

(Xeno)metabolomics for the evaluation of aquatic organism's exposure to field contaminated water



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ABSTRACT

Environmental (xeno)metabolomics offers a major advantage compared to other approaches for the evaluation of aquatic organism's exposure to contaminated water because it allows the simultaneous profiling of the xenometabolome (chemical xenobiotics and their metabolites accumulated in an organism exposed to environmental contaminants) and the metabolome (endogenous metabolites whose levels are altered due to an external stressor). This approach has been widely explored in lab exposure experiments, however in field 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.

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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 transport 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

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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 to fill this knowledge gap. Particularly, environmental (xeno)metabolomics offers a major advantage compared to other approaches because, by comparing a control group with an exposed group, both the xenometabolome or exposome (chemical compounds and their metabolites present in an organism as a result of environmental exposure and that are not naturally expected in the studied organism) and the metabolome (endogenous metabolites whose levels are altered due to an external stressor) can be simultaneously profiled [2]. This approach has been widely explored in lab exposure experiments, where exposure conditions can be closely controlled, while in field studies environmental (xeno)metabolomics has only been started in the last few years, despite being a powerful tool for the assessment of chemical and biological health status of an ecosystem. Its popularity for the analysis of biological samples directly from the field is rapidly increasing and it is anticipated to escalate as metabolomics becomes a more routine tool for environmental monitoring [13]. In fact, the application of this approach in the environmental field started 10 years ago, but it has been in the last 5 years when it has gained a lot of attention from the scientific community.

Both, xenometabolomics and metabolomics in environmental science shares the goal of obtaining biomarkers of (chemical) exposure and/or effect. In line with these definitions, a biomarker in experimental biology has been "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention" [14,15]. This definition only refers to endogenous compounds altered by an external cause. However, xenobiotic' metabolites or transformation products generated by its metabolism could also be named biomarkers, as they can be considered "endogenous metabolites" though derived from a xenobiotic source [16]. Thus, in this review we will define biomarkers derived from xenobiotic sources (and not normally present in the studied organism) as "biomarkers of exposure", while endogenous biomarkers (normally present in the studied organism) will be named "biomarkers of effect".

This paper presents studies addressing different (xeno)metabolomics approaches for the evaluation of aquatic organisms exposed to contaminated water, where bioaccumulated xenobiotics, biomarkers of exposure and biomarkers of effect have been studied, together with their current limitations and future perspectives.

2. Analytical methods applied

2.1. Sample pre-treatment

A total of 22 studies have been published in the last decade (from 2011 to 2020), where (xeno)metabolomics approaches have been applied for testing the effects of exposure to contaminated water in aquatic organisms (Table 1). In these scientific articles,

liver has been the tissue of preference for analysis (9 articles) followed by the whole organism (5 papers). Other tissues such as gonads or digestive gland have also been analysed (3 articles) as well as biofluids like plasma or serum (4 papers) or skin mucus (1 article). An in-vivo extraction with Solid Phase MicroExtraction (SPME) from muscle has also been performed in one occasion to conduct a metabolomics study. Depending on the matrix type, different sample pre-treatments have been used.

2.1.1. Liver

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

Several works [17–22] have applied in their studies with liver samples the extraction the method proposed by Wu et. al [23] followed by a clean-up procedure for lipids extraction reported by Bligh & Dyer [24]. This method consists on the extraction of homogenized tissue with methanol (MeOH) (4 mL/g) and water (H₂O) (0.85 mL/g) in an orbital shaker (or enhanced with a tissuelyzer), followed by the addition of chloroform (CHCl₃) (2 mL/g) and H₂O (2 mL/g) forming two phases (with a final proportion of MeOH:H₂O:CHCl₃, 2 : 1.425 : 1). After this step, dryness followed by reconstitution with deuterated water (D₂O) buffered with sodium phosphate (pH 7.0–7.4) is usually performed for the analysis. Polar compounds remain in the aqueous phase, while lipids are found in the chloroform, therefore obtaining less interferences in the analysis of the polar analytes.

