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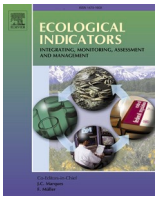

Perspective Article

Relevance of cell subcompartmentalization techniques to predict adverse effects of metals in bivalves and fish

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ABSTRACT

Subcellular fractionation is an interesting technique to study metal cell compartmentalization and helps on evaluating the impact of a contaminant in an organism. It provides a better understanding about the fate and behaviour of metals within cell compartments, being then able to identify if metals experience a detoxification process or, on the contrary, they are trophically available. Having this information about metals and metalloids is crucial in the context of risk assessment, as it provides valuable information about their behaviour throughout the food chain at different trophic levels.

Coastal and marine environments are often affected by dangerous spillages. Pollutants tend to accumulate in water, soils and sediments, where they can become readily available to species, such as bivalves and fish. These species are often used as bioindicators as they can provide information about the trophic transfer and/or the accumulation and of different pollutants. After a bibliographic search, the protocols used to study the subcellular fractionation on bivalves and fish exposed to metals have been highlighted. This literature mini review focuses on the different protocols used for studying this issue and the improvements that subcellular fractionation has brought to the topic. Nonetheless, future research needs and perspectives are pointed out as they can improve the robustness of using such techniques for risk assessment.

1. Introduction

Metals and metalloids present in aquatic environments, dissolved in water or bound to abiotic compounds, can be absorbed by aquatic animals, directly from water and sediment, or accumulated throughout the food chain. Metal uptake and bioaccumulation induce toxic effects on the organisms that can then be assessed by many observable and / or measurable changes (biomarkers) at molecular, biochemical, cellular, physiological, or behavioural levels (Fernandez de Oliveira et al., 2018; Lomartire et al., 2021). Biomarkers quantify these variations in whole organisms, their tissues or other biological parameters. The use of biomarkers allows to assess exposure responses to metal pollutants and can also help understanding toxic action mechanisms. Combined with chemical analyses, this approach contributes to the evaluation of the health status of organisms at a specific time.

Once entered the body, metals will not always accumulate in the same tissues. An internal compartmentalization of the metal occurs, as the dissolved metals can be found in the cytosolic cellular fraction (called "soluble" fraction), potentially associated with ligands (detoxification proteins, metalloproteins, enzymes). Metals are also found associated with cell organelles as lysosomes, with granules, or cellular debris in the "insoluble" fraction. Different methods have been developed to allow the differentiation between the number of metals, which are bio-accumulated in the soluble and/or insoluble parts of an organism's tissue, thus providing information on the cellular distribution of a particular contaminant (Mouneyrac et al. 2001; Wallace et al. 2003; Damiens et al. 2006; Buffet et al. 2011; Hanana et al. 2018).

Advanced methods of subcellular fractionation, which allow the study of the different fractions inside a cell, deepen the knowledge on metal subcellular distribution. Thus, it helps to estimate the amount of metal-based pollutant not detoxified, as well as its trophically available part. This information is essential as certain levels of the trophic chain, such as bivalves (uptake from organic matter, sediments, water) or some fish (uptake from organic matter, water), can accumulate a certain

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quantity of metals, which are then transferred to predators, moving upwards in the food chain and leading to bioaccumulation. This led to a better understanding of the fate and behaviour of the metals throughout the food chain (Wallace et al. 2003; Geffard et al. 2010; Wang et al. 2016).

The aim of this mini review was to compile the different studies that used subcellular fractionation in risk assessment mediated by metal-based pollutants. After a brief description of the current methodologies developed to analyse metal subcellular distribution, this mini review cites some of the techniques mostly used to study bivalves and fish exposed to metal-based pollutants and their current limitations, as well as starting to point out relatively new techniques that can improve the knowledge about this topic.

