Chapter 3

Mussel as a Tool to Define Continental Watershed Quality

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Abstract

Bivalves appear as relevant sentinel species in aquatic ecotoxicology and water quality assessment. This is particularly true in marine ecosystems. In fact, several biomonitoring frameworks in the world used mollusks since several decades on the base of contaminant accumulation (Mussel Watch, ROCCH) and/or biological responses called biomarker (OSPAR) measurements. In freshwater systems, zebra and quagga mussels could represent alternative sentinels, which could be seen as the counterparts of mussel marine species. This chapter presents original studies and projects underlying the interest of these freshwater mussels for water quality monitoring based on contaminant accumulation and biomarker development measurements. These sentinel species could be used as a tool for chemical/biological monitoring of biota under the European water framework directive and for the development of effect-based monitoring tools.

Keywords: *Dreissena*, biomonitoring, bioaccumulation, biomarkers

1. Introduction

The continental ecosystems are subjected to many stresses related to the human activities through the emission of a large number of molecules whose toxicological and ecological effects are currently poorly studied. There needs to be further development of tools not only for highlighting the exposure of nontarget organisms to these molecules but also for assessing possible risk to the stability of their population and, by domino effect, to the biodiversity and ecological functioning of the ecosystems. Water quality evaluation, management, and protection represent a strong challenge of our society to maintain the sustainability of freshwater ecosystems and protect the biodiversity and ecosystem services for human benefits such as the potabilization or recreational uses. This challenge results in particular in a better management of all the new chemical products likely to be introduced into the environment (program recording of chemical substances REACH) as well as the evaluation of its ecological state (European framework directive on water 2000/60/CE). Currently, the texts of the European Community dated October 23, 2000, set a number of environmental objectives in order to reach the "chemical and ecological good state" of freshwaters. Although environmental quality standards appear in European texts, this monitoring program is primarily based on the evaluation of the level of contaminants in water and on the biological diversity of the aquatic communities. In addition to the biocenotic approaches, many researches focus on biological responses evaluated at sub-individual and individual levels, gathered under the generic term of "biomarkers." Among these biomarkers, a very close attention is paid to the processes whose disturbances may induce a chain reaction of damage on the population and community levels. These biomarkers include physiological functions such as the energy metabolism, the reproduction, or the immune system. The development of biomarkers is conducted on a large number of species known as "environmental sentinels" with respect to criteria related to their sedentary lifestyle, their large distribution, knowledge on their biology and ecology, and the feasibility of using them for experimental exposures. Among these species, bivalves appear to be a genuine tool for the detection of the chemical and biological contamination of the environment [1]. Indeed, the filter feeders have the ability to accumulate and concentrate the contamination in a time- and dose-dependent manner that allows to recall events of pollution prior to the sampling point. The studies are mainly based on the marine species of economic interest as the mussels (*Mytilus* sp.), the clams (*Ruditapes* sp.), and the oysters (*Crassostrea* sp.). However, at the continental level, a model organism, zebra mussel *Dreissena polymorpha* (Pallas, 1771), proves its interest and could represent the freshwater counterpart of *Mytilus* sp. [2]. Zebra mussel is an invasive species originated from Ponto-Caspian region in central Europe. It has spread around the world and became invasive in Europe and North-America freshwater ecosystem, where it has been observed in the great lakes at the end of the 1980s. *D. polymorpha* is characterized by a large abundance and widespread distribution, great filtration capacities leading to high levels of xenobiotic accumulation, and a good tolerance of environmental stressors. Being sedentary, their individual responses may be correlated to the quality of the site. Moreover, dreissenids present a lifetime of several years, can be sampled all year around, and their biology and ecology are well known [3]. Therefore, zebra mussel constitutes a valuable bioindicator species, largely used as freshwater biomonitoring tool [4–7].

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2. Interest for bioaccumulation capacities

Bioaccumulation is defined as the accumulation of a target compound in an organism relative to its concentration in the surrounding environment. It depends on several intrinsic (e.g., detoxification abilities, lipid content, maturity, or sex of the organism) and environmental (e.g., water temperature or pH) factors that have to be considered in bioaccumulation assessment. The ability of species to bioaccumulate contaminants could represent an indirect evaluation tool of water quality allowing going beyond the limitations associated both to the sporadic nature of water sampling and to potent high dilution of contaminant in the water matrix. In fact, contrary to water matrix, using attached filter-feeding organisms makes contamination measurements representative of their living environment. Measurements in appropriate biological matrices such as mussels will (i) limit the variability (temporal integration) of measurements as compared to measurements on water samples, (ii) convey the degree of contamination of water bodies more reliably, and thus facilitate comparisons. Zebra mussel, *D. polymorpha*, has a high filtration activity and filters between 0.018 and 0.402 L/mussel/h [8] that may give them a capacity to accumulate environmental contaminants.

2.1. Pharmaceuticals

There are only a few studies dealing with the uptake and bioaccumulation of pharmaceuticals in freshwater mussels; more studies are available for fish. Quantification of pharmaceuticals at trace levels in mollusks is often analytically challenging, due to the complexity of the biological matrix, requiring suitable extraction and purification steps combined with selective and sensitive detection techniques. Various methods are employed for the extraction of pharmaceuticals in bivalves: pressurized liquid extraction [9, 10], microwave-assisted extraction [11, 12], sonication [13], or QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction [14, 15]. The last one has the advantages of a low solvent consumption combined with rapid extraction and clean-up steps. Most current analytical methods for the separation and detection of pharmaceuticals are based on gas chromatography-mass spectrometry (GC-MS) or more frequently on liquid chromatography (LC) coupled to ultraviolet (UV), diode array (DAD), fluorescence (FL) detectors, or mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Due to large differences in their physicochemical properties (e.g., polarity and solubility), efficient extractions of all the targeted pharmaceuticals are difficult to obtain, especially in multiresidue methods. As a consequence, the few studies related to the bioaccumulation of pharmaceuticals in freshwater mussels generally focused on a very small number of target compounds [15–17]. Li et al. [18] studied the distribution of 22 antibiotics in 11 mollusk species sampled from the Bohai Sea of China, with concentrations up to 1575.10 μg/kg for quinolones, 76.75 μg/kg for sulfonamides, and 36.21 μg/kg for macrolides. Du et al. [19] studied the bioaccumulation of several selected pharmaceuticals in two mussel species (*Pondhorn mussel* and *Paper pondshell* mussel) and fish, among other matrices, collected from a lower-order effluent-dependent stream in Texas, USA. They concluded that pharmaceuticals accumulated to higher concentrations in invertebrates in comparison to fish. Fluoxetine, carbamazepine, and diphenhydramine were quantified at concentrations

