Can proteomics contribute to biomonitoring of aquatic pollution? A critical review

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Abstract

Aquatic pollution is one of the greatest environmental problems, and therefore its control represents one of the major challenges in this century. In recent years, proteomics has emerged as a powerful tool for searching protein biomarkers in the field of pollution biomonitoring. For biomonitoring marine contamination, there is a consensus that bivalves are preferred organisms to assess organic and inorganic pollutants. Thus, the bivalve proteome was intensively studied, particularly the mussel. It is well documented that heavy metal pollution and organic chemicals altered the structural proteins causing degradation of tissues of molluscs. Also, it is well known that proteins involved in stress oxidative such as glutathione and enzymes as catalase, superoxide dismutase or peroxisomes are overexpressed in response to contaminants. Additionally, using bivalves, other groups of proteins proposed as pollution biomarkers are the metabolic proteins. Even though other marine species are used to monitor the pollution, the presence of proteomic tools in these studies is scarce. Concerning freshwater pollution field, a great variety of animal species (fish and crustaceans) are used as biomonitors in proteomics studies compared to plants that are scarcely analysed. In fish species, proteins involved in stress oxidative such as heat shock family or proteins from lipid and carbohydrate metabolism were proposed as candidate biomarkers. On the contrary, for crustaceans there is a lack of proteomic studies individually assessing the contaminants. Novel scenarios, including emerging contaminants and new threats, will require proteomic technology for a systematic search of protein biomarkers and a greater knowledge at molecular level of those cellular pathways induced by contamination.

Abbreviations

Two-dimensional gel electrophoresis (2-DE)
Isobaric tags for relative and absolute quantitation (iTRAQ)
Mass spectrometry (MS)
Two-dimensional difference gel electrophoresis (DIGE)
Matrix-assisted laser desorption ionisation (MALDI)
Time-of-flight mass spectrometry (TOF)
Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS)
Liquid chromatography mass spectrometry (LC/MS)
Data-independent acquisition (DIA) strategies
Data-dependent acquisition (DDA) strategies

1. Introduction

Most human activities have a negative impact on aquatic ecosystems causing a serious environmental problem (EEA, 2018). Emissions and waste from industries, unregulated household waste, mining, aquiculture, pesticides and harmful chemicals in agriculture and livestock end up polluting marine and freshwater ecosystems around the world. Thus, large quantities of pollutants are accumulated in coastal ecosystems (Islam and Tanaka, 2004; Halpern et al., 2008), lakes, rivers, aquifers, and groundwater (Vörösmarty et al., 2010; Behmel et al., 2016) posing a great threat to life in these environments. Therefore, water quality control and management are an imperative issue for the health of ecosystems and human populations (UNESCO, 2009).

Biomonitoring is an ecological, simple, and economical tool which can be used to carry out the pollution control on aquatic ecosystems. Biomonitoring is the assessment of a chemical toxic into a living organism at level of molecules, organelles, cells, tissues, organs, organisms, populations, communities, ecosystems and biomes. Quantitative variables related to chemical, biochemical, morphological, physiological, behavioural or abundant concentrations indicate the pollutant toxicity. They seem to indicate certain characteristics of the environment related to the level of pollution in the environment (Bargagli, 1998; Markert et al., 2003). Thus, there are hundreds of studies on biomonitoring of water quality, most of them focus on the bioconcentration or bioaccumulation of pollutants in organisms from marine or freshwater environments (*e.g.* Debén et al., 2015; García-Seoane et al., 2018), as well as those focus on studies at the community level (*e.g.* Pandey et al., 2018). However, there are few studies at the molecular level that use biochemical biomarkers, such as proteins, to biomonitor water pollution.

The final concentration of pollutant in the aquatic organisms is the result of the balance between the concentration of the chemical substance in the organism and the water. However, studies on the bioaccumulation of pollutants in aquatic organisms have several

limitations such as kinetic balance that have not yet been solved as reviewed Schöne and Krause Jr (2016). For instance, *Mytilus trossulus* and *Mytilus californianus* exposure to low levels of Cd resulting high kinetic differences in metal uptake and release (Lares and Orians, 1997). Additionally, the direct bioaccumulation of the pollutants in the organisms, does not enough information over toxic effects in organisms (Wang, 2016). On the other hand, biochemical alterations would be useful because they can provide information on the toxic effects of pollutants. Many protein biomarkers can be developed to detect and quantify a specific pollutant. Indeed, it is known that several proteins act as biomarkers in a particular aquatic organism in response to a chemical as discussed in this review. As we mentioned earlier, there are few studies on biochemical biomarkers in aquatic pollution compared to the high number on bioconcentration or bioaccumulation ones. This may be because there are a high variability of biochemical alterations depending on the type of organism and pollutant being studied. In addition, they are rarely used as complementary techniques. Studies using biomarkers provide the concentrations of pollutants in the environment but not the bioconcentrations in the organisms used. Therefore, the presence of pollutants in the environment and biological responses should be studied thoroughly to expand the range of protein biomarkers as well as search new biomonitoring organisms for which protein biomarkers have been not identified. Integrating all these data could help us to stablish the toxic mechanisms in the organisms.