2. Importance of compartmentalisation analysis to better understand effects of metal-based pollutants on bivalves and fish

Cells are composed of well-defined compartments, each specialized in a metabolic function. The location of metals inside an organism's cells is key to identify its target compartment and how it can affect the exposed organism, as the effect may vary depending on the fate of the metal-based pollutant in the cell. When a non-essential metal is internalised or when an essential metal is in excess in cells, different processes are solicited to control the intracellular homeostasis of each metal to minimize its adverse impact in cellular processes: regulation of metal accumulation (bioavailability decrease, reduced metal uptake…) or excretion. The late process involves ligand synthesis as metallothioneins, peptides as glutathione, or granules (Ruttkay-Nedecky et al., 2013). Knowing the partitioning of metals, i.e., the distribution of the different forms in which a metal will be found in a given environment (cation, hydrated cation, charged complex, inorganic non-charged complex, organometallic complex), will provide a better understanding of its bioavailability.

Performing a subcellular fractionation experiment is a first step that can help to discriminate the subcellular distribution of a metal. Wallace et al. (2003) developed such a method to study subcellular compartmentalization of cadmium (Cd) and zinc (Zn) on clams. It consists in isolating various fractions which can contain metals by differential centrifugation. Wallace et al. (2003) described the separation of three main fractions corresponding to three main subcellular compartments: the metal-sensitive fraction (MSF) including organelles and enzymes (heat-sensitive proteins); the biologically detoxified metal fraction described as metal-rich granules (MRG) and heat-stable proteins (HSP, metallothioneins); and the trophically available metal fraction (TAM) representing the one readily assimilated by predators with organelles, enzymes and metallothioneins. This protocol was afterwards mainly applied to study metal-based pollutant compartmentalization on aquatic species. Giguère et al. (2006) refined the protocol on fish showing that up to six subcellular fractions can be obtained and defined as sensitive to metals (e.g 1) mitochondria; 2) lysosomes and microsomes, 3) cytosolic enzyme; 4) detoxified NaOH-resistant granules; 5) cytosolic heat stable protein and peptides; 6) cellular debris: cell membranes, intact cells and connective tissue, nuclei). Recently, Urien et al. (2020) optimized each step of the protocol on fish liver and showed the need of a protocol adaptation at the species organ level. This method, thus, provides crucial information about the fate of the metal within the organism. However, it does not provide the precise localization of the metals within the different subcellular compartments. Identifying the cellular target of a pollutant is not an easy task, even when that is a common objective of ecotoxicological studies.

Subcellular fractionation applied to organs can be an important source of information on the partitioning tissue metal concentration, when coupled with the use of biomarkers targeting different cell's compartments (Beaumelle et al. 2017). This way, it also enables the estimation of the metal quantity that can have adverse effects on the studied organism. The cellular response will thus be better characterized. Despite these advantages, the study of the relationships between internal compartmentalization of metals and induction of biological responses still needs to be improved using specific biomarkers (Jones et al. 2009; Colacevich et al. 2011; Beaumelle et al. 2017).

Another aspect that should not be neglected is that subcellular compartmentalization analysis allows the precise estimation of the amount of metals that becomes trophically available and which can thus be transferred to the upper trophic levels. This means that by studying organisms lying at the base of the trophic chain, it could be possible to predict some of the metal transfers and effects on consumers of higher levels. Sánchez-Marín and Beiras (2017) compared the trophic transfer of lead (Pb) from a clam *Dosinia exoleta* (Linnaeus, 1758) and a mussel *Mytilus galloprovincialis* (Lamarck, 1819) to the common prawn *Palaemon serratus* (Pennant, 1777), showing a different cellular distribution of Pb between both bivalves. Using subcellular fractionation experiments allowed the authors to reveal that MRG-associated Pb was not available for trophic transfer to predators as the hermit crab *Clibanarius erythropus* (Latreille 1818). Thus, this method applied to carefully selected bioindicator species along the food webs can be used to assess metal contamination in polluted environments. Additionally, subcellular fractionation helps to track the distribution of metal within the cell over time, helping to characterize its evolution during accumulation, metabolization and potential detoxification processes (Thit et al. 2016; Scola et al. 2021).

In addition, the use of this method can also help to unravel the fate and behaviour of pollutants of different natures, which have not been deeply studied to this date, such as rare earth elements (REEs). Only a few studies that investigated REEs have used subcellular compartmentalization to characterize their effect on organisms (Shen et al. 2014; Fu et al. 2014; Cardon et al. 2019).