inferior to 22 μg/kg, whereas elevated concentrations of the antidepressant sertraline and its primary metabolite desmethylsertraline were observed in both mussel species at levels higher than 130 μg/kg. Indeed, as they could exhibit equal or higher bioaccumulation capacities, it is relevant to include metabolites and transformation products in bioaccumulation studies [15, 19]. Bioconcentration factors (BCFs) have been calculated for the anti-inflammatory drug diclofenac in different studies. BCF of 10 have been displayed for blue mussels exposed during 8 days in aquarium to diclofenac at 1 μg/L [20], and comprised between 4 and 13 in another study where diclofenac was introduced in mesocosms at concentrations between 0.05 and 5 μg/L [15]. This study shows the accumulation in *D. polymorpha* as a function of exposure time, and highlights the presence of one of its transformation products in this bivalve species. The bioaccumulation of the contraceptive hormone levonorgestrel was also examined in *D. polymorpha* [21]. The lowest concentration (0.312 μg/L) was 100-fold bioconcentrated within 4 days. A decrease of the BCF was observed within 1 week for the highest tested concentrations (3.12 and 6.24 μg/L).

2.2. Protozoa pathogens

Protozoa such as *Toxoplasma gondii, Cryptosporidium parvum*, and *Giardia duodenalis* represent a threat to human health because they are infectious at low doses and their (oo)cysts can remain infectious in the environment for months due to a robust wall [22, 23]. *T. gondii* is the agent of toxoplasmosis and infection is mainly acquired by the ingestion of food or water that is contaminated with oocysts. *C. parvum* and *G. duodenalis* are intestinal protozoa found in mammals, fish, and birds. (Oo)cysts can be transmitted via drinking and recreational waters contaminated by agricultural and urban runoff [24]. Protozoa are difficult to detect in environmental samples. Actually, no standardized method has been developed for the detection of *Toxoplasma* oocysts in water samples. On the contrary, *Cryptosporidium* oocysts and *Giardia* cysts are currently detected in samples from 100 L of filtered water, using indirect immunofluorescence after immunomagnetic separation (ISO 15553:2006). Limitations have been identified in this method: it requires large volumes of filtered water and high parasite concentrations, it is costly and time-consuming, and thus does not allow for rapid routine detection. Moreover, filtration and purification techniques from water supplies can yield variable results depending on water quality, sampling period, place, and quantity [24].

Several studies have demonstrated the interest of using aquatic organisms to assess and monitor the quality of water bodies [25] and *D. polymorpha* can represent a tool to reveal protozoan contamination. Indeed, during a laboratory experiment, zebra mussels were exposed to various *C. parvum, G. duodenalis*, and *T. gondii* (oo)cyst concentrations for 1 week, and bioaccumulation by mussels proportionally to ambient contamination was observed. This study showed for the first time *T. gondii* accumulation by zebra mussel [26]. In a second in vivo experiment, *D. polymorpha* were exposed to 1000 *T. gondii* oocysts per individual and per day for 21 consecutive days, followed by 14 days of depuration time in protozoa-free water. *T. gondii* was detected in all organs in a time-dependent manner, but oocysts were found in greater amount in the hemolymph and mantle tissues compared to the other organs (gills, gonads, and foot).

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This study also shows that mussels accumulate protozoan proportionally to water contamination. Moreover, *T. gondii* was still present in the mussel tissues for up to 14 days postinoculation, reflecting the integrative character of *D. polymorpha* [27]. A field study was also carried out in order to test protozoa detection under environmental conditions. *D. polymorpha* were caged for two seasons (autumn and spring) for 1 month upstream and downstream of wastewater treatment plants (WWTPs). Concerning the study realized in autumn, *T. gondii* was detected in mussels caged downstream of two WWTPs. In spring, *T. gondii* was detected upstream of one WWTP. These results highlight the interest to use *D. polymorpha* caged as a new effective tool in sanitary biomonitoring of water bodies [28].

2.3. Cyanobacteria

The eutrophication of aquatic ecosystems, associated to the climate change, enhances the frequency and the severity of cyanobacterial proliferations. Cyanobacteria are photosynthetic organisms producing endotoxins such as neurotoxins, hepatotoxins, dermatotoxins, and cytotoxins, threatening target organisms and humans [29]. The World Health Organization derived a guideline value of 1 μg/L in tap water for the hepatotoxins microcystins (MCs), the most reported cyanotoxins in fresh waters worldwide. In addition to being exposed to cyanotoxins through drinking, humans can also be exposed by ingestion or inhalation of contaminated water during recreational activities [30, 31], or by trophic transfer of MCs as demonstrated between invertebrates and vertebrates [32]. The bioaccumulation of MCs and their effects on organisms are overall quite well documented. However, some cyanobacteria and diatom strains may also produce the neurotoxin BMAA (β-N-methylamino-L-alanine) [33–35], a nonproteinogenic amino acid that has been associated with neurodegenerative diseases like amyotrophic lateral sclerosis/Parkinsonism dementia complex (ALS-PDC) that occurred on the Guam Island [36]. The bioaccumulation of BMAA in marine organisms is little documented [37–39]. However, BMAA accumulation and impact on freshwater organisms during laboratory exposure or field investigations remains poorly investigated [35, 37].