Other studies have followed a similar procedure to this biphasic extraction with little modifications. The biphasic system was obtained by analysing separately polar compounds via Nuclear Magnetic Resonance (NMR) and non-polar via High Resolution Mass Spectrometry (HRMS). In both Glazer [25] and Park [26] studies, they performed the extraction by vortex agitation with MeOH:H₂O, followed by addition of CHCl₃ and H₂O. Then, samples were incubated in an ice-bath and centrifuged (with a final proportion of MeOH:H₂O:CHCl₃ of 2 : 1.425 : 1 (Glazer) and 2 : 1.75 : 1 (Park)). In the case of Glazer and Park, only the polar fraction was analysed.

2.1.2. Whole body/other tissues

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

For some analysis, the same strategy proposed for liver (biphasic system) have been followed with little modifications. Cappello et.al. [27], for gill tissue, used ultraturrax homogenization with MeOH, followed by the previously exposed bi-phasic separation (with a final proportion of MeOH:H₂O:CHCl₃ of 2 : 1 : 4 for Melvin [28] et.al. and 2 : 1.425 : 1 for Cappello et.al.).

For the rest of studies, the authors have preferred bead beating extraction for the whole body, incubation [7] or simply vortex agitation [29].

In the case of bead beating, different bead materials have been employed, such as zirconium (Huang et.al. [30], both 100 mg) or ceramic beads (Jeppe et.al [31]). These studies used solvents with different polarities and the biphasic extraction. Huang et.al [30] applied an additional clean-up procedure with Nunc 96-well plates while Jeppe et.al [31] only analysed the polar fraction.

Studies analysing digestive gland carried out an easier extraction protocol. Campillo et.al. [7] incubated digestive gland with a mixture of acetonitrile (ACN):10mM KH₂PO₄, (3 : 1, v/v) and Dumas et.al. [29] preferred to vortex samples following the biphasic extraction (MeOH:H₂O:CHCl₃ of 1 : 0.8 : 1).

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 KH ₂ PO ₄ , 3:1, v/v at pH 7.4, incubation , centrifugation (15000 g, 20 min, 4 °C), liquid-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 (4 mL/g) and H ₂ O (0.85 mL/g) by orbital shaker , addition of CHCl₃ (2 mL/g) and H ₂ O (2 mL/g), vortex (60 s), centrifugation (1000 g, 4 °C, 15 min), polar phase dried and reconstituted (600 µL D ₂ O buffered 100 mM 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 (4 mL/g) and H ₂ O (0.85 mL/g) by orbital shaker , addition of CHCl₃ (2 mL/g) and H ₂ O (2 mL/g), vortex (60 s), centrifugation (1000 g, 4 °C, 15 min), polar phase dried and reconstituted (600 µL D ₂ O buffered 100 mM 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 (4 mL/g) and H ₂ O (0.85 mL/g) by mechanical tissuelyzer , addition of CHCl₃ (2 mL/g), tissuelyzed (20 min), addition of H ₂ O (2 mL/g), centrifugation (3200 g, 4 °C, 20 min), polar phase dried and reconstituted (600 µL D ₂ O buffered 100 mM 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 (4 mL/g) and H ₂ O (0.85 mL/g) by orbital shaker , addition of CHCl₃ (2 mL/g) and H ₂ O (2 mL/g), vortex (60 s), centrifugation (1000 g, 4 °C, 15 min), polar phase dried and reconstituted (600 µL D ₂ O buffered 100 mM 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 (4 mL/g) and H ₂ O (0.85 mL/g) by orbital shaker , addition of CHCl₃ (2 mL/g) and H ₂ O (2 mL/g), vortex (60 s), centrifugation (1000 g, 4 °C, 15 min), polar phase dried and reconstituted (600 µL D ₂ O buffered 100 mM sodium phosphate pH 7.4)
[22] Zhen 2018	River + WWTP effluents	Fish	Liver cells	NMR & MS	Non-target H-NMR & GC-MS	Homogenization of cells in MeOH (15 min), tissuelyzed , addition of CHCl₃ (0.24 mL), homogenized (20 min), addition of H ₂ O (0.22 mL), homogenized (15 min), centrifuged (3000 g, 15 min), polar phase led to dryness and reconstituted (0.1 M sodium phosphate buffered D ₂ O containing 20 µM TSP), lipidic part led to dryness and reconstituted (CDCl ₃ :CD ₃ OD (2:1) containing 1 mM TMS)
[25] Glazer 2018	Estuary PCB-contaminated area	Fish	Liver	MS	Target LC-MS	Snap-frozen sample extracted with MeOH:H ₂ O (1 mL:212.5 µL), vortexed 60 s, addition of CHCl₃ (0.5 mL), vortexed 60 s, incubated in ice 10 min (shaking every 60 s), addition of 0.5 mL CHCl ₃ and 0.5 mL H ₂ O, vortexed 60 s, centrifuged (1000 g, 15 min, 4 °C), polar fraction vacuum dried and reconstituted in ACN:H ₂ O (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	Lipophilized sample (1 g) extracted with MeOH (1.6 mL) and H ₂ O (0.6 mL), vortexed (5 min), addition of H ₂ O (0.8 mL) and CHCl₃ (0.8 mL), vortexed (5 min), incubated in ice bath (15 min), centrifuged (1000 g, 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 (4 mL/g) and H ₂ O (0.85 mL/g) by Ultraturrax , addition of CHCl₃ (2 mL/g) and H ₂ O (2 mL/g), vortex, centrifugation (2000 g, 4 °C, 5 min), polar phase dried and reconstituted (100 µL D ₂ O buffered 240 mM sodium phosphate pH 7.0)
[28] Melvin 2018	Contaminated area with metalloids	Fish	whole body	NMR	Non-target H-NMR	Homogenization with ice-cold MeOH (400 µg/L) with Ultraturrax , sonication, incubation (1 h, -20 °C), addition of CHCl ₃ (800 µL), H ₂ O (200 µL), vortexed, centrifuged (16000 g, 4 °C, 10 min) and stored at -80 °C.
[29] Dumas 2020	WWTP eluted extracts from effluent	Mussels	Digestive gland	MS	Non-target MS	Addition of 0.25 mL MeOH and 75 µL H ₂ O to 30 mg sample, vortex 60 s, addition of 0.24 mL CH ₂ Cl ₂ and 0.12 mL H ₂ O, vortex 60 s, centrifuged (2000 g, 15 min, 4 °C) and 50 µL supernatant led to dryness and reconstituted with 0.2 mL ACN:H ₂ O (5:95, v/v) and filtered with 0.2 µm PTFE syringe 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 (10000 rpm, 30 s), supernatant collection, CHCl₃ addition (200 µL), centrifugation (10000 rpm, 30 s), mix of both extracts , One portion derivatized with phenylisothiocyanate, Other Nunc 96-deep well plate extraction with 5 mM CH ₃ COONH ₄ in MeOH, divided in 2 aliquots, one diluted with MeOH (lipid analysis) and other with H ₂ O (Bile acids, FAs and hexoses).