Proteomics is a useful and effective tool for searching protein biomarkers in the field of pollution biomonitoring. Proteomics and other OMICs techniques have the potential to identify protein biomarkers as well as protein patterns which would become more robust and specific markers of a particular pollutants (Armengaud, 2016). A proteomic approach could help us to develop a broader view of the protein alterations, identifying the biomarker candidates. Proteome analysis could provide an overall picture of structural and functional cellular information and the response mechanism against aquatic pollution. These proteins

biomarkers after validation process, are useful as alternative biomarkers for routine application (Schmit et al., 2018). All this information would enable to develop commercial kits based on protein markers. In recent years, there are two main proteomic approaches established: protein separation in gel and mass spectrometry analysis (Tholey and Becker, 2017). Electrophoretic and chromatographic techniques based on prior protein digestion are called bottom-up approach. The other strategy, top-down, is based on analysis of intact protein by means of separation and mass spectrometry. Specifically, the second choice in combination with small protein biomarkers (below 30 kDa) could be easily and routinely implemented to identify and quantify protein biomarkers (Tholey and Becker, 2017). Additionally, the intense analysis of proteins would contribute to the identification of more specific and sensitive markers of pollution. These novel tools about the knowledge of complete proteomes open up new avenues for environmental sciences. Currently, environmental proteomics is being developed as a key branch of environmental sciences, providing more information about molecular responses to contaminants in living organisms (Li et al., 2018).

In the present review, the recent proteomic literature in the field of water pollution using biomonitors was provided. The aim was to identify the main aquatic species and the proteomic techniques used to search new protein biomarkers as well as to propose new strategies. A discussion about the constraints of several species due to limitations in databases or technical problems was conducted in the following sections.

2. Search and selection of literature

A systematic search of literature was performed using different tools, webs and databases: Google Scholar, Pubmed.gov (NCBI), Scopus and Web of Science (Clarivate Analytics). The keywords used for the search were: "contamination", "pollution", "biomonitors", "bioindicators", "aquatic", "animals" and "plants" combined with "biomarker", "proteome", "proteomics" and "protein biomarker". Only original papers published from

2010 to 2020 were considered and all were carefully checked. The most appealing articles were included in Tables 1 and 2 which yielded a total of 30 and 19 articles for marine and freshwater environments, respectively.

3. Biomonitors of marine pollution

The most abundant candidate species for biomonitoring marine pollution are invertebrates which constitute a huge group of macroscopic species, highlighting among them the bivalve molluscs for their key role in the ecosystems and for their great value to aquaculture industry (Campos et al., 2012). Bivalves are used for biomonitoring due to ubiquitous distribution, easy accessibility, high filtering capacity as well as high resistance to a wide range of pollutants (Suárez-Ulloa et al., 2013). Even emerging pollutants such as microplastics (Ward et al., 2019), nanoparticles (Zha et al., 2019) and other organic pollutants (Rodil et al., 2019) have been studied on bivalves.

Indeed, mussels are widely used to monitor the coastal water pollution (*e.g.* heavy metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, etc.) as reviewed Beyer et al. (2017). Accordingly, mussel watch programs define different environmental quality parameters. For this purpose, mussels distributed stations throughout different locations and for fixed periods of time were analysed (Jernelov, 1996). Currently, the scientific community is developing an intensive research in order to standardize protocols for monitor of pollution using mussels even in emergent pollutants (Li et al., 2019). In addition, there is an increasing concern because bivalves as edible species can be a health risk for humans if the metal pollutants concentration, such as Cd, Pb, Hg and As is too high (Ruiz-Fernández et al., 2018). Indeed, there are several guidelines of different countries and associations with recommendations of these trace elements (Stanković et al., 2011). For instance, for Cd contamination, the salinity on the bioaccumulation and depuration are important factors to safe oyster *Crassostrea gigas* consumption (Sun et al., 2018). In contrast, little is known about effects of emerging pollutants and no maximum residue levels have yet been set. Few effects

of these contaminants are known. For example, microplastics that could cause negative effects on bivalves, affecting filtration activity, feeding behaviour and reproductive problems (Zhang et al., 2019a). Fortunately, it was demonstrated that depuration of mussels can be an effective method to reduce plastic fibres (Birnstiel et al., 2019). Additionally, other environmental contaminants (*e.g.* polychlorinated biphenyls and polycyclic aromatic hydrocarbons) are affecting the health marine organisms with risk of adverse effects on human health, because they can increase serious viral contaminations (Fiorito et al., 2019).