Even though monitoring programs are in place to evaluate the concentration of cyanotoxins in fresh waters, prompt management of ecological and public health risk remains difficult due to the high spatiotemporal variability of the cyanobacterial proliferations. Freshwater mussels, and particularly *D. polymorpha*, are known to ingest phytoplankton and cyanobacteria and accumulate cyanotoxins at the laboratory [40, 41] and in the field [42–44]. Therefore, the use of sentinel species to integrate and reveal early MC and BMAA contamination at low cyanobacterial or phytoplankton densities may allow a preventative management of contaminated sites. As MC accumulation by *D. polymorpha* has been largely demonstrated, we primarily focused on the capacity of the mussel to accumulate BMAA using a laboratory approach. The mussel *D. polymorpha* was exposed to exogenous BMAA at 500 μg/L for 3 weeks, and we followed up both free and protein-bound BMAA in tissues. The free amino acid BMAA was detected from day 2 to day 21 and really high concentrations (reaching 283 μg/g dry weight) were quantified in mussels at the end of the contamination experiment. BMAA was therefore taken up by *D. polymorpha* and the internalized concentration of the toxin, detected as its free form, increased linearly over time ($r^2 = 0.9635$) while no steady state was reached (**Figure 1**).

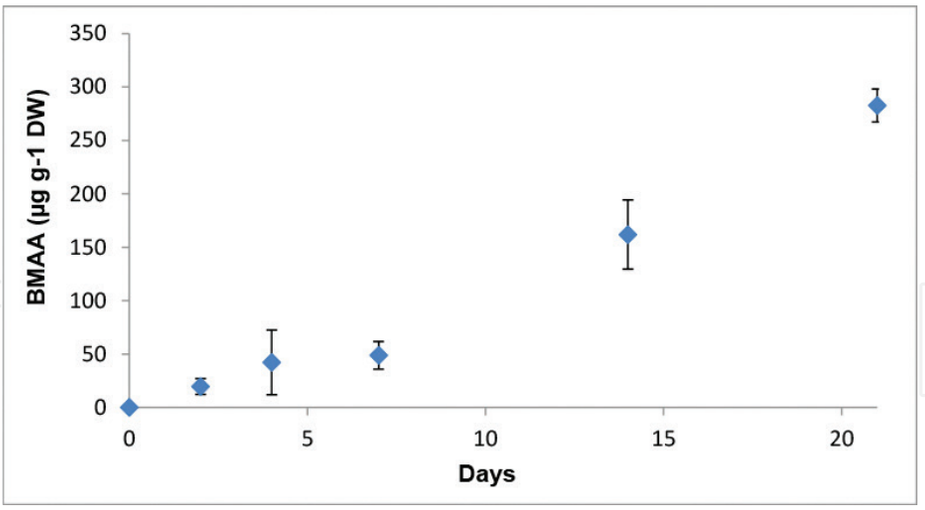

Figure 1. Concentration of free BMAA in *D. polymorpha* with fitted linear regression after free exogenous exposure for 21 days at 500 µg/L. Error bars denote standard deviation of $n = 5$ (except for D21, $n = 2$).

It is conceivable that this freshwater species would have continued to accumulate BMAA from the medium after a longer exposure time. Concentrations of total BMAA in mussels of day 2 and day 21 were not significantly different from the concentrations of free BMAA in the same individuals (data not shown). Thus, the incorporation into or in association of BMAA to proteins was not evident in this study.

Overall current projects also include other freshwater bivalves as potent predictive sentinel species of the presence of MCs and BMAA in freshwater environments through laboratory and *in situ* approaches. The results that will be achieved in these projects will facilitate the long-term tracking of the contamination of ecosystems by cyanotoxins, which will provide an advance in the knowledge about the ecodynamic of cyanotoxins and the main conditions of human exposure. This step forward could constitute a decision-making tool for public health authority in charge with the management of food safety risks.

3. Interest for biomarker measurement

Different biological responses have been developed as biomarkers on freshwater mussels. Biomarkers related to energy metabolism could be an interesting prognostic tool bearing in mind the close relationships existing between the energy balance and health of individuals. As well, the reproduction function is a key element for the preservation of the species in their environment and can be disrupted by numerous stresses of anthropogenic origin. Furthermore, organisms are subjected to various pathogens. Thus, interaction with hemocytes, a key component of the immune system, undoubtedly occurs. Exploring these three relevant functions will permit to develop early warning and sensitive diagnosis tools with an ecological relevance which seems to be promising for the early assessment of the ecosystem degradation resulting from damaging contaminants.

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3.1. Energy and metabolism

All the biochemical and physiological processes involved in the vital cycle of the organisms depend strictly on the energy metabolism. Besides, within the framework of an evaluation of the quality of the environment, the variations at the molecular level (e.g., enzymatic activities) are one of often first answers to the stress; they thus represent useful points in the confirmation of the toxic effects before these last ones are perceptible at superior biological levels of organization (cellular, tissular, and physiological). Biomarkers related to energy metabolism could then constitute interesting prognostic tools considering the casual relationships linking the energy balance and the health status of individuals. A very large number of biological (adenylate energy charge, energy reserves, cellular energy allocation, scope for growth, etc.) responses to stress linked to the metabolism and energy of living organisms may be investigated. While the concept is clear, linking cellular or biochemical responses to the individual and population level remains relatively scarce. However, several studies underline the relevance of cellular responses to extrapolate effects at higher levels of biological organization in *D. polymorpha* under laboratory [45] and environmental conditions [46]. Zebra mussel and all other animals obtain the energy necessary for life from an external source. So, in heterotrophic organisms, the acquisition of energy is controlled by feeding and the subsequent breakdown of food to release the energy contained (assimilation). Feeding rate/clearance was studied in different species of mussels (particularly blue mussel) exposed to various contaminants [47–49]. Generally, feeding appears unaffected and/or less sensitive compared to biochemical responses. Inversely, during the study of different zebra mussel populations, among several genes involved in cellular metabolic activities, detoxification process, oxidative stress, and digestive functions, digestive enzyme genes expression appears particularly relevant to discriminate the sites according to the contamination level [50]. This study has also pointed out different sources of variability in gene expression (individual size, season, trophic resources, and origin of mussels) which are inevitable in natural fluctuant environment. Accordingly, in *D. polymorpha*, we decided to investigate the breakdown of food's mechanisms and particularly the digestive capacity. Digestion in bivalves occurs through a biphasic process involving an extracellular digestion phase of ingested food particles followed by an intracellular digestion phase. The first step occurs within the stomach under the mechanical and chemical actions of the crystalline style (CS), a gelatinous rod saturated with digestive enzymes. The CS protrudes from the style sac into the stomach and revolves against a cuticular structure, the gastric shield, promoting the grinding of food materials and their mixing with the enzymes released by its former dissolution. This extracellular digestion phase allows a preliminary breakdown of food particles into molecules small enough to enter the digestive diverticulae where digestion is completed intracellularly within phagocytic digestive cells. In this way, amylolytic and cellulolytic activities in the crystalline style and the digestive diverticulae of the freshwater bivalve *D. polymorpha* were characterized [51]. In the same time, to avoid the influence of biotic parameters (age, size, etc.), we proposed to use individual from the same population transplanted to the study sites (see part 5). Thus, during active biomonitoring carried out with *D. polymorpha*, in the catchment of the River Vesle, upregulation of digestive activities in digestive gland was observed in mussels exposed to various chemical contaminants and thus potentially facing an increase in their