Table 1 (Continued)

Article	Stressor	Organism	Tissue	Analysis	Target / Non-target	Sample treatment summary
[31] Jepe 2017	Contamination of animals exposed to different sediments	Mosquito	Whole organism	MS	Target GC-MS and target LC-MS	Bead beating with ceramic lysis beads (6800 rpm, -10 °C), ice-cold MeOH (330 µL) and H ₂ O (110 µL), addition of ice-cold CHCl₃ (110 µL), shaken (15 min, 2 °C), addition of H ₂ O (220 µL), centrifuged (14000 g, 0 °C, 5 min) 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 120 s at 30% in ultrasonic probe) with MeOH. Evaporated to dryness, redissolved in H ₂ O with EDTA 1%. Extraction with Oasis HLB , elution with MeOH, dryness and reconstitution with MeOH:H ₂ O (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 ₂ O (10 s), frozen, desorbed in vortex agitation (90 min, 1000 rpm) with ACN:H ₂ O (80:20)
[35] Heffernan 2017	Contaminated bays	Turtles	Plasma	MS	Non-target HRMS	Plasma samples (1 g) mixed with ACN (3 mL) and H ₂ O (1 mL), 0.2 g NaCl, 1 g anhydrous MgSO ₄ and ceramic homogenizer, manually shaken , centrifuged (3700 rpm, 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 ₂ O (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 ₂ O (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 5 mM CH ₃ COONH ₄ in MeOH (250 µL), shaken (30 min), eluted to a Nunc 96-deep well plate by centrifugation (100 g, 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 (30 s), centrifuged and supernatant diluted with 20% H ₂ O. 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 ₄ OH MeOH, Ethyl acetate, dryness and reconstitution with MeOH:H ₂ O (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 ₂ O (1:19, v/v, 150 µL).
[40] Wagner 2019	Influent (post-secondary clarification) and Effluent Wastewater (EWW) with PFOs addition in EWW	Crustacean	Whole organism	MS	Target H-NMR and target LC-MS/MS	Sonication (15 min) with MeOH:H ₂ O (80:20, 200 µL), incubation (1 h, 4 °C), centrifugation (13000 g at 4 °C for 20 min), N ₂ dryness, resuspension with Mobile phases