3. Literature overview: Use of cell compartmentalization techniques in bivalves and fish exposed to metal-based pollutants

To better visualize where research focused on the use of subcellular fractionation during ecotoxicological studies on metals, a literature search was carried out. This search applied the keywords: *compartmentalization, subcellular, fractionation, partitioning, distribution, metal, aquatic, species/organisms, fish, bivalves, clams,* and their various combinations, finding more than fifty studies related to these topics over the last twenty-five years. The search included a wide variety of studies on different aquatic organisms exposed to metals and evaluating or not subcellular partitioning of these contaminants. Since the early 2000s, the increase in the number of these studies has led to a better understanding of the processes by which metal-based pollutants are sequestered into different cell organelles. These advances allowed the study of the mechanisms by which metals bioaccumulate in different species, as well as the fraction of the metal available for the trophic transfer.

Table 1 gathers studies focused on different aquatic species (mainly bivalves and fish, but also those which might be relevant), metals, techniques and biomarkers from the last 25 years, which were critical for advancing in the study of bioaccumulation and trophic transfer of metals in different organisms. It shows that studies that used subcellular compartmentalization (marked with an *) to investigate metal-based contaminants were not such a minority (21 out of 52 articles in total) compared to those focused on a more simplistic compartmentalization process. Indeed, subcellular fractionation is a technique increasingly used in ecotoxicological studies, as it provides a lot of information on the fate of contaminants. The literature search on studies that use compartmentalization techniques to evaluate the distribution of metals in bivalve and fish tissues showed that the main protocol used was the one developed by Wallace et al. in 2003 (Campbell et al. 2005; Rainbow et al., 2006; Wang et al. 2016; Zimmermann et al. 2017; Cardon et al. 2019). This protocol has been used on whole organisms, but also on specific tissues, such as: gills, digestive tissues, liver, or muscles. For

Table 1
Bibliographic compilation of the different studies focused on the study of accumulation of metals in bivalves, crustacea and fish using simplistic or subcellular (*) compartmentalization techniques.