energy expenditure (general adaptation syndrome) [52]. However, this upregulation has not been recorded at all contaminated sites, lower levels of pollution or higher levels of food availability probably explaining the low need for organisms to "invest" in their digestive activities (least energy requirement). These results highlight adjustment of digestive enzymatic activities according to intake and energy requirements of the organism, factors which, if not taken into account, constitute potential confounding sources in the interpretation of response measured. According to literature data, digestive carbohydrate activity of bivalve species shows a great adaptability to variations in food availability, quantity, and quality. However, if food availability exerts a primary control on digestive activities, the latter could also vary in response to other factors, both external (e.g., temperature) and internal (e.g., reproductive state). These "regulation" factors exhibit seasonal variations, which, logically, should be reflected on digestive enzyme activities [52]. Nevertheless, if seasonal amylase and cellulase activities were well recorded in the digestive gland of zebra mussels, no seasonal activities were recorded in their crystalline style. The absence of seasonality in the crystalline-style activities could be related to the innate nature of the style, for example, a secretion organ whose weight does not vary according to the seasons. Inversely to digestive gland, the crystalline-style activity (downregulated) appeared to be more associated with the location and metal exposure level, allowing the discrimination of the most chemically impacted site from the others. Thus, the high discrimination potential of crystalline-style enzymes activities, in association with their low seasonal dependency, makes these parameters very promising biomarkers to develop in biomonitoring studies using *D. polymorpha*. In this way, zebra mussels were transplanted during 2 months along a metal and organic pollution gradient in spring 2008 (Seine River, France; PIREN-Seine program) [53]. Amylolytic and cellulolytic activities measured in the CS displayed similar patterns of response with lower activities at the downstream site compared to the upstream site (20–30% lower). By contrast, digestive enzyme activities in the digestive gland displayed only slight variations during the whole period of exposure, confirming the interest of the crystalline style as matrix in biomonitoring program.

3.2. Reproductive process

There is a large body of literature on ecophysiology and reproductive biology of dreissenid species [54]. The reproductive function is a key element for the preservation of species, implying a lot of responses which may be studied in reproductive ecotoxicology at different levels of biological organization, for example, from toxic effects on biological macromolecules to the consequences on the reproductive capacity of organisms and the maintenance of their population in the environment. Until today, literature in dreissenids mainly focused on disturbances of gametogenesis and on biomarkers related to the integrity and functionality of gametes. Numerous stressors of anthropogenic origin are susceptible to modify the reproductive physiology of mussels, either by direct or by indirect effects. In dreissenid species, existing data on the disturbances of the reproductive cycle by environmental factors, such as water temperature, food availability, or chemical contamination, highlighted early or delayed sexual maturation as well as asynchronous development between congeners in exposed mussels [55–58]. Many studies also revealed that gonads were the primary organs infected by trematodes of the family *Bucephalidae* in dreissenid mussels, known as the first intermediate host. The development of cercariae in sporocysts coincides with the host sexual

maturation and causes in infected mussels an energetic depletion allowed to the reproductive tissue, leading to disturbances of their reproductive cycle as well as in the most severe cases to complete castration and host sterility [59, 60].

The gametogenesis and reproductive effort in dreissenid species has mainly been investigated by either nonhistological examinations, with the measure of the reproductive tissue biomass (gonad) compared to the rest of soft tissues (somatic) and the definition of a *gonado-somatic index*: GSI (the difficulty for discriminating the reproductive tissue and the digestive gland renders this methodology quite imprecise); either histological examination of gonads with the definition of various reproductive stages (*resting, development, prespawning, or postspawning stages*) and the possible measurements of morphological parameters by computer-assisted image analysis systems (e.g., oocyte number in follicles, oocyte diameter, etc.). Although informative, histological examination of the gonads is both time-consuming and experimenter-dependent. Recently, a novel strategy has been developed for a rapid and sensitive determination of an *index of sexual maturity* in male zebra mussels. This index considers the proportion of germ cells in male gonad according to their DNA content ((sub)-haploid, diploid, or tetraploid cells) measured by flow cytometry. This *index of sexual maturity* was used to describe the reproductive cycle in a control population of zebra mussels over a year. This biomarker also highlighted the reprotoxicity of carbamazepine, an anti-epileptic compound which was experimented in a mesocosm study in caged zebra mussels [61]. Initiation of gametogenesis, proliferation, and differentiation of immature germ cells determines both the quantity and quality of mature germ cells or gametes implied in fertilization and *de facto* in the reproductive success of zebra mussels [62]. Gametes represent "cells of choice" for the development of biomarkers in ecotoxicology, by relating the exposure of adult mussels with the long-term consequences in offsprings [63]. Until today, spermatozoa of dreissenid mussels have mainly been studied in comparison with oocytes for the development of biomarkers like sperm mortality (or membrane permeability), sperm motility (duration, velocity, etc.) with computer-assisted sperm analysis (CASA) system or fecundity [64]. Other authors investigated the integrity or the functionality of specific organites, like acrosomal integrity or mitochondrial membrane potential, and demonstrated the sensitivity of zebra mussel's spermatozoa to the toxicity of commercial formulations of pesticides (Bayluscide, Round-Up Ready-to Use Plus®) [65]. In their study, Seaver *et al*. [66] observed in spermatozoa of zebra mussel that *in vitro* irradiated with a physical agent (UV-B) decreased acrosomal integrity, incorporation of sperm into egg cytoplasm, and first zygotic cleavage. These defects in first zygotic cleavages, as also observed by these authors with the irradiation of eggs to UV-B, could be related to genomic damage in gametes. To our knowledge, the use of gametes (spermatozoa and oocyte) for evaluating the genotoxicity of water contaminants has not yet been tested in dreissenid mussels. Other authors also highlighted the sensitivity of early-life stages in zebra mussels to the toxicity of various water contaminants and proposed their use for the development of biomarkers [67].