3.1 Proteomic analysis for biomonitoring marine pollution

Currently, the development of OMICs technologies and bioinformatics for data interpretation allow advances in many scientific fields. Research marine proteomic studies have been carried out with numerous contaminants or natural toxins using both field and laboratory experiments (Suárez-Ulloa et al., 2013). Moreover, a proteomic approach may lead to a deeper understanding of gene expression and protein translation but also protein interactions or modifications (phosphorylation, denaturation, methylation, etc) in living organisms (Ambrosio et al., 2018).

Traditional biochemical methods as well as gel-based proteomics are intensively used to search protein biomarkers of marine contamination (see Table 1). In fact, in Figure 2 we can see that of the articles reviewed for this study the most used proteomic methodology is the 2-DE method followed by MALDI TOF. However, these methods analyse a small subset of proteins resulting highly biased methods with a poor quantification power (Aebersold and Mann, 2016). Overall, these studies highlight the need of robust MS-based proteomics methods for identification and quantification of proteins in biological tissues, therefore, their application to biomonitoring the marine contamination has a significant potential. Another more sensitive gel-based method such as protein labelling with fluorescent dyes (fluorescence difference gel electrophoresis, DIGE) must also be considered.

178 These various proteomic methods have been applied in various toxicological studies
179 of marine organisms with animal studies being more common than plant studies due to
180 technical reasons related to the extraction of complex proteins (Figure 1). Indeed, vegetal
181 tissues have low concentrations of proteins in a high presence of interfering substances
182 (Righetti and Boschetti, 2016). Another important limitation is that only the model plant
183 species (i.e. Arabidopsis, rice, maize and wheat) have an extensive and curated protein
184 database in contrast to non-model ones (Patole and Bindschedler, 2019). Fortunately,
185 proteomic technologies and bioinformatics advances will be addressing these technical
186 problems in a short period of time.

187 Proteomic analyses have been applied to diverse marine animals such as annelids (*e.g.*
188 Roohi-Shalmaee et al., 2019), crustaceans (*e.g.* Jebali et al., 2014; Vellinger et al., 2016) and
189 fishes (*e.g.* Borcier et al., 2019), but the most extensive proteome have been reported in
190 molluscs for biomonitoring the aquatic environment and ecosystem health. According to
191 (Campos et al., 2012), a significant number of proteomic researches using 2-DE and MS/MS
192 showed that bivalves could be considered as aquatic environment sentinels. Therefore, it can
193 assume that the protein expression of bivalves was strongly affected by pollutants, based on
194 proteomic studies (Table 1). Thus, from the proteomic data of bivalve molluscs, it can be
195 observed that the bivalve cellular structures of different tissues are degraded by the action of
196 contaminants mainly by metal pollution. For example, Liu et al. (2012) have indicated that
197 Cd (20 and 200 μg L-1 for 15 days) altered the expression of structural proteins (i.e. actin
198 and tubulin) in marine bivalves using 2-DE followed by MS/MS. In oysters, other metals
199 such as Cu, Pb and Zn until a concentration of 100 μg L^{-1} for 4 days affected the tropomyosin
200 decreasing for 4.1, 100.9 and 6.7 times, respectively (Muralidharan et al., 2012). Moreover,
201 these metal pollutants also affected endogenous proteases as cathepsin which can degrade
202 other proteins and lead to a tissue destruction (Xu et al., 2016). For this reason, urban sewage
203 which likely includes metal pollutants also altered proteins related to cytoskeleton of oysters

as noted above (Flores-Nunes et al., 2015). Other non-metal pollutants as anthracene or benzo α-pyrene caused alterations in structural proteins in bivalves as clam *Ruditapes decussatus* (Sellami et al., 2015). All these structural proteins were detected by gel-based proteomic technologies and most identified by MALDI-TOF/TOF, as discussed above.

Following on protein alterations in the proteome of bivalves, environmental pollutants also provoke alterations in cell redox balance increasing the oxidative status of proteins. The generation of free radicals such as reactive oxygen species (ROS) could cause important toxicity at cellular level (Table 1). To counter this adverse effect, antioxidant molecules as glutathione and other enzymes as catalase, superoxide dismutase or peroxidases are expressed (Braconi et al., 2011). In *M. galloprovincialis*, a recent study has shown a level change ratio for GST (Glutathione S-transferase) with a fold change of 3.5 in response to 30 $\mu g\ L^{-1}$ of polychlorinated biphenyls for 3 weeks (Ambrosio et al., 2018). Other studies have revealed that these family of antioxidant enzymes can be considered as suitable proteomic biomarkers (Leung et al., 2011) for the control of Cd and H_2O_2. Other emergent contaminants such as CuO nanoparticles and Cu^{+2} (10 $\mu g\ L^{-1}$ for 15 days) or microplastics (800 $\mu g\ L^{-1}$ for 52 days) varied the level of proteins involved in oxidative stress, affecting its cellular detoxification efficiency (Gomes et al., 2014; Green et al., 2019). Moreover, a recent study with mixtures of contaminants clearly revealed the alteration of the GSTs level, suggesting the activation of a defence mechanisms used to survive in polluted environments (Azevedo et al., 2015).