Authors	Year	Biomarker or tracer	Studied metals	Species	Studied element
Bivalves					
Goudard et al.*	1998	Direct measure	Am, Pu, Tc, Cs	*Crassostrea gigas*	Hepatopancreas, visceral masses, liver, pyloric caeca and digestive glands
Mouneyrac et al.	1998	MT	Cd, Cu, Zn	*Crassostrea gigas*	gills/digestive gland/remain
Mouneyrac et al.	2000	MT	Ag, Cd, Hg	*Macoma balthica*	soft tissues
Wallace et al.*	2003	MT	Cd, Zn	*Macoma balthica*	organelles/enzymes
Shi et Wang*	2004	MT	Cd, Zn	*Mactra veneriformis, Ruditapes philippinarum*	Soft tissues
Bonneris et al.*	2005a	MT, MDA	Cd, Cu and Zn	*Pyganodon grandis*	Gills and digestive gland
Bonneris et al.*	2005b	MT	Cd, Cu and Zn	*Pyganodon grandis*	Gills and digestive gland
Campbell et al. *	2005	MT, MDA	Cd	*Pyganodon grandis*	Gills and digestive gland, liver tissue
Damiens et al.	2006	MT	Cd, Cu	*Crassostrea gigas*	tissue
Rainbow et al.*	2007	MTLP, MRG, cellular debris, organelles, HSP	Ag, Cd, Zn	*Marcia hiantina* *Perna viridis* *Saccostrea cucullata*	DG: digestive gland, M: adductor muscle, R: remaining soft tissues
Cooper et al.*	2010	Direct measure	Cd	*Pyganodon grandis*	gills, digestive gland, (mantle, foot and remaining organs)
Rainbow et al.*	2010	MRG	Ag, Cd, Zn	*Mytilus edulis* *Ruditapes philippinarum,* *Aequipecten opercularis Crassostrea gigas*	digestive glands and adductor muscles
Buffet et al.	2011	MT	Cu	*Scrobicularia plana*	soft tissues
Freitas et al.	2012	MT + ENZ	Al, Cr, Ni, Cu, Zn, Cd, Pb, Hg and As	*Cerastoderma edule*	Tissue
Khati et al.	2012	MT	Cd, Cu	*Perna perna*	Gills
Cooper et al.*	2013	MT	Cd, Cu, Zn	*Pyganodon grandis*	Gills and digestive gland
Buffet et al.	2014	LDH and MT, GST, CAT, SOD, TBARS, CSP and AChE	Ag NPs	*Scrobicularia plana*	soft tissues
Mouneyrac et al.	2014	CAT, GST, SOD, LDH and CSP activities	Ag, Au, Cu, Cd, Zn	*Scrobicularia plana*	soft tissues
Merschel and Bau	2015	Direct measure	La, Sm, Gd	*Corbicula fluminea*	Shell
Bonnail et al.	2016		As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb, Se, Zn	*Corbicula fluminea*	soft tissues
Wang et al.*	2016	MT	Cu, Zn, Cd, Pb	*Mitromorpha iridescens*	Digestive glands
Bonnail et al.	2017		La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu	*Corbicula fluminea*	soft tissues
Zimmermann et al. *	2017	MT + organelles	Ag	*Dreissena polymorpha*	soft tissues
Guo and Feng	2018	REVIEW	Ammonia, Metals, Organic pollutants	*Corbicula fluminea*	REVIEW
Hanana et al.	2018	LPO/ DSB/ COS/ GST	Sm, Y	*Dreissena polymorpha*	soft tissues/digestive glands
Oliveira et al.	2018		Zn, Mn, Fe	*Anodontites trapesialis*	
Bonnail et al.	2019	GST, GPx, effect -DNA, histopathological damage	As, Cd, Co, Cu, Fe, Ni, Pb, Zn	*Corbicula fluminea*	digestive gland/gills/gonad
Crustaceans					
Goudard et al.*	1998	Direct measure	Am, Pu, Tc, Cs	*Homarus gammarus*	Hepatopancreas, visceral masses, liver, pyloric caeca and digestive glands
Cardon et al.*	2018	ENZ (CS, CCO, LDH, APHO)		*Daphnia magna*	Kidney/mitochondria
Cardon et al.*	2019	Mitochondria	Y	*Daphnia magna*	Kidney/mitochondria
Cardon et al.*	2020	cytosol	Y	*Daphnia magna*	muscles < liver < gills < intestine
Fish					
Qiang et al.	1994	Direct measure	La, Gd, Y	*Cyprinus carpio*	int. organs > gills > skel. > muscle
Hao et al.	1996	Direct measure	La, Ce, Pr, Nd and Sm	*Cyprinus carpio*	int. organs > gills > skel. > muscle
Goudard et al.*	1998	Direct measure	Am, Pu, Tc, Cs	*Anguilla anguilla*	Hepatopancreas, visceral masses, liver, pyloric caeca and digestive glands
I.-Georgudaki and Kotsanis	2001	tissue damage	Cd, Hg	*Oncorhynchus mykiss*	Liver/kidney/spleen/stomach/gastric caeaca
Campbell et al. *	2005	MT, MDA	Cd	*Perca flavescens*	Gills and digestive gland, liver tissue
Kraemer et al. *	2005	Direct measure	Cd, Cu and Zn	*Perca flavescens*	liver
Del Barga et al.	2006	React. Oxyg. Spec. interfering cell macrom.	Algal extracts	*Oncorhynchus mykiss*	Blood cells
Giguere et al. *	2006	Direct measure	Cd, Cu, Ni and Zn	*Perca flavescens*	Liver
Suiçmez et al.	2006	Direct measure	Pb	*Oncorhynchus mykiss*	Liver/gills
Zhang and Wang*	2006	HSP	Cd, Se, Zn	*Terapon jarbua*	Tissue
Mol	2011	Direct measure	Fe, Zn, Cu, Cd, Sn, Hg, Pb	*Oncorhynchus mykiss*	Canned fish
Rasanen et al.	2012	Protein expression	PAH's	*Oncorhynchus mykiss*	Liver
González et al.	2014	enzymes (GSH…)	La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu	*Review*	int. organs > gills > skell > muscle
Mayfield and Fairbrother	2015	Direct measure	Sc, Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu	*Oncorhynchus mykiss*	Body (small)/ carcass + fillet (big)
Rosabal et al.*	2014	Direct measure	Ag, As, Cd, Ni, Pb, and Tl	*Anguilla anguilla*	Liver
Brix et al.	2016	Direct measure/gamma radioat.	Ag, Cu, Ni	*Oncorhynchus mykiss*	Gills

