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Review

Critical knowledge gaps and relevant variables requiring consideration when performing aquatic ecotoxicity assays

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ABSTRACT

The increasing diversity and complexity of contaminants released in the environment continuously lead to new challenges when applying ecotoxicity assays. This paper comprises a review concerning exposure assessment and highlights important variables that should be taken into account when investigating aquatic media toxicity under both laboratory or field conditions. Thus, to reflect as much as possible what occurs in nature, ecotoxicity assays must carefully consider these variables in their experimental design. This includes contaminant properties, the selected bioindicators and biomarkers, the dose mode/regime, concentration *vs.* load, exposure to single *vs.* multiple contaminants and exposure of single *vs.* multiple species. Many of these, however, are not usually taken into account, leading to critical knowledge gaps in this area, discussed in detail herein.

1. Introduction

Aquatic ecotoxicology has become much more than the simple application of laboratory assays in evaluating the effects of a certain specific compound on only one selected bioindicator, an organism or group of organisms that reflects environmental quality information (Markert et al., 2003) and on its biochemical, physiological, or histological indicators, or biomarkers (Forbes et al., 2006). Although this one-to-one approach is still valid, particularly during investigations prior to releasing new xenobiotics into the industrial chain, toxicity assays have also become useful in assessing system investigations, including mixture evaluations concerning multiple species in complex aquatic environments.

The increasing complexity of environmental issues associated to the release of emerging contaminants has led to continuous new challenges and should be taken into account in the design of new ecotoxicity assays. In addition, an increasing demand for better analytical tools is also noted, in order to adequately detect and quantify an increasing number of compounds discharged routinely at very low concentrations into recipient water bodies (Caban et al., 2016; Kaczala and Blum, 2016).

Recent and novel ecotoxicology investigations focus on a high number of biomarkers and organisms at different levels of the trophic chain, populations, communities and ecosystem (Angel et al., 2010; Ashauer et al., 2006; Costa et al., 2010; Hallgren et al., 2014, 2012; Lazarus et al., 2015; Mattsson et al., 2015; Ranjan and Yasmin, 2015). Currently, new ecotoxicity assays are designed taking account the increasing number of variables known to affect organism responses. These include discharge regime or dosage mode (continuous, intermittent, episodic, spraying and accidental spills), aiming to simulate different events such as sewage and industrial effluents discharges (point sources); stormwater runoffs from urban and agricultural areas (diffuse source) and atmospheric fallouts (Angel et al., 2010; Bejarano and Farr, 2013; Kaczala et al., 2012, 2011; Reinert et al., 2002). In addition ecological risk assessments have incorporated ecotoxicity tests as a further line of evidence (Mendes et al., 2017; Pan et al., 2016), alongside chemical and ecological data.

Both laboratory and field-scale assays present advantages and disadvantages that should be evaluated during the experimental design stage. Laboratory assays, for instance, can be cost-effective and allow for better variable control compared to field-scale studies, and are, thus, suitable to investigate different exposure scenarios, such as pulsed versus continuous contaminant exposure and recovery and acclimatization periods, among others (Angel et al., 2010; Ashauer et al., 2006; McCahon and Pascoe, 1990). However, lab-scale toxicity tests are, in principle, simplistic and conservative and, depending on the experimental setup, results may not be environmentally relevant and the

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conclusions may not apply to real-life scenarios (Diamond et al., 2006; Panter et al., 2000).

Field assays, in turn, are more realistic and allow for the identification of contaminant effects on non-target organisms. In addition, they also allow for assessments regarding interactions between different variables related to the physical, chemical and biological complexity of real aquatic environments (Lazarus et al., 2015). However, these types of studies are often difficult to undertake, due to weak experimental condition control and frequent logistic problems and high costs (Angel et al., 2010; Ashauer et al., 2006; McCahon and Pascoe, 1990).

Thus, it is clear that, in order to realistically support ecotoxicology assay interpretations concerning environmental conditions, differentiated perspectives are required. Importantly, the response given by a selected species to a range of concentrations of a certain contaminant is only the first approach to the problem, not the final one.

In this regard, a number of physical, chemical and biological variables which are not always taken into account considerably affect organism responses (Diamond et al., 2006). Therefore, the following variables/groups of variables are addressed in the present review which may aid in assessing and further understanding the major gaps in the field of aquatic ecotoxicology: (1) Contaminant characteristics and their entry routes and pathway; (2) Investigated species (bioindicators) and biomarkers; (3) Dose mode; (4) Contaminant concentration vs. load related to the size of the experimental unit; (5) Exposure to single vs. multiple contaminants and (6) Exposure of single vs. multiple species to the same contaminated water. Variables (1), (2) and (3) have been extensively investigated in aquatic ecotoxicology and were included herein to maintain (4), (5) and (6) in perspective and highlight the ways they are related to each other. Variables (4), (5) and (6), on the other hand, were selected due to surprisingly few literature data in this regard and even fewer studies discussing their overall toxicity response contributions.