Finally, another significant group of proteins altered by pollution were the proteins involved in the metabolism (Table 1). Hence, proteins involved in carbohydrate, fat and protein metabolism are also valuable biomarkers in bivalve organisms. Variations in metabolism as a result of mechanisms of biotransformation or detoxification of pollutants can be also used to monitor the contaminant trace and the ATP synthase beta subunit was proposed as protein biomarker (Muralidharan et al., 2012). Heavy metal pollution caused an

important damage to amino acid and carbohydrate metabolism representing a 2% and 11% of the total altered proteins in oysters. Particularly, the glycolytic pathway was disturbed by metal pollution (Cd, Cu, Pb and Zn with a concentration of 100, 50 and 5 μg L^{-1} for each metal and during 4 days) as a result triose phosphate isomerase has been highlighted in proteomic studies (Thompson et al., 2012). It has been reported that in the case of exposure to CuO nanoparticles and Cu^{+2} for 15 days, most of the metabolic proteins were up-regulated (i.e. ATP synthase F0 subunit 6 and cytochrome C oxidase subunit III) in the digestive gland of *M. galloprovincialis* (Gomes et al., 2014). Furthermore, post-translational modifications of proteins should be investigated in order to assess the functionality of proteins. At present, little is known about the effect of aquatic pollution on post-translational modifications.

As we mentioned previously, other marine invertebrates also were studied using proteomic techniques. As example, the effect of aliphatic and aromatic hydrocarbons on crab *Carcinus maenas* was detected through proteins involved in several biological process as the chitin catabolism, proteolysis, exoskeleton biosynthesis, protein folding, stress response and transport (Jebali et al., 2014). This finding demonstrates a strong response of these chemicals on the crab proteome revealing a group of 19 protein biomarkers (Jebali et al., 2014).

4. Biomonitors of freshwater pollution

In general, the choice of aquatic bioindicator has to meet some criteria such as easy to recognized, high abundance and wide distribution as well as sensitive to a wide range of pollutants (Le et al., 2016). As can already be seen in Figure 1, there are far fewer biomonitoring studies of freshwater pollution than of marine pollution and in fact, there is no consensus on sentinel organisms in the freshwater environment. The most common organisms used to biomonitoring freshwater pollution are species of fish, crustaceans and molluscs but there are mainly two organisms that stand out. The bryophyte *Fontinalis antypiretica* (Debén et al., 2015), and the zebra mussel *Dreissena polymorpha* (Binelli et al., 2015). The use of *F. antypiretica* would be appealing as a simple, reliable, and economic tool for

256 biomonitoring inorganic (i.e. heavy metals and metalloids) and organic contaminants (i.e.
257 PCDD, PCDF, PAHs and PCBs). Likewise, another bryophyte species *Sphagnum palustre* was
258 recently used by Capozzi et al. (2018) for biomonitoring microplastics in freshwaters
259 environments. On the other hand, there are many published studies about zebra mussels in
260 recent years, but it is an invasive species. As recommended by Binelli et al. (2015) it should
261 not be introduced in new areas but it would be practical to use its positive peculiarities in the
262 ecosystems where it is already established or use it in laboratory experiments. But none of
263 these organisms are considered to be sentinels, as is the case with the mussel in the marine
264 studies.

265 *4.1. Proteomic analysis for biomonitoring freshwater contamination*

266 As in marine pollution, a wide range of proteomic methodologies were used up until now.
267 Proteomic technologies, including innovative combinations of different techniques, were
268 applied to pollution studies (Figure 2, Table 2). One of the first techniques was the 2-DE
269 which allows the separation of a high number of proteins followed by their identification by
270 MS spectrometry. The main drawbacks of the 2-DE technique are its time-consuming and
271 its poor reproducibility. Therefore, there is a clear trend towards gel-free alternatives such as
272 SWATH-MS, LC-MS/MS or iTRAQ as reflected in Table 2. Even though accurate and
273 sensitive mass-spectrometry-based methods enable the proteome analysis of aquatic
274 biomonitor selected, a large-scale study of proteomes requires a high-quality spectral library
275 even in the case of data-independent acquisition (DIA) strategies. Currently, data-dependent
276 acquisition (DDA)-based proteomics are the preferred methods due to the researcher do not
277 need to know about the specific proteins and the molecular mechanisms involved in the
278 biological process (Aebersold and Mann, 2016). In this sense, great efforts are also being
279 carried out to generate high-quality libraries for DIA MS in non-model organisms based on
280 fragmentations and retention time of proteins (Searle et al., 2020). When fully development,

281 the proteomic technologies will reach a maximum applicability in the biomonitoring
282 freshwater contamination.