(*continued on next page*)

Table 1 (*continued*)

Authors	Year	Biomarker or tracer	Studied metals	Species	Studied element
McEneff et al.	2017	Direct measure	As, Cd, Cr, Co, Cu, Fe, Pb, Mn, Mo, Ni, Se, Sn, V, Zn	*Oncorhynchus mykiss*	Muscles/skin
Varol et al.	2017	Direct measure	As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn	*Oncorhynchus mykiss*	Muscles
Cardon et al.*	2018	ENZ (CS, CCO, LDH, APHO)		*Oncorhynchus mykiss*	Kidney/mitochondria
Saïbu et al.	2018	Changes in the protein peaks	Zn, Cu, Cd	*Oncorhynchus mykiss*	gills
Urien et al. *	2018	Direct measure	As, Cd, Cu, Se and Zn	*Cephalorhynchus commersonii*	Liver, gonads
Cardon et al.*	2019	Mitochondria	Y	*Oncorhynchus mykiss*	Kidney/mitochondria
Khadra et al. *	2019	Direct measure	Hg, Se	*Perca flavescens*	Gonads, liver
Sandhu et al.	2019	MC2R: Cortisol levels	Cd	*Oncorhynchus mykiss*	Plasma/head kidney
Santos et al.	2019		Cu	*Oncorhynchus mykiss*	Larvae
Cardon et al.*	2020	cytosol	Y	*Oncorhynchus mykiss*	muscles < liver < gills < intestine

AChE: acetylcholinesterase, CAT: catalase, ChE: cholinesterase, GSH: glutathione, CSP: caspase, GPx: glutathione peroxidase, GSTs: glutathione-S-transferases, HSP: heat-sensitive proteins, MDA: malondialdehyde, MRG: metal-rich granules, SOD: superoxide dismutase, TBARs: thiobarbituric acid reactive substances, CS: citrate synthase, CCO: cytochrome *c* oxidase, LDH lactate dehydrogenase, APHO: Acid phosphatase, MTLP: metallothionein like proteins, MT: metallothionein, LPO: lipid peroxidation, DSB: DNA strand breaks, COX: prostaglandin cyclooxygenase, MC2R: melanocortin 2 receptor.
Ag: Silver, Ag NPs: Silver nanoparticles, Al: Aluminium, Am: Americium, Ammonia, As: Arsenic, Au: Gold, Cd: Cadmium, Ce: Cerium, Co: Cobalt, Cr: Chromium, Cs: Caesium, Cu: Copper, Dy: Dysprosium, Er: Erbium, Eu: Europium, Fe: Iron, Gd: Gadolinium, Hg: Mercury, Ho: Holmium, La: Lanthanum, Lu: Lutetium, Mn: Manganese, Mo: Molybdenum, Nd: Neodymium, Ni: Nickel, PAH's: Polycyclic aromatic hydrocarbons, Pb: Lead, Pr: Praseodymium, Pu: Plutonium, Sb: Antimony, Sc: Scandium, Se: Selenium, Sm: Samarium, Sn: Tin, Tb: Terbium, Tc: Technetium, Tm: Thallium, V: Vanadium, Y: Yttrium, Yb: Ytterbium, Zn: Zinc.

example, the study by Cooper et al. (2013) demonstrated a differential accumulation of cadmium (Cd) in the gills and digestive gland cells of *Pynagodon grandis* (Say 1829) using subcellular fractionation. They showed in the gills that Cd accumulated preferentially in the granule subcellular fraction whereas in digested glands a greater accumulation of Cd was observed in the heat-denatured fraction. Another study by Khadra et al. (2019) highlighted differences in methylmercury (Me-Hg) subcellular partitioning between hepatic and gonadal cells of the Yellow Perch *Perca flavescens* (Mitchill, 1814). More than 70 % of the Me-Hg were found in the sensitive fraction (heat-denatured proteins and mitochondria) of the liver, compared to the 44 % of Me-Hg found in the cytosolic fraction of gonadal cells. This way, studies have shown a differential fdistribution of metals depending on the organism and the nature of the metal (Wallace et al. 2003; Wang et al. 2016; Cardon et al. 2019).