2. Important variables to address in aquatic ecotoxicity assays

2.1. Contaminant characteristics, entry routes and pathways

2.1.1. Physico-chemical characteristics

The search for understanding on the nature of the contaminant and its physical and chemical properties are usually the driving force behind ecotoxicological assays. Several contaminant characteristics affect aquatic organism responses, such as the following (Ashauer et al., 2006; Bejarano and Farr, 2013; Boxall et al., 2013; Breitholtz et al., 2006; Hallgren et al., 2012; Landrum et al., 2012; Lazarus et al., 2015; Mattsson et al., 2015):

- Bioavailability: the amount of a certain substance which enters the body is able to present an active effect;
- Biodegradability: the capacity for biological degradation of organic materials by living organisms down to base substances (water, carbon dioxide, methane, basic elements and biomass);
- Recalcitrance: resistance to degradability (bio or otherwise);
- Volatilization: the conversion process of a chemical substance from a liquid or solid state to a gaseous or vapor state;
- Polarity: the separation of electric charge leading to a molecule or its chemical groups displaying an electric dipole moment, with a negatively charged and a positively charged portion;
- Water solubility: a measure of the amount of chemical substance that can dissolve in water at a specific temperature;
- Hydrophilic-lipophilic partition: a measure of the degree to which a compound is hydrophilic or lipophilic, determined by calculating values for the different regions of the molecule;
- Octanol-water partition coefficient (log K_{ow}): defined as the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase of a two-phase octanol/water system;
- Hydrolysis: any chemical reaction in which a molecule of water ruptures one or more chemical bonds;
- photolysis: a chemical reaction in which a chemical compound is broken down by photons;
- Chemical oxidation: transfer of electrons from an oxidizing reagent to the chemical species being oxidized, in the present case, aiming to convert contaminants to non-toxic or stabilized molecules;
- Half-life in water and in sediments: the amount of time it takes for a given amount of a certain compound to decrease to half of its initial value;
- Sorption kinetics: a measure of the adsorption or dessortion with respect to time in a certain environmental compartment (e.g. sediment);
- Bioaccumulation in the food web: the gradual accumulation of substances in organisms belonging to different trophic niches.

2.1.2. Exposure variables

Exposure variables include water quality parameters (pH, temperature, salinity), contaminant sources and their bioavailability; biochemical pathways, uptake rates; tissue accumulation (mechanisms of action) and metabolites and by-products, among others (Ahmed et al., 2015; Angel et al., 2010; Ashauer et al., 2006; Benzer, 2017; Landrum et al., 2012; McCahon and Pascoe, 1990; Reinert et al., 2002; Thorpe et al., 2003). Contaminant accumulation and, in some cases, biomagnification, throughout the food chain is also related to contaminant physico-chemical characteristics, including half-life, biodegradability and solubility (Breitholtz et al., 2006; Hallgren et al., 2012; Handy, 1994; Mattsson et al., 2015; Xu et al., 2008), as well as certain biological characteristics, such as lipid content (Ashauer et al., 2006; Olsson et al., 2000; Slootweg et al., 2015).

2.1.3. Contaminant effects on the target organism

Contaminant effects can be classified as reversible, irreversible, additive or cumulative, depending on the contaminant's classification and mechanisms of action (Ashauer et al., 2006; Boxall et al., 2013; Handy, 1994; Reinert et al., 2002; Rossier et al., 2016). These mechanisms differ among species, and are related to contaminant exposure degree, duration, frequency, recovery period, uptake and depuration rate, accumulation (critical body threshold) and life stage (Angel et al., 2010; Ashauer et al., 2010; Diamond et al., 2006; Landrum et al., 2012; Naddy and Klaine, 2001; Rainbow, 2007; Segner et al., 2003).

2.1.4. Contaminant entry routes and pathways

The manner in which contaminants enters the assessed bioindicator also influences organism response (Ashauer et al., 2010; Canli and Atli, 2003; Cedergreen et al., 2005). The most common entry routes are through (i) spiked or naturally contaminated water (Allert et al., 2013; Ashauer et al., 2010; Hassanin et al., 2002; Knudsen et al., 2011; Landrum et al., 2012; Ondarza et al., 2012; Salomão and Marques, 2015, 2014); (ii) food or diet, through spiked or naturally contaminated food (Allert et al., 2013; Breitholtz et al., 2006; Landrum et al., 2012; Lazartigues et al., 2013; Mandiki et al., 2005; Mattsson et al., 2015) and; (iii) intraperitoneal or intramuscular exposure through injections (Costa et al., 2010; Hallgren et al., 2009; Kumar, 2012; Ng et al., 2001; Purdom et al., 1994; Solé et al., 2000; Verslycke et al., 2002). The most natural exposure pathways are the water and food contamination, which are frequently adopted in aquatic ecotoxicity assays. However, the sensibility or response of the organism to one or more contaminants can vary depending on the exposure route (Breitholtz et al., 2006). In addition, water exposure usually results in greater effects compared to the dietary route (Allert et al., 2013; Madsen et al