Previšić et al. [32] sonicated aquatic invertebrate samples with a sonication probe, cleaning the extract with a SPE procedure. The use of SPE can help to reduce the number of interferences, enhancing the possibilities of detecting xenobiotics in the sample. However, there is a potential loss of other compounds that may be not retained in the cartridge.

Whole body or tissues sampling is an invasive technique as they require scarifying the animals. For this reason, other studies have proposed different non-lethal and less invasive options to study the (xeno)metabolome such as the analysis of external parts of the organisms performing an in-vivo solid phase extraction. In this sense, Roszkowska et al. [33] inserted a C18-coated blade in field fish muscles, which was desorbed with ACN:H₂O (20:80) and directly analysed by MS. This strategy shows an interesting non-slaughtering sampling alternative, which avoids sacrifice and allows applying (xeno)metabolomics in tissues.

2.1.3. Biofluids

An alternative less-invasive approach is the extraction of biofluids such as plasma or skin mucus. This type of sample is easy to obtain and the organism can be safely returned to the environment after taking it, avoiding animals' sacrifice. Plasma (or serum) has been pointed out as an interesting biofluid in the

metabolomics analysis because it offers information regarding the health status of the organism as it contains many endogenous metabolites. Moreover, xenobiotics, as for instance polar contaminants, are also found there and they can be distributed to other tissues through it. Finally, it makes possible to study contaminants mixtures accumulated in wild individuals as well as their effects in vulnerable populations, as sacrifice is not necessary.

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

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

and kidney with the same treatment, performing samples extraction by sonication with MeOH, applying then the same clean-up procedure.

Mosley et al. [39] studied skin mucus of fathead minnows with a simple blotting with glass-fiber filter strips, which were eluted with ice-cold MeOH, vacuum dried and reconstituted with ACN:H₂O (1:19).

As a take home message of this section, full body extractions with bead beater have been preferred by most of the authors for analysing whole body samples using a polar and non-polar biphasic system (water:methanol:chloroform) also described for liver tissues. This approach helps reducing matrix effect of these complex matrices and therefore its use is recommended. Finally, a deproteinization step in biofluids analysis is mandatory when analysing them directly. This is usually achieved by using organic solvents such as acetonitrile (ACN) or ice-cold methanol, which allows to precipitate and separate the proteins from the rest of the fluids. Moreover, the use of ACN as solvent avoids the requirement of working with freeze-dried solvents, preventing incomplete precipitation and further problems as column damage.

2.2. Analysis by HRMS vs NMR

Two analytical platforms are mainly used to carry out metabolomics studies: Nuclear Magnetic Resonance (NMR) employed in 9 studies [17–22,27,28,40], low- or high-resolution Mass Spectrometry (MS or HRMS) in 16 [7,21,35–40,22,25,26,29–33], whereas both instrumental set-ups were applied in 3 out of 22 papers.

The pros and cons of NMR and HRMS for metabolomic applications have been widely discussed in the literature [41]. On the one hand, NMR are non-invasive and non-destructive procedures, and they have high reproducibility and greater elucidation power for unknown compounds than HRMS. On the other hand, MS and HRMS have higher sensitivity, achieving the detection of lower concentrations for target and non-target compounds. Consequently, in a non-target analysis more compounds can be detected using MS than NMR. Concretely in the articles reported in Table 1 up to 208 compounds were detected using MS techniques, while between 3 and 31 compounds were observed when NMR was selected as analysis technique. In reference to xenometabolomics applications, none of the articles reviewed used NMR to monitor xenobiotics, most likely due to their lower sensitivity.