3.3. Hemocytes

To be relevant, biomarkers must gather several properties such as being flexible according to the various pressures exerted by the environment and associated with a specific mode of action which is measurable by significant and reproducible techniques. Moreover, the early

analyses of these biomarkers have to predict the effects at higher levels of organization, that is, the population. In this context, the hemocytes responses show a particular interest since they are key cells involved in the major physiological functions in invertebrates. Indeed, the hemolymph is a remarkable mussel tissue. With an opened circulatory system, hemolymph circulates through the bodies allowing to bring the nutrients to the various organs. Drawn from the adductor muscle of the bivalves after having carried out a little breach on the shell, hemolymph sampling is not lethal for the individuals and represents a clear advantage for biomonitoring. Circulating cells, the hemocytes, are present in the cellular fraction of the hemolymph and have been recently characterized [68]. These cells, mainly granulocytes and hyalinocytes, provide various functions involved in the essential physiological activities such as the transport and the digestion of the nutrients, breathing, excretion, repairs as well of the tissues and of the shell and, of course, immune defenses [69]. Contrary to vertebrates, which have an *acquired immunity* (or specific), the immunity of the invertebrates is only composed of the *innate immunity* (or not specific) ensured by the hemolymphatic compartment (hemocytes and plasma). In a global context, hemocytes allow the measurement of various markers involved in the physiological processes usually studied in ecotoxicology. Current studies are based mainly on the evaluation of the biomarkers of genotoxicity (measurements of DNA damage in hemocytes [70]), of cellular parameters (oxidative activity, mortality [71]), and immune defense (capacity of phagocytosis [72]). However, all these responses suggest the implication of another component of the immune system and complementary to the cellular responses of the organism, the humoral-mediated responses in plasma [73]. Humoral responses are regrouped in different effectors such as the lysosomal enzymes, lectins, protease inhibitors, or antimicrobial peptides [74]. The phenoloxidase cascade [75] and the fast production of reactive oxygen species (ROS) also take part in organism homeostasis. Both systems interact to give an effective answer relative to the various types of aggressions which the organism undergoes.

A recent study by Juhel et al. [45] highlights the effect of cyanotoxins on the immune functions of *D. polymorpha*. The authors underline a reduction in the full number of hemocytes per milliliter of hemolymph as well as a modulation of the subpopulations of hemocytes, an inhibition of the phagocytosis capacity and an increase in the concentration of lysozyme after 21 days of *in vivo* exposition to various strains of cyanobacteria. However, this study raises the problematic of the complexity to define the real immune effects based only on cellular approaches. Currently, very few studies take into account the entire hemolymphatic compartment, for example, cells and plasma, in the analysis of the biomarkers. However, the evaluation of the interactions and the relations between these two types of responses would permit to understand the functioning of this complex tissue which maintains actively the homeostasis of organism (for review see [76]). Indeed, considering that a panel of enzymes and hydrolytic molecules can influence the cellular responses, it seems paramount to study the humoral compounds in parallel. Considering the central role of hemolymph in the physiology of the organisms, the comprehension of the different functions it provides in relation to other major biological functions is also necessary to ensure relevant conclusions in ecotoxicological studies. However, to date, very little information is available on the hemocyte implications on the physiological responses other than those of immune functions and defenses. This lack of

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knowledge is certainly due to difficulties in studying simultaneously both cellular and acellular compartment, because it necessarily implies a global and multi-markers approach which is difficult to perform with the techniques commonly used.

4. Interest of *D. polymorpha* in aquatic biomonitoring framework

For being usable as early warning system, a sentinel species should be able to accumulate contaminants at much higher levels than those in the environment and/or to show variations in biological parameters at one or several levels of organization. In fact, even if analyses of bioaccumulated pollutants have the great benefit of traducing the bioavailable fraction of xenobiotic, they give no information about the potential biological effects. Therefore, the relationship between exposure to pollutants and associated adverse effects is of growing importance in environmental-risk assessment (ERA) and management. In this way, a sentinel species could be designated as a necessary tool of choice for monitoring both the contamination levels and the associated risks for organisms. The zebra mussel was extensively used as model organism for the quality assessment and the biomonitoring of freshwater systems for large amounts of well-known mineral and organic trace pollutants (for review, see [2]). However, more recently zebra mussel appeared also particularly relevant for the assessment of chemical (pharmaceutical, part 2.1) and biological (protozoa and algae toxins, parts 2.2 and 2.3) emergent contaminants as underlined in this chapter. Similarly, an increasing development of biological responses, called as biomarkers, under controlled laboratory conditions (*in vivo* and *in vitro* approaches considering particularly hemocytes and gill cells) and field studies ([2] and part 3 of this chapter) is observed. Regarding field studies, several passive biomonitoring approaches based on autochthonous populations underline a significant modulation of biological responses not only by environmental parameters (e.g., temperature, conductivity, etc.) but also by intrinsic parameters (e.g., size, age, genetic background, life history according to contamination, and potential adaptive responses) [50, 7]. These confounding factors could induce a misunderstanding in the data interpretation according to the toxicity of pollutants. Also, as discussed in section 4, zebra mussel seems to coexist with another *Dreissena* species and it remains difficult to distinguish between the two species. These potent physiological discrepancies could put into questions the biomarker response interpretation.

