**34 compounds**
[29] Dumas 2020	WWTP eluted extracts from effluent	**Mussels**	Lab	NO	**39 compounds**
[30] Huang 2016	Exposition to Exogenous endocrine compound, Performance chemicals, PhACs and PCPs, Petroleum derivative, heavy metals and EWW	**Fish**	Lab	NO	**208 compounds**
[31] Jeppe 2017	Contamination of animals exposed to different sediments	**Mosquito**	Field (Wild animals)	NO	**177 compounds**
[32] Previšić 2020	WWTP effluent impact in river	**Insect larvae**	Field (caged)	YES (5 compounds)	**32 compounds**
[33] Roszkowska 2019	Contamination of Athabasca river by pulp and paper mill	**Fish**	Field (Wild animals)	YES (42 compounds)	**137 compounds**

[35] Heffernan 2017	Contaminated bays	**Turtles**	Field (Wild animals)	YES (13 compounds)	**10 compounds**
[36] Al-Salhi 2012	WWTP effluents	**Fish**	Lab	YES (236 compounds)	**8 compounds**
[37] Simmons 2017	Contamination of Hamilton Harbour by WWTP	**Fish**	Field (Wild animals)	YES (15 compounds)	**159 compounds**
[38] David 2017	WWTP effluents	**Fish**	Lab	YES (54 compounds)	**10 compounds**
[39] Mosley 2018	WWTP infl + effl	**Fish**	Lab	YES (4 compounds)	**30 compounds**
[40] Wagner 2019	Influent (post-secondary clarification) and Effluent Wastewater (EWW) with PFOSs addition in EWW	**Crustacean**	Lab	NO	**18 compounds**

Figure 1. Number of times that a pathway has been reported as disrupted in the experiments carried out with aquatic organisms exposed to contaminant mixtures (natural and spiked waters) presented in Table 2. In red, pathways reported as altered in more papers (>8 papers), followed by orange (4-8 papers), yellow (3 papers), blue (2 papers) and green (only 1 paper).