An important drawback of MS is that the sample treatment required is usually more challenging than in NMR and some compounds could be lost during those extraction procedures. Solid samples (e.g. liver or other tissues) must be extracted with solvent in order to be in a liquid phase for their analysis by liquid chromatography, and further converted in gas phase (usually by Electrospray) for their ionization and MS detection. In the case of liquid samples (e.g. plasma or serum), their deproteinization is mandatory before liquid-chromatography analysis for avoiding any clogging and damage of the chromatographic column due to protein precipitation. By applying this step, the poor ionization efficiency, the potential detector saturation or any matrix effects caused by proteins or phospholipid species in MS detectors [42] are reduced, as well as the potential loss of compounds of interest.

As we have previously stated, a critical comparison of NMR and MS is out of the scope of this manuscript, and can be checked more deeply in the literature [41]. However, considering NMR and MS (MS/MS or HRMS) benefits and drawbacks, the use of HRMS is encouraged, as it allows the analysis of low concentrated compounds, both endogenous and xenobiotic compounds, which cannot be studied with the less sensitive NMR instruments.

2.3. Target / non-target strategy

In the previous sections, sample treatment and instrument selection have been exposed. In this subsection, the data acquisition strategy followed for the analysis of metabolites and contaminants is discussed. There are two main approaches regarding compound preselection, target and non-target strategies. Target analysis is based on the identification and quantification of a previously selected set of compounds, depending on their expected relevance in the experiment. For instance, in the case of MS/MS based target analysis, both parent and daughter ions are selected before acquisition experiments. Normally, a validated method based on the comparison with corresponding analytical standards is applied in order to quantify these compounds. However, information about any other ion from the matrix not included in the acquisition list will not be obtained *a posteriori*. This strategy has been followed in 7 of the papers published [7,17,25,27,28,30,31] (Table 1).

Non-target strategies are based on the full-scan data acquisition (in the case of MS based approaches) and statistical data treatment to mark features that better explain the differences between groups of samples. In this strategy, the lack of information before acquisition makes more challenging the identification of the compounds that vary the most between the groups (filters are usually applied to decrease the dataset size), but offers the possibility to move from the classical hypothesis-driven research to a data generating hypothesis-driven approach, more interesting in metabolomics experiments [43]. This powerful tool allows performing post-target analysis of the acquired data, clarifying the highlighted results, or even revealing new hypothesis previously not observed, which is very useful in xenometabolomics studies in order to search and identify chemicals. This strategy has been used in most of the studies reviewed in this paper, in 16 scientific articles (Table 1). It is based on the comparison of areas between a control group of a certain organism and a group of organisms exposed to a particular stressor. It is mainly a qualitative technique, but quantification of compounds can be performed after identification if chemical standards are available.

The main bottleneck of non-target strategies, as stated before, is the elucidation process for highlighted compounds. On-line spectral databases (e.g. METLIN [44], Massbank [45] or MZCloud, among others) are very useful for the identification of compounds and are constantly being expanded. However, not all the compounds detected are registered and available to check in these databases (i.e. TPs of some contaminants).

Some in-silico software (e.g. SIRIUS 4 [46]) have also appeared for fulfilling this gap, allowing to search possible candidate identities for compounds included in databases (e.g. PubChem [47]) without spectral information. Comparing experimental tandem mass spectra information with in-silico spectra prediction, a candidate list can be shortened, reducing elucidation time for molecules not included in databases.

Other studies have employed both target and non-target strategies with more than a single analytical platform (combining NMR with MS, or LC-MS with GC-MS) to obtain widened information [17,21,28,31]. However, with the continuous increase in sensitivity of HRMS instruments combined with their versatility, the use of a single instrument in full-scan acquisition (in the so-called data independent or data dependent acquisition modes) have made possible to perform both kind of analysis in the same run. As previously pointed out and shown in the literature, full scan acquisition offers information about both contaminants (which can be impacting the metabolism) and endogenous metabolites present in the sample that are up or down regulated because of the chemical stress. Therefore, HRMS allows the possibility of

performing *a-posteriori* suspect analysis of compounds, which had not been targeted before acquisition.