An active biomonitoring approach appears as an interesting strategy with the view to improving the usefulness of biomarkers and particularly defining reference and threshold values allowing (i) a qualification of the toxicological effects of water quality on organisms and (ii) a suitable comparison between the monitored sites. In fact, the caging of individuals from the same population (and *a fortiori* the same species) in different sites allows (i) to limit or avoid influence of intrinsic parameters and (ii) to expose organisms for a time-limited period, so recent contamination can be detected. As for contamination level and/or biological effects associated, several studies had underlined the interest of the active biomonitoring approach using the zebra mussel, *D. polymorpha* [28, 52, 53, 77, 78]. In the purpose of improving the ecological relevance of biomarkers as early-warning responses, many studies proposed to apply a multi-biomarkers approach considering physiological processes particularly those implied

to maintain an individual and/or its population (e.g., reproduction, energy, and immunity) as indicated in part 3 of this chapter. Therefore, the proposition of an integrated tool is mandatory to ensure the use of zebra mussel (together with developed biological responses) in environmental-risk assessment and management in order to establish environmental quality standards and to favor ecosystem protection. Through the process, several developments and approaches were proposed including "Integrated Biological Response" (IBR) to summarize biomarker responses [79]. This approach takes into account "only" biomarker measurements that may be limiting for an environment quality assessment requiring data from a variety of multidisciplinary sources. Then, the weight-of-evidence (WOE) approach including multiple parameters such as for instance bioaccumulation, biomarkers, bioassays, or life history traits appears particularly relevant (see part 6.2 of this chapter).

5. The usefulness of another dreissenid species, *D. rostriformis bugensis*

Despite its high invasive potential, zebra mussel populations have been recently reported to decline such as in the Rhine River [80] or in the Seine River where some populations seem to have disappeared since a dozen years (personal observations). In the meantime, the closely related quagga mussel *D. rostriformis bugensis* [81] became in turn invasive in Europe and North America. In Western Europe, the first observation of quagga mussels was made in 2006 in the Hollands Diep [82, 83] and since 2008 in the Meuse River [81, 84]. In some sites, *D. rostriformis* seems to have become gradually the dominant species [85], as it has been observed in some German and Dutch rivers [86]. Some researchers tried to explain the dominance shift from zebra to quagga mussels through biological, ecological, and ecophysiological studies. The two species are indeed characterized by differential physiological performances. They disclose different temperature, salinity and low oxygen tolerance levels, byssal thread attachment, growth, respiration rates, assimilation efficiency, or reproduction [87–89]. Quagga mussels reveal a lower tolerance to high temperature, although there is a better adaptability to lower temperature, allowing a development in deeper waters. A higher physiological adaptability of quagga mussel than zebra mussel is also highlighted with higher filtration and lower respiration rates, decreasing the energetic cost in quagga [88, 90]. A higher assimilation efficiency allows quagga mussel to maintain higher growth and fecundity rates even at low food levels [8]. Both species also reveal different reproductive strategies. Quagga mussel spawn earlier in the season and in deeper waters, but zebra mussel releases more eggs than quagga [87, 88, 90]. Quagga mussel seems to invest less energy into reproduction than zebra mussel: its lower fecundity is offset by an earlier maturity [90]. Quagga larvae settle at a larger size as they grow more rapidly. Quagga higher somatic growth rate and size [91] are supposed to contribute to a better stress survivorship. A differential sensitivity to chemicals could also be involved in the replacement of a species by the other. Some studies focused on differential xenobiotics accumulation between the two species, mostly on metals and given scarce or inconclusive information [92–95]. Schäfer et al. [80] also showed differences between *D. polymorpha* and *D. rostriformis* in the bioaccumulation potential of pesticides from resuspended sediments. They showed that quagga mussel accumulates more organochlorine pesticides, with greater DNA damage and lower stress protein hsp70 content. These results suggest a difference of sensitivity to genotoxic

stress between the two species that may be related to "threshold" levels of DNA damage or to differential capacity of DNA repair [80]. More recently, Potet et al. [96] exposed zebra and quagga mussels to nickel and chromium at two temperatures in laboratory conditions. They measured a set of 14 biomarkers that revealed differential bioaccumulation patterns, filtration activity, and cellular antioxidant and detoxification responses between the two species, with more marked effect of metals in *D. polymorpha*.

In most of the comparative studies, the morphological criterion is chosen to identify the two species. However, the two species show high morphological similarities [97, 98] that prevent from morphological consistent species identification [99]. Mussel's "angularity" has been proposed anyway to improve morphometric identification with discriminating thresholds for zebra and quagga mussels [100]. Molecular genetic markers have then been developed to differentiate *Dreissena* species [97, 98, 101], but most of them are based on restriction fragment length polymorphism (RFLP) of cytochrome-C oxidase subunit I (COI gene). Such mitochondrial genetic tool does not allow identifying potential interspecific hybrids [97]. If natural hybridization has been reported only once by Voroshilova et al. [97] using allozyme patterns, no evidence exists that the two species are unable to hybridize in natural environment. In ecotoxicological studies, a reliable identification of *Dreissena* species is a key step to ensure the efficiency of the use of such freshwater sentinel species and their associated biomarkers to reflect exposure and toxic effects of xenobiotics. It is currently essential to deepen our knowledge on the levels of responses and sensitivity of these two species with a view to their use in biomonitoring projects.

6. Perspective: toward new markers and an integrative approach

Contamination of aquatic environment is multifaceted as it combines chemical, physical, and biological pollutions, which could be influenced by a wide range of (a)biotic factors. Both short- and long-term cascading negative effects of such pressures on ecosystems still remain difficult to evaluate considering the complexity and the diversity of exogenous inputs in the aquatic environment. While the contamination level in a particular site can be quite readily determined through chemical analysis as defined by the presence of "substances that would not normally occur or at concentrations above the natural background," the assessment of the "pollution status" also integrates the notion of chemicals bioavailability and biological impacts induced by contaminants within the considered environment [102]. Assessing the ecological health of an ecosystem should thus address the following questions, initially enunciated for metallic contaminants by Chapman et al. [103], but which can be generalized to all environmental contaminants: (1) do chemicals/biologicals accumulate in biota above background (or reference) concentrations? (2) once accumulated, are they bioreactive? and if so, (3) what are the incidence and severity of the induced effects (acute, long-term sublethal, individual, population effects, etc.)? Thus, a relevant and successful water quality assessment has to be based on combined analyses integrating the greatest possible number of these various parameters. This also includes the sanitary status of aquatic organisms which are chronically exposed to a cocktail of pollutants in their environment. Despite a large number of publications on invertebrate

biomarkers, little of them reached a level of validation allowing to be recommended as efficient tools for an accurate evaluation of the quality of the aquatic compartment. Nonetheless, new integrative approaches could permit to remove scientific (define the reference levels) and technical (integration of several responses) obstacles encountered in environmental-risk assessment strategies. The use of OMICS tools in field studies has gained great interest for some years as it gives broad information on pollutant modes of actions, considerably increasing knowledge and an understanding of such mechanisms. Finally, clustering data in a weight-of-evidence approach represents a powerful and practical tool to facilitate the decision-making processes of environment managers within the framework strategies.