283 At this moment, proteomic tools can provide an appealing approach to study the
284 freshwater environmental stressors on organisms. As we previously said, the species used for
285 biomonitoring freshwater pollution are highly heterogeneous and therefore we did not find
286 many articles on the same organism. Initially, plants might seem to be an ideal organism for
287 pollution biomonitoring in the rivers and lakes as we already observed with bryophytes but
288 from a proteomic perspective the situation changes. The heavy metal tolerance was deeply
289 investigated from a proteomic point out of view only in crop species, proving that metals are
290 mainly absorbed by roots and accumulate in other parts, producing important oxidative stress
291 inside the cell. In green macroalgae, it is known that the main mechanisms to counteract the
292 metal effect are the cellular inclusion of metals, synthesis of metal chelators and activation
293 of antioxidants (Moenne et al., 2016). Regarding proteomic studies, there are only a few
294 works with algae species. Recently, research conducted by Khatiwada et al. (2020) using
295 SWATH-MS, revealed that facilitator superfamily transporters, Cd/Zn transporting ATPasa
296 and heavy metal transporting P1B-ATPase were overexpressed in the single-celled algae
297 *Euglena gracilis* in response to contamination of Hg, Pb and Cd in a concentration of 5-90,
298 500-10000, and 50-1000 mg L^{-1}, respectively, during 7 days.

299 As mentioned before, the most frequent aquatic animals used to monitor the
300 environmental quality are species of fish, crustaceans, and molluscs (Table 2), highlighting
301 fish in terms of the cutting edge of the proteomics tools used. From these organisms, several
302 tissues such as muscles, liver, gills, kidney, brain, or skin can be investigated, and oxidative
303 stress is one of the main mechanisms studied (Zhang et al., 2019b). Certainly, pollutants in
304 the river often induce oxidative stress, inducing devastating effect on the fish proteins. As
305 oxidative stress is an imbalance between the production of reactive oxygen species by radicals
306 and antioxidants in the cell system, cellular antioxidants such as superoxide dismutase,

307 catalase and glutathione peroxidase are often differentially expressed to achieve the balance.
308 Additionally, heat shock proteins are also involved in the regulation of the redox potential
309 via repairing damaged proteins or degrading them when the situation is irreversible. In this
310 sense, the freshwater catfish *Rita rita* has been proposed to monitor riverine pollution, due
311 to the expression of a reference proteome which included a set of heat shock proteins
312 (Mohanty et al., 2015). Moreover, the proteome of goldfish (*Carassius auratus*) subjected to
313 herbicides and fungicides (8.4 and 42 μg L^{-1}, respectively) mixture and high temperatures (22
314 and 32 ºC) produced significant changes in response to cellular stress (Gandar et al., 2017).
315 In the same line, alteration of heat shock proteins and catalase was confirmed in *Gobiocypris*
316 *rarus* exposed to benzotriazole (0.05, 0.5 and 5 μg L^{-1}) (Liang et al., 2017).

317 The cellular metabolic pathways are reorganized in fish species in response to
318 inorganic and organic compounds of contaminated environments (Table 2). Within energy
319 metabolism, the main pathways studied are lipid and carbohydrate metabolism and it has
320 been reported that organic contaminants in aquatic environments could be bioaccumulated
321 in the lipids of organisms with adverse effects on transport, biosynthesis and β-oxidation of
322 fatty acids. The proteins responsible for fatty acid metabolism comprise those involved in
323 the hydrolysis of triacylglycerol, intracellular transport of fatty acids, fatty acid oxidation from
324 mitochondrial and peroxisome (Olivares-Rubio and Vega-López, 2016). These facts have
325 been proved in the proteome of white sucker (*Catostomus commersonii*) under the effects of
326 mining dust in Athabasca river (Canada) (Simmons and Sherry, 2016). Concerning
327 carbohydrate metabolism, proteins such as alpha-enolase, triose phosphate isomerase B,
328 glyceraldehyde-3-phosphate dehydrogenase and pyruvate carboxylase were altered by DDT
329 and Cd (500 and 1 mg L^{-1}, respectively) in blood cells of European eel *Anguilla anguilla*.
330 Indeed, these proteins have been point out as potential biomarkers proteomic index to detect
331 pollutants (Roland et al., 2016). In the case of rainbow trout (*Oncorhynchus mykiss*) exposed to
332 an herbicide (diquat dibromide; from 0.12 to 10 mg L^{-1} during two 24 h pulses), an

enrichment analysis of liver proteins differentially expressed showed that they were strongly linked to glycogen degradation. A malfunction of the glycogen metabolic pathway means that the prolonged swimming of the fishes without glycogen and triglycerides could lead to greater predation (McCuaig et al., 2020). On the other hand, the systemic damage as the result of pollutants in living organisms could be assessed studying the sub-proteome and consequently, the proteomic analysis becomes focused on a particular organ tissue (Gouveia et al., 2019). It has been reported that the fish neuroendocrine system is clearly affected by pollutant chemicals from the environment, as well as theirs effect could be monitored by protein biomarkers related to its system (Martyniuk et al., 2012).