As an example, Gago-Ferrero et al. [48] performed suspect analysis of more than 2000 compounds by means of UPLC- HRMS in a single run and in full-scan acquisition mode. This wide-scope screening is based on a previously defined set of compounds (2316 different substances) and, only those detected were further quantified in wastewater using a calibration curve (target analysis). The same data also allows to perform non-target analysis, opening the possibility to widen the number of identified compounds. The same approach has been applied to organisms in the XENOMETABOLOMIC project (CTM2015–73179-JIN, AEI/FEDER/UE). A target method using HRMS was developed for the analysis of a mixture of relevant contaminants in mussels [11]. The compounds included in the method were quantified in mussels from Ebro Delta (Spain) [49]. Later on, a non-target approach was applied allowing the identification of other significant contaminants previously not included in the method [50]

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

3. (Xeno)metabolomics approaches in environmental studies

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

3.1. Xenometabolomics and biomarkers of exposure

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

3.1.1. Lab exposure to real contaminated waters

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

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

effluent (BPA, 1,7-dimethylxanthine, cotinine and triclosan transformation products).

3.1.2. Field exposure

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

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

3.2. Metabolomics and biomarkers of effect

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

3.2.1. Lab exposure to real contaminated waters

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

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

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

of fluoxetine, strong effects on the metabolome profile were observed in combination with the effluent, while for PFOS the same effect was not observed probably due to possible interactions affecting bioavailability.

Al-Salhi et.al. [36] exposed rainbow trout to WWE during 10 days, when some of the fish were sampled at the end of the exposure period and two subgroups were transferred to clean water for depuration during 4 and 7 days respectively. They observed a significant increase in the plasma concentration of the bile acids cyprinol sulfate, taurocholic acid and lysophospholipids, and a decrease in the lipid sphingosine. These findings showed the disruption of bile acid and lipid homeostasis with probable consequences for cellular signalling and maintenance of cell membrane integrity. However, after 11 days of depuration, with around 90–100 % of xenobiotics elimination, all markers returned to normal levels and the initial health status of organisms was nearly recovered.

David et.al. [38] exposed *Rutilus rutilus* to 100 % wastewater effluent observing mortality of 8 out of 60 fish. The plasma of survivors was collected and analysed by nanoflow Ultra High Performance Liquid chromatography– High Resolution Mass Spectrometry (nUHPLC-HRMS). The authors observed alterations in tryptophan metabolism, bile acid metabolism as well as serotonin metabolism and sphinganine signalling disruption. Mosley et. al [39] also exposed sexually mature fish to different WWE dilutions (5, 20 and 100 %), observing 52 altered pathways related with energy, amino acids, oxidative stress, nitrogen, vitamins and phospholipid metabolism among others, and including biotransformed xenobiotics.

Zhen et.al. [22], exposed liver cells to 75 % concentration extract of wastewater effluent and some other river waters up- and downstream. They also found alterations in energy metabolism, oxidative degradation and amino acid metabolism. This study demonstrated the utility of cell-based metabolomics for assessing the biological effects of contaminant mixtures.

Water has been deeply explored as the main source of contamination of aquatic environments. However, lipophilic and less polar compounds tend to be present in sediments at higher levels than in water. In the study by Williams [20] European flounders were exposed to contaminated estuarine sediments in a mesocosms study. They observed alterations in liver immune response by means of transcriptomic analysis of the fish samples. They also found eighteen altered tentative metabolites using a NMR-based metabolomics although their identity was not further confirmed. Dumas et. al [29] performed a SPE extraction from a WWTP effluent to trap lipophilic compounds, and exposed mussels to the extracts. They found alterations in some amino acid metabolisms, but also in purine, pyrimidine, pyruvate and glutathione metabolites.

3.2.2. Field exposure

In order to study the effects of contaminants exposure in the field (an uncontrolled exposure scenario) two different strategies have been followed. Wild animals can be sampled in specific sites in the environment so that their metabolome reflect the stressors present in their natural habitat, including different contaminants. Alternatively, the organisms can be placed in a natural site inside cages for a specific period of time in order to reduce specimen mobility, which is especially critical in the case of fish for instance. That way, organism metabolome profile reflects more accurately the contamination impact of a particular area under study.

Most works published so far have dealt mainly with fish captured from aquatic environments but also midge has been studied, an insect that lives in wetlands. Field exposure has been studied in 13 out of the 22 papers considered in this review

(Table 2), 7 of them used caged organisms to perform their study and 6 sampled wild organisms.