6.1. Genomics and proteomics in ecotoxicology

Although next-generation sequencing has made great progress over the last decade, genome sequencing of animal models is still far to be a trivial task. This is due to their large genome and the difficulty for assembling too numerous short reads without any scaffold from a closely related species. Furthermore, the delineation of coding open-reading frames and their functional annotation is difficult and subjected to an important bias toward easy annotation of conserved genes but detrimental to species-specific genes. Till now, no complete genome is available for any *Dreissena* and genetic markers are relatively scarce [104–106]. The genomes of marine mussels such as *M. galloprovincialis* are only partially known [107, 108]. Transcriptomics is fully complementary to genomics and adds value in highlighting genes of interest if their expression is regulated. However, this approach has till now been really poorly used for characterizing *Dreissena*. Very few studies based on cDNA microarray have been published, specifying the molecular mechanisms at the early stage of underwater adhesion of the zebra mussel [109], or the effect of seasonal and environmental variations on the physiology and metabolism of *D. polymorpha* [110]. Today, cDNA microarray has been advantageously replaced by RNAseq. High-throughput sequencing of cDNA allows comparing the transcriptome from several conditions and highlighting the most modulated genes in terms of expression levels. In addition, RNAseq data may be used to construct a six-reading frame RNA-translated database that can be used for discovery proteomics. This concept is the basis of numerous proteogenomics analysis of various animal models or even nonmodels [111]. For example, the amphipod *Gammarus fossarum* has been scrutinized in detail to document the reproductive system of amphipods [112] or to understand the response to endocrine disruptors [113].

Till now, *Dreissena* has been worked out in terms of proteomics only with 2D-PAGE traditional approach. For example, [114] analyzed *D. polymorpha* exposed to benzo(α)pyrene and focused on 28 proteins. A set of 16 proteins were found more abundant and 12 were noted as less abundant in exposed mussels. They could be identified after MALDI-TOF/TOF mass spectrometry measurements on their tryptic peptides. Such methodology is known to focus only on the most abundant and soluble proteins [115], thus explaining the relatively low number of protein hits identified by this approach. For sure, high-throughput shotgun proteomics based on next-generation mass spectrometers should allow today documenting thousands of proteins and delineating candidate biomarkers after the analysis of specific exposure conditions. Importantly, these candidate biomarkers require a strict validation consisting in monitoring

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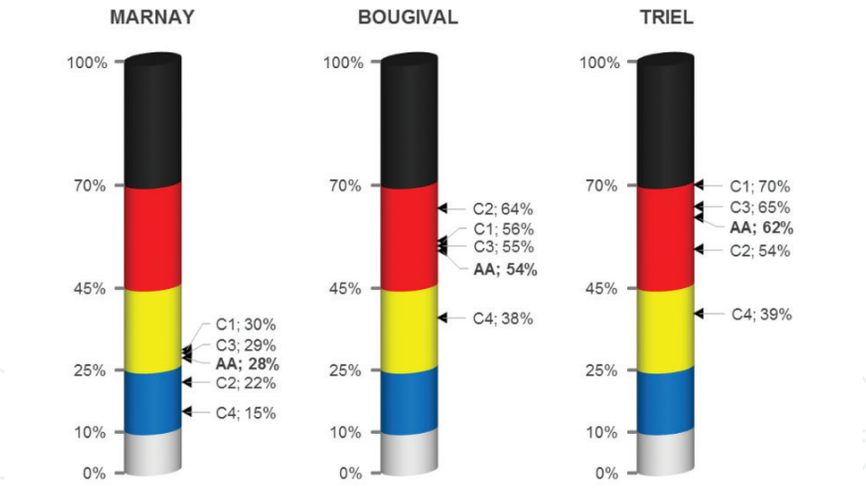
the candidate biomarkers for a large cohort of animals and various conditions [116]. Such monitoring is ideally carried out with targeted proteomics based on the selected reaction monitoring (SRM) quantitative approach. SRM mass spectrometry assay allows high-throughput multiplex analysis, with fewer samples required. It is efficient in terms of time and cost and is able to reliably detect different proteins across a broad dynamic range of concentrations as recently reported for *G. fossarum* [117]. Thus, in our opinion, OMICS tools are today pertinent for obtaining major insights into the most important molecular mechanisms of mussels.