The crustaceans are other studied group in the field of freshwater biomonitoring as sentinel organism due to their sensibility for assessing the water pollution. Crustaceans offer numerous advantages in terms of their small size, abundance in the freshwater ecosystem and their position in the food web. Recently, it has been demonstrated that several enzymes of crustaceans involved in antioxidant system response, detoxification mechanisms and damage occurrence responded to polluted environments (Bertrand et al., 2018). Another study analysed the impact of chlordecone, an organochlorine insecticide, on the proteome of the crustacean *Macrobrachium rosenbergii* resulting a possible endocrine disruptor compound in chemical concentrations of 0.2, 2 and 20 μg L^{-1} for 30 days (Lafontaine et al., 2017). Using LC-MS/MS, *Daphnia magna* was studied to assess organic contamination in freshwater. A set of 288 proteins from energy metabolism and oxidative stress were altered by the impact of the contamination in Gulpo river (South Korea) (Chatterjee et al., 2019). In other study conducted with *Carcinus maenas,* the CBR-NHR-218 nuclear hormone receptor, two components of the ABC transporters and the aldehyde dehydrogenase, were proposed as biomarkers (Montes Nieto et al., 2010). As described in fish species, a polluted environment can produce oxidative stress by redox unbalance. This stress caused by a polluted environment in aquatic ecosystems of Doñana National Park (Spain) could be analysed by

means of redox proteomics using crayfish (*Procambarus clarkii*) as reported Fernández-Cisnal et al. (2017), who detected cysteine oxidized forms in ferritin and nucleoside diphosphate kinase. At a more advanced level, targeted quantitative proteomics by mass spectrometry can also provide valuable information for detecting a mixture of pollutants in a freshwater ecosystem. Based on current protein databases, a selected reaction monitoring mass spectrometry-based methodology in amphipod crustaceans corroborated the methodology to assess the aquatic contamination (Gouveia et al., 2017).

Finally, for biomonitoring of environmental quality, bacteria are not very common in proteomic studies and they are focused on the use of microorganisms to destroy or reduce the environmental pollutants through bioremediation. In this sense, recent proteomic studies could provide a better understanding of molecular mechanisms to improve efficiently the bio-removal of pollutants (Wei et al., 2019; Yu et al., 2020).

5. Final remarks and future trends

There is a rising concern about water pollution caused by industrial and agricultural activities. These scenarios are in constant evolution and the type of the contaminants is constantly changing. Emerging contaminants and new threats require new approaches, identifying novel biomarkers in aquatic organism. As shown in the Figure 3, the application of proteomics is increasingly relevant in aquatic pollution searching protein biomarkers in novel sentinel organisms. Additionally, protein biomarkers represent a valuable tool for early detection of pollutant exposure and even early effect evaluation. The proteomic approach is one of the most dynamic and fast developing field to analyse and decipher protein expression. However, the establishment of biomonitoring protocols implies a great effort due to the seasonal and spatial variability of communities as well as individual variability. Past researches have taken the initial steps using lab experiments to create models, but more complex systems with interactions among pollutants and uncontrolled environmental conditions must be considered in the future.

385 Nevertheless, one of the main difficulties is the lack of a curated protein database for
386 these living organisms. To overcome this limitation, new proteomic mass spectrometry
387 technologies are being developed to detect and quantify the proteins increasing the precision
388 and accuracy. In recent years, DNA sequencing towards precise genomes are implementing
389 proteome data building an accurate reference protein database. It is becoming increasingly
390 the number of non-model aquatic organisms with enough proteomic data, thereby opening
391 new avenues for proteomic approach.

392 In the following years most of studies should be focused on the study of protein
393 isoforms, post-translational modifications and protein interactions which could reveal
394 further information on the stress response to chemical pollution. The analysis of alternative
395 protein isoforms and protein interactions would provide insight into the relation between
396 proteins and their functions. Additionally, post-translational modifications of proteins,
397 particularly phosphorylation, which play key roles in the regulation of protein functions and
398 signalling networks of biological systems is therefore a major challenge.

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CAPTIONS TO FIGURES

Figure 1. Number of articles published during the last 10 years using plants (A) and animals (B) for marine ■ and freshwater ■ pollution biomonitoring; those studies that employed proteomic tools are highlighted ■.