The protocol of Wallace et al. (2003) did not include an efficient estimation of the purification of each fraction, which could contaminate each other, by rupture of the membranes of the organelles during the homogenization step. In many studies, no purification degree of the different fractions was performed (Goudard et al. 1998; Kraemer et al. 2005; Giguère et al. 2006). Nevertheless, a particular effort to validate the quality of the fractions is noticeable in recent studies such as, for example, performing enzymatic assays on each fraction (Cardon et al. 2018; Urien et al. 2018, 2020). Khadra et al. (2019) developed an enzyme-validated subcellular partitioning protocol for subcellular fractionation of liver and gonads of *P. flavescens* (Yellow Perch). In this study, measurement of specific enzyme activities (citrate synthase, mitochondrial biomarkers as cytochrome *C* oxidase, or cytosolic biomarker as lactate dehydrogenase) estimated the purification degree of each fraction and validated the subsequently compartmentalization results. Metallothioneins are also commonly quantified in the heat-stable protein fraction (BDM). Then, their concentrations are correlated to metal levels in the same fraction and they were analysed as detoxification biomarkers in order to compare results between organs or organisms (Wallace et al. 2003; Shi and Wang 2004; Bonneris et al. 2005a, Bonneris et al., 2005b; Campbell et al. 2005; Cooper et al. 2013; Wang et al. 2016).

4. Current methodologies and limitations

As seen previously with the literature search, bivalve and fish studies that used subcellular compartmentalization for a better understanding of metal localisation, were mainly based on the subcellular fractionation protocol adapted from Wallace et al. (2003) (e.g. for example, Jones et al. 2009; Beaumelle et al. 2015; Zimmermann et al. 2017; Cardon et al. 2020). However, considering recent advances on the ecotoxicology field, these subcellular fractionation protocols would need additional development if they want to be competitive with other state of the art techniques.

Subcellular fractionation is more complex than regular cellular compartmentalization. Table 2 gathers the main advantages and disadvantages of their use. Subcellular fractionation is more technologically advanced and requires an interdisciplinary in-depth knowledge in the fields of biology and chemistry. It is a method with a high degree of accuracy if verifications are made to ensure that the studied fractions are those required for a robust understanding of the cellular distribution of a metal. It is, therefore, necessary to control the accuracy of the method to *i)* estimate the purification degree of each fraction *ii)* validate the protocol used and *iii)* verify the biological interpretation of the compartmentalization analysis.

A first control of the protocol is the efficiency of the lysis step. Several studies have shown that without a reliable homogenization step, a significant part of the unhomogenized sample reached the debris, varying between species [as demonstrated between *Chironomus riparius* (Meigen 1804), *Daphnia magna* (Straus, 1820) and *Onchorynchus mykiss* (Walbaum, 1792) (Cardon et al., 2018); *Chlamydomonas reinhardtii* (Dangeard 1888) (Lavoie et al. 2009)], but also between tissues (Simon et al. 2005), hence leading to bias when comparing such conditions.

Secondly, the different fractions could be studied with several techniques, such as transmission electron microscopy, as reported by Bustamante et al. (2006) and Simon et al. (2005). However, another possibility to test subcellular fractionation methods (and a more

Table 2
Main advantages and disadvantages of cellular compartmentalization vs subcellular fractionation.

Techniques	Advantages	Limitations
Cellular compartmentalization	Simpler technique	Less sensibility Less accuracy Does not allow a deeper study of the sub fractions Limitated use of biomarkers
Subcellular fractionation	More sensible High degree of accuracy Allows the study of sub fractions Use of different biomarkers	More technologically dependant Requires verification

common one) is to use several biomarkers, based on the abundance of enzymes/proteins in specific fractions, in order to have an integrated approach on the biological markers associated with the different compartments. Such tests present some advantages, as they are relatively fast and easy.