6.2. Weight of evidence

The WOE approach is based on the packaging of a wide variety of data within several lines of evidence (LOEs) in which the contamination level—assessed through chemical analyses—is combined to bioavailability (bioaccumulation) analysis, and biological responses (biomarkers) on key species and/or model organisms (bioassays) at different levels of biological organization, from the molecular to the community level [118, 119]. The resulting environmental diagnosis is then based on the calculation of a hazard index for each LOE, which is next set out on an evaluation grid allowing a clear and rapid classification of hazard [118, 120, 121]. A global hazard evaluation is also proposed through the compilation of all calculated LOE indexes within a single one that is also finally assigned to a hazard class. The WOE approach is applicable to various matrices such as effluent, water, and soil, as well as for more global environmental diagnosis like aquatic and terrestrial ERA. The WOE model developed by Piva et al. [121] was mainly applied to the quality assessment of harbor area using fish species (European eel, *Anguilla anguilla*) and/or mussels (Mediterranean mussels, *M. galloprovincialis*) as bioindicator organisms. These studies undoubtedly demonstrated the relevance and the performance of the procedure to diagnose ecosystem health status in chronically impacted area (e.g., industrial harbors, natural crude oil, and gas seepage) [122, 123] as well as in accidental pollution events as demonstrated by its use in the Mussel Watch program following the Costa Concordia wreck [124]. Mussels were relevantly used in these studies for their suitability in translocation (caging) procedures and their ability to reflect environmental pollution levels through bioaccumulation measurements (see part 2) and biomarkers analysis (see part 3). The elaboration of each hazard index within the WOE approach relies on the initial calculation of ratio-to-reference (RTR) values. It thus supposes that reference levels are available for every end point integrated in the model. In the abovementioned studies, the reference levels were determined examining the biological responses in control organisms maintained in clean water under laboratory conditions or transplanted at a reference site. However, the laboratory (controlled) conditions are far removed from those during *in situ* exposure as control organisms are not submitted to any variations of their environment that naturally occur in field (e.g., temperature variations, general physico-chemistry of the water column, etc.) and which could modulate the biological responses of the organisms with no link with the contamination status of the environment. Transplantation of organisms at a reference site is also commonly used to avoid such bias. However, a "perfect" reference station would assume that (i) the site is geographically close enough to reflect the natural state of the studied environment; (ii) the exposure conditions are exactly similar to those at the other sites (in terms of temperature, physicochemistry, etc.); and (iii) that there is absolutely no anthropogenic contamination which could induce any modulations in the biological responses, even in a limited

way. There is no doubt that the determination of such station in each studied area is not realistic—if not utopic—and that the use of control organisms (at a reference station or maintained in laboratory conditions) to set the reference levels integrated in the WOE approach generates a bias in the ecological health status assessment.

An alternative was proposed by Barjhoux et al. [125] to address these concerns. Briefly, the study proposes an application of the WOE strategy to a freshwater system: the Seine River (France), well known to be submitted to heavy anthropogenic pressures through important industrial, agricultural, and urban activities. The three studied sites were located upstream (Marnay, in a non-urbanized area) to downstream from Paris conurbation (Bougival and Triel, respectively, situated at 40 and 80 km from Paris). The dataset selected for WOE integration included (i) chemical contamination levels, (ii) bioavailability (bioaccumulation) measurements, (iii) biological effects in field-transplanted organisms (biomarkers in gammarids), and (iv) (eco)toxicological responses assessed using laboratory bioassays. The strength of the quality assessment proposed in this study lies on the use of the same population of gammarids for bioaccumulation and biomarkers measurements. Reference and threshold values were established using modeling developments quantifying the natural variability of the studied markers in relation to identified confounding factors. These reference/threshold levels were integrated in the WOE approach as they clearly enhance the reliability of *in situ* methodology and allow its implementation at a large spatial and temporal scales [126, 127]. The calculated WOE indexes clearly reflected the anthropogenic gradient along the Seine River,



Summary of the hazard classes attributed to the HQs values within each LOE

HQs	MAR_C1	MAR_C2	MAR_C3	MAR_C4	MAR_AA	BOU_C1	BOU_C2	BOU_C3	BOU_C4	BOU_AA	TRI_C1	TRI_C2	TRI_C3	TRI_C4	TRI_AA
$ChemHQ_{water}$	Moderate	Moderate	Moderate	Moderate	Moderate	Severe	Severe	Severe	Major	Severe	Severe	Severe	Severe	Major	Severe
$ChemHQ_{sed}$	Severe	N/A	Severe	Moderate	Severe	Severe	N/A	Severe	Severe	Severe	Severe	N/A	Severe	Severe	Severe
BioavHQ	Slight	Slight	Slight	Slight	Slight	Major	Major	Major	Slight	Major	Major	Moderate	Moderate	Slight	Moderate
BiomHQ	Moderate	N/A	Moderate	Slight	Moderate	Severe	N/A	Major	Major	Major	Severe	N/A	Severe	Major	Severe
$ToxHQ_{water}$	Absent	N/A	Absent	Absent	Absent	Moderate	N/A	Absent	Absent	Slight	Major	N/A	Slight	Absent	Slight
$ToxHQ_{sed}$	Slight	N/A	Slight	Slight	Slight	Slight	N/A	Moderate	Slight	Moderate	Severe	N/A	Severe	Slight	Major

Figure 2. WOE indices and associated hazard classes integrating the results of each LOE calculated for the three stations during four sampling campaigns (C1–C4) and annual average (AA) values. The hazard class attributed to each LOE hazard quotient (HQ) is summarized in the table below. $ChemHQ_{water/sed}$, water/sediment contamination HQ; BioavHQ, bioavailability (bioaccumulation) HQ; BiomHQ, biomarker HQ; $ToxHQ_{water/sed}$, bioassay-based HQ on water/sediment samples. Note that in the C2 campaign, only data on water contamination and bioavailability are evaluated. The integral version of the article is available at: http://link.springer.com/article/10.1007/s11356-016-6993-6. The figure was reproduced with permission of SpringerNature publisher.

with values increasing from upstream to downstream of Paris (**Figure 2**: [125]). The results also highlighted some seasonal variations in the hazard class attributed to each site with the winter campaign showing lower level of perturbation than the three other campaigns.

Accordingly, the use of external reference values and thresholds eliminated the need for a reference site in the study area, which could be very problematic in large rivers subjected to multiple and diffuse pressures. The results of this study also reveal that at the upstream site, generally used as a relative reference or control site in previous investigations in this area, the low contamination levels nonetheless resulted in low but significant biological effects. The WOE approach applied in this study proved to be efficient and relevant in terms of both global environmental hazard diagnosis and seasonality analysis. The in-depth characterization of the baseline levels and relevant effect thresholds for environmentally relevant end points is thus a challenge and might be vigorously pursued and developed further over the coming years to lead to homogenized ERA procedures between the various environmental institutions. In particular, several research programs are in progress to define basal reference levels, effect thresholds, and confounding factors of the biological responses in *D. polymorpha* (from molecular to population) in order to routinely include this promising species in WOE approaches dedicated to freshwater environment quality diagnosis.

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