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

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

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

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

Skelton et. al [21] applied non-target NMR to fish caged in three different zones of a river impacted by WWTPs. One of them was taken as a control group and the other two as exposed from contaminated areas near the effluents, one highly urbanized and the other with huge farm and agricultural impact. The fish were caged upstream, in the effluent mouth and downstream. They pointed out differences in amino acid metabolism, phospholipid, cholesterol, and energy metabolism affected by the WWTPs.

Davis et. al [18] exposed fish to different distances from a pulp and paper mill outflow as well as a control site. The authors observed differences in amino acid, creatine, and taurine metabolism, as well as energy metabolism alterations and liver toxicity biomarkers.

From all these manuscripts, we consider important to enhance the manuscript that Davis et. al published in 2016 [19]. The authors caged different fish in 18 sites from 5 lakes (Great Lakes basin) and performed PCA analysis of endogenous compounds. Then, with these variations, the authors carried out a Partial Least Squares (PLS) regression with contaminants levels analysed in a target way, pointing out those who better explain differences observed in endogenous metabolites. This model highlights xenobiotic compounds affecting the metabolism and helps to discriminate contaminants without effects observed in the dataset. The authors concluded that up to 52 % of detected contaminants were not correlated with changes in endogenous metabolites. This interesting approach can help xenometabolomics field to observe the impact of xenobiotics in the metabolome, helping to prioritize anthropogenic contaminants with real relevance in metabolome alterations.

Previšić et. al [32] in a non-target analysis of the whole body of *Hydropsyche* sp. larvae observed alterations in 32 compounds, with

disruptions in fatty acid metabolism (energy metabolism), phospholipid metabolism and oxidative stress.

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

In the case of Simmons et. al [37], they combined taking wild animals with caged ones, as they exposed caged goldfish to a possible WWTP contaminated area at different distances downriver and took wild fish from the Jordan Harbour (near the point of the caged ones). All the plasma samples were analysed by means of a multi-targeted set of metabolites. They observed that some contaminants were detected in both caged and wild animals at similar levels. The authors also discussed that the magnitude of fold change was higher in wild organisms due to their long-term and constant exposure (gemfibrozil as an example). For the ones caged near the WWTP, they highlighted 47 altered biological functions compared to fish caged in a reference site. They found liver necrosis and metal ion transport functions activated, as well as depletion of the synthesis of cyclic adenosine monophosphates (AMPs). Additionally, in the other sampling points, an increase in amino acids, accumulation of lipids and glyceride, inhibition of steroid synthesis or increasing in glutathione concentration were some of the effects observed. Overall, the expression of plasma metabolites and proteins in caged goldfish agreed well with those in the wild goldfish, suggesting that the combined use of omics approaches and caged surrogates is a useful way to predict the molecular effects of contaminants in wild fish [37].

Park et. al [26] exposed *Danio rerio* to water from different zones (reference sites and other contaminated with different xenobiotics) in order to study its liver, as well as wild fish captured in the study sites. The highlighted compounds observed in the environmental fish also shown differences in lab-exposure conditions, but with smaller ratios. The authors observed that lab exposure experiments have lower impact in the metabolism of exposed organisms than others captured directly from the environment.

Glazer et. al [25] also performed a target analysis of liver samples from adult fish collected from a PCB-contaminated area (Acushnet River Estuary) and from pristine site (Scorton Creek). They found alterations of one-carbon metabolic pathway and amino acid imbalance.

Roszkowska et. al [33] also captured wild individuals and performed in-vivo SPME sampling in their tissues with a PAN-C18-coated blade. Different metabolites related with lipid metabolism were observed by means of this technique designed to study less polar contaminants spectrum.

Heffernan et. al [35] took blood samples from turtles from different zones of Australia, an offshore control site and two coastal areas exposed to urban/industrial and agricultural activities and performed a non-target strategy. They pointed out alterations in some nucleotides, fatty acids and vitamin related compounds.

Jeppe et. al [31] studied midge captured in different wetlands where sediments shown diverse contamination. They pointed out alterations in methionine metabolism, glycolipid metabolism and sugar metabolism by metals and total petroleum hydrocarbon contaminated zones. Mitochondrial electron transport and urea cycle metabolism were individually related to bifenthrin contamination, as well as sugar metabolism related to all contaminant inputs.