Figure 2. Bar chart highlighting proteomic methodology used in revised studies about pollution biomonitoring for aquatic environment from 2010 to 2020.
Figure acronyms: LC-Q-TOF=Liquid chromatography-quadrupole-time of flight; SWATH-MS= Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry; SDS-PAGE= Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RP-HPLC= Reverse Phase High Performance Liquid Chromatography; LFQ= Relative label-free quantification of shotgun; 2D-UPLC= Two dimensional by ultra performance liquid chromatography; PLGS= Protein Lynx Global Server; 2D-DIGE= Two-dimensional difference gel electrophoresis; 2-DE= Two-dimensional gel electrophoresis; LC-MS= Liquid chromatography mass spectrometry; Nano-LC-MS/MS= Nano- liquid chromatography-tandem mass spectrometry; iTRAQ= Isobaric tags for relative and absolute quantitation; MALDI TOF/TOF= Matrix-assisted laser desorption ionisation - tandem mass spectrometry (TOF)

Figure 3. Advantages and drawbacks of pollution biomonitoring from a proteomic point of view

Figure

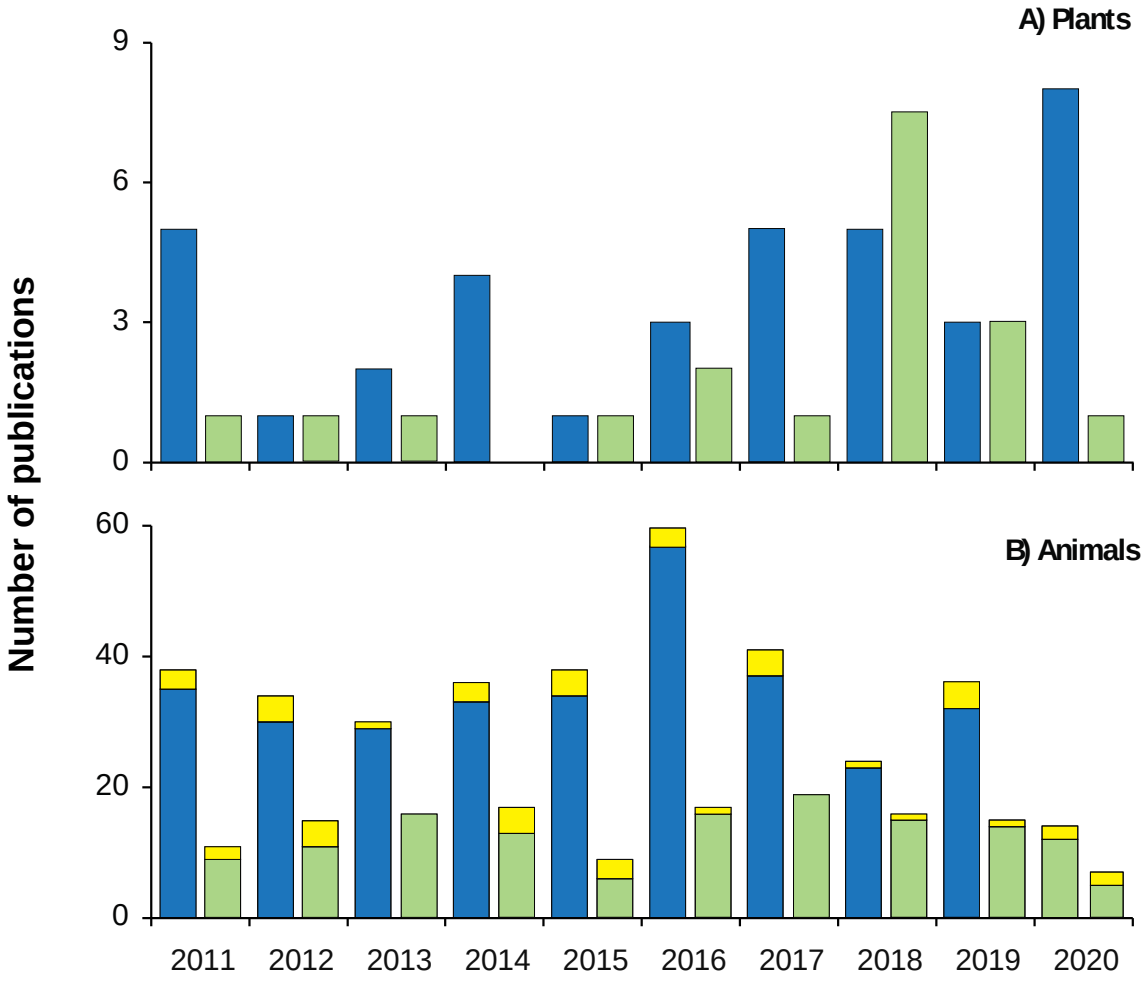
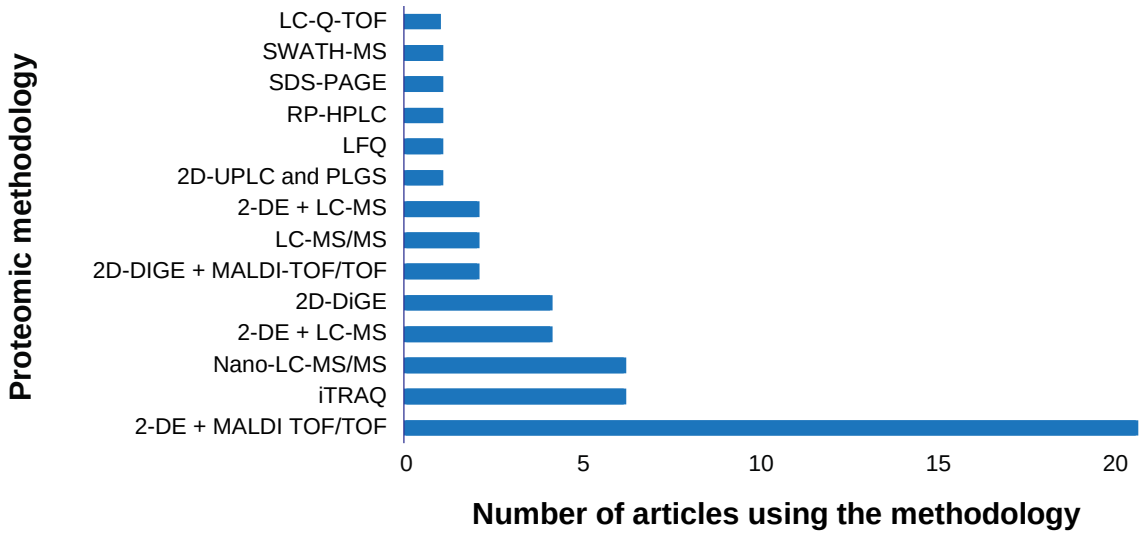