In the studies in which this control is performed, only metallothionein (MT) or metallothionein like proteins (MTLP) are measured and mainly through polarography. MT are cysteine-rich, heat-stable, low molecular weight proteins that bind metals, contributing to intracellular metal homeostatic regulation and metal detoxification processes. The synthesis of these proteins is often induced when organisms are exposed to metal polluted environments, thus being of vital importance when studying these processes, as MT levels can be directly related to the metal concentration in the different cell fractions. Nonetheless, some studies reported differences regarding the relationship between MT induction and levels of bioaccumulated metals and consequently their potential toxic effects. These limitations on the technique depend on multiple factors, such as: loss of sample during its manipulation, the detection limit of polarography, the distribution of pollutants among fractions and if their content would result in MT induction. The chemical partitioning of the metals is key, as their different forms may bind, or not, to MT, thus obtaining possible unreliable results. Tissue-specific induction of the protein, organelle functions, isoforms of MT and many other experimental factors such as: temperature, pH or salinity must also be considered (Beaumelle et al. 2017).

Other enzymatic biomarkers could also be addressed. As an example, an enrichment of activities of the cytochrome *c* oxidase, enzyme specific to mitochondria and acid phosphatase (specific to lysosomes) were verified in the respective fractions, to ensure good efficiency of the protocol by Taylor and Maher (2012). In addition, an increase in LDH (lactate dehydrogenase) activity after the lysis step helped to verify the accuracy of this step, as this enzyme is present in the cytosol (Philipp Seib et al. 2006; Rosabal et al. 2014). Cardon et al. (2018, 2019, 2020) did extensive work on optimizing subcellular fractionation protocols in freshwater species. Thus, they compared subcellular fractionation of three aquatic species: *D.magna, C. riparius* and liver of *O. mykiss* selecting the most accurate protocols, by comparing fraction-specific enzymatic activities between species. Several enzymatic biomarkers were used by the same authors to verify each of the fractions obtained such as: citrate synthase and cytochrome *c* oxidase for the mitochondrial fraction, LDH for cytosol separation and acid phosphatase for the lysosomal fraction. They have shown that the mitochondrial matrix is more likely recovered in the cytosolic than in the mitochondrial fraction, both for *C. riparius* and *D. magna*. However, since the mitochondrial fraction belongs to the metal sensitive fraction (MSF), results could be underestimated and do not reflect expected detoxification processes. In the same manner, an important part of the lysosome fraction was found in the cytosol of *O. mykiss and D. magna*, hence posing questions of bias in the interpretation. Even more, one may wonder if the classification of this fraction differs between studies (as some consider that it is part of the metal-rich granules part; Nassiri et al. 2000; Bustamante et al. 2006; Frelon et al. 2013), whereas others stated that their components are released into the cytosol after toxicity (Eaton and Qian 2002; Marasinghe Wadige et al. 2014).

The debris fraction can be separated into two subfractions, the so-called cellular debris and metal-rich granules fractions, by using a treatment with sodium hydroxide (NaOH) of heating followed by centrifugation (Wallace et al., 2003). However, these treatments do not allow subsequent enzymatic activity measurement and validation of those two sub-fractions remains difficult. Furthermore, the heat treatment has been demonstrated to cause metal transfer between HDP and HSP fractions (Bragigand and Berthet 2003; Geffard et al. 2010).

5. Research directions and needs

To assess the global effect of a metal (adverse effect and behaviour within the organism), the coupling of subcellular compartment technique and biomarkers analysis appears to be a relevant strategy. In the context of Adverse Outcome Pathway (AOP), such a combination of techniques allows linking molecular events to predictions at the individual level (trophically available part).

Enzymatic assays represent relevant methods to verify subcellular fractionation efficiency and present many advantages. However, they could also have limitations since it is well known that many proteins have multiple subcellular locations, often with different biochemical/physiological roles depending on the site within the cell, usually associated with different isoforms. In addition, protein location is often dynamic, depending on conditions and signalling. This indicates that a potential source of uncertainty exists and should be taken into consideration especially in aquatic species, whose biology is not fully characterized.