3.3. Metabolic alterations

After profiling the metabolome, further data analysis is usually undertaken to find out the most altered metabolic pathways and draw biological conclusions. In this review, despite the experiments correspond to different exposure conditions, organisms, and analytical platforms, some metabolic pathways can be pointed out as the most commonly disrupted by chemical contamination, showing important alterations in organism that may be related to pathologies. Fig. 1 presents these metabolic pathways reported as disrupted due to the exposure to contaminants (according to Table 2). The sixteen pathways reported can be ranked based on the number of times that a certain pathway has been presented in a paper as disrupted. The top 3 are hold by energy metabolism, followed by aminoacid and phospholipid metabolism (Fig. 1 in red colour). Considering the total number of papers included in Table 2, energy metabolism has been reported in 57 % of the papers published and aminoacid metabolism in 50 %. Therefore, these two metabolic routes are highlighted as the most commonly altered (nearly half of the experiments). Similar pathways were previously reported as the most affected ones when aquatic organisms are exposed to a single contaminant [1]. Phospholipid metabolism has been mentioned in 29 % of the papers. After them, other altered pathways were oxidative degradation and liver toxicity in 21 % and 18 % of the papers respectively (Fig. 1 in orange), nitrogen and taurine metabolism in 11 % of the papers (Fig. 1 in yellow), bile acid metabolism, carnitine metabolism, cholesterol metabolism, sphinganine metabolites, steroid biosynthesis and vitamin metabolism in 7 % of the papers (Fig. 1 in blue), creatine metabolism, serotonin

metabolism and repair capacity in 4 % of the papers (Fig. 1 in green).

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

4. Conclusions and future research

Conclusions

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

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

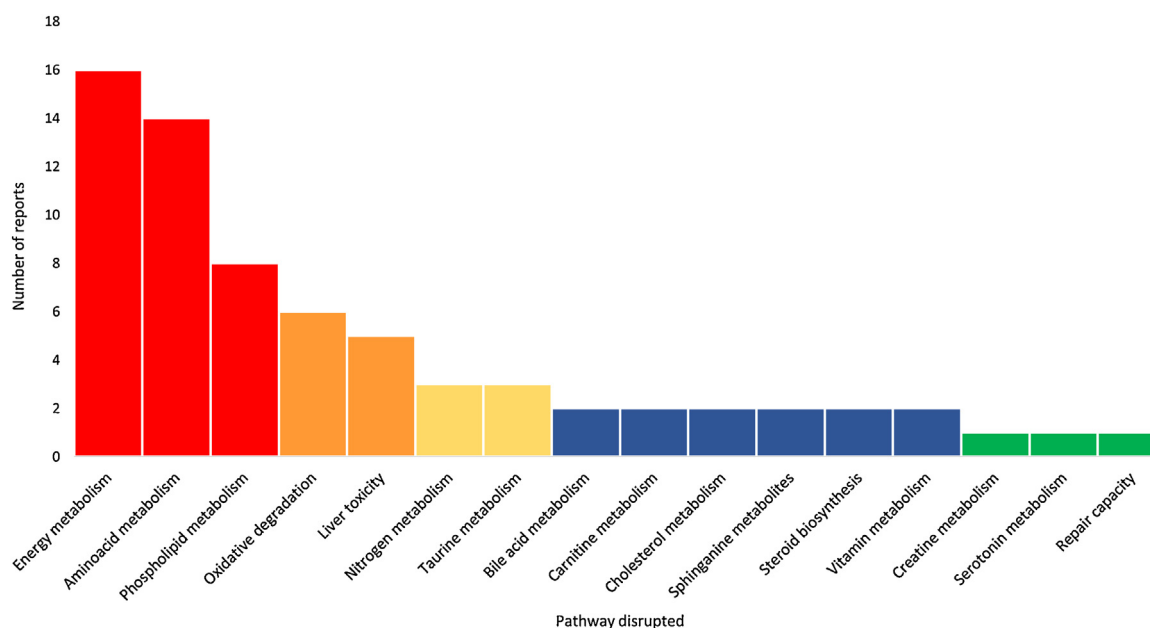


Fig. 1. Number of times that a pathway has been reported as disrupted in the experiments carried out with aquatic organisms exposed to contaminant mixtures presented in Table 2. In red, pathways reported as altered in more papers (>8 papers), followed by orange (4–8 papers), yellow (3 papers), blue (2 papers) and green (only 1 paper) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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

Declaration of Competing Interest

The authors report no declarations of interest.

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