Figure 1

Figure

1



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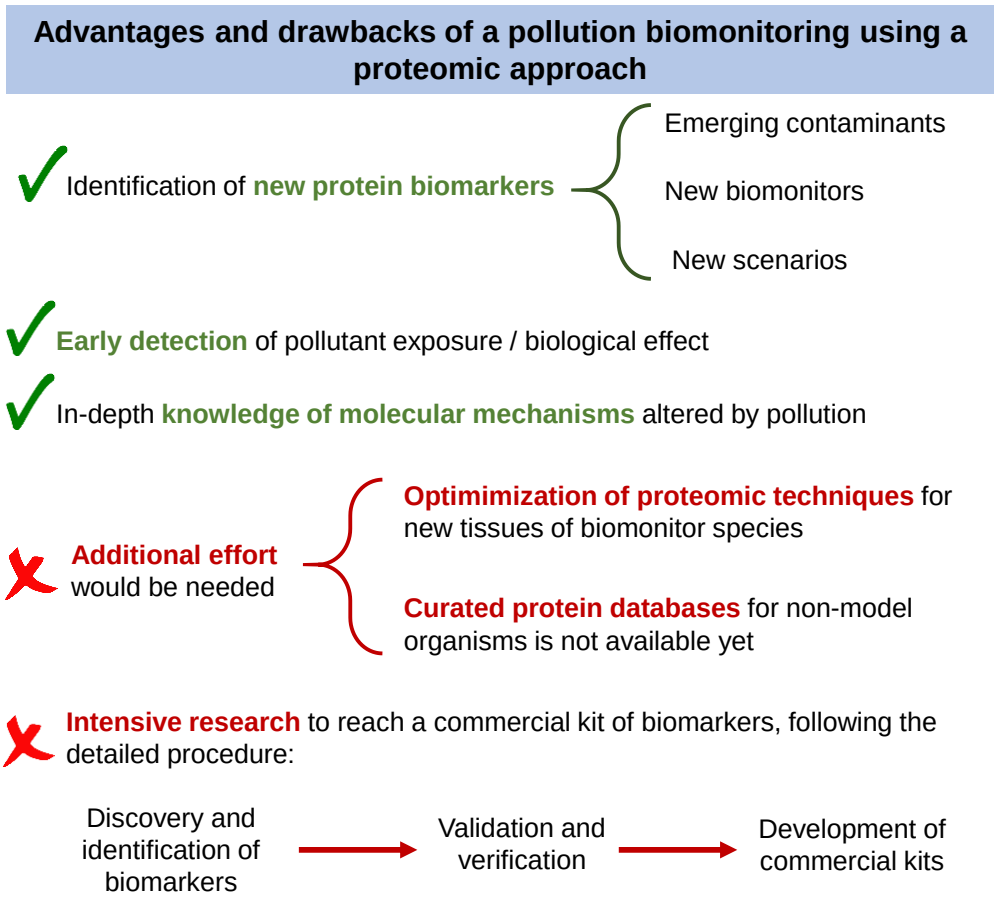
3 **Figure 2**

4

Figure



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2 **Figure 3.**

3

Graphical Abstract