The potential of omic methodologies to get a full signature profile of subcellular fractions could help to obtain a clear overview of the consequences at the protein (proteomic), metabolite (metabolomics), or gene expression levels (transcriptomics) (Revel et al. 2017). These approaches involve highly advanced and reliable analytical technologies, mainly nuclear magnetic resonance and mass spectrometry, as they provide high degrees of both sensitivity and selectivity. Coupled with bioinformatics methods, they could help to investigate global cellular responses as well as their subcellular localization. Thus, spatial proteomics correspond to the systematic and high-throughput study of protein subcellular localisation, allowing the determination of their specific biochemical and interaction environment (Gatto et al. 2019). This field relies on a wide range of subcellular separation techniques, most employing differential centrifugation or separation along density gradients, and the subsequent analysis of protein profiles in these subcellular fractions (reviewed in Gatto et al. 2010). Through metabolomics studies, subcellular compartmentation of metabolism is also more and more considered, helping to unravel metabolic network activity and functionality (Klie et al 2011; Fürtauer et al. 2016).

In this context, metallomic approach aims to combine high-sensitivity analytical tools [inductively coupled plasma (ICP MS), high resolution mass spectrometry (HR MS)] and highly resolutive separation techniques [size exclusion (*SEC*), liquid chromatography (LC), capillary electrophoresis (CE)]. It helps to deepen the distribution of bioaccumulated metals and metal-binding molecules (proteins, metabolites) among biological structures allowing to define their localization, identity, quantity, function in single cells, organs or organisms (Lavradas et al., 2016, Dragun et al. 2020). Several bioindicators species (such as: fish or bivalves), have not yet been well-characterized regarding their metallome variations in response to environmental contaminants (Lavradas et al., 2016). Less than 15 studies emerge doing a literature search based on the keywords “metallomic” “fish” and “bivalves”, demonstrating the open field to explore. As the metallome is influenced by genetic and environmental factors, it would be relevant to combine proteomic, metabolomic and transcriptomic approaches. Under stress conditions, it would help to finely characterize molecular differences in expression profiles between bivalves or fish tissues. The objective is to link molecular event variations to the adverse effect observed at higher levels of organization. In this way, research on fish or bivalves are even less numerous. A toxicogenomic approach on a fish model, the stickleback *Gasterosteus aculeatus* (Linnaeus, 1758) has been led by Santos et al. (2010) to investigate the signalling pathways mediating the effects of exposure to copper (Cu). Genomic and metabolomic data closely aligned supported the conclusions that copper induces a shift to anaerobic metabolism. On the other hand, developing experiments, combining such omic approaches and subcellular fractionation, could afford better insight into the understanding of subsequent biological processes and the identification of reliable subcellular markers. In this line of reasoning, recent studies aim to combine cellular cytosol fractionation and proteomic approaches in different organs of fish. Different isoforms of the same metal-binding biomolecules were

thus evidenced by Krasnići et al. (2019), in hepatic and gill cytosols of Vardar chub *Squalius vardarensis* (Karaman 1928). Dragun et al. (2020) also highlighted striking organ-specific differences in metal/nonmetal cytosolic distributions in the liver and gills of Prussian carp *Carassius gibelio* (Bloch, 1752) from the Croatian river Ilova. Therefore, the integration of whole subcellular fractionation to metallomic approaches appears highly relevant for a better understanding of the metal behaviour within the cells.

6. Conclusion

This mini review highlights the inputs of subcellular fractionation in the understanding of internalisation and toxic mechanisms of metal-based pollutants, at the individual level and for food web purposes. Some limitations of the method, mainly the lack of qualitative validation of the fractions and many steps which increase the risk of loss of pollutant, can bias the biological interpretations and are important issues to be considered. The identification of fraction-specific markers (proteins, enzymes) appears as the most valuable method to improve fractionation quality, benefiting from several advanced analytical tools. Finally, in the context of filling the gap between molecular and cellular responses (e.g., subcellular fractionation) of exposed organisms, impact on population (through trophic transfer) and next generation, the concept of Adverse Outcome Pathways (AOP) appears as a suitable approach linking multiple levels of biological organization (Völker et al., 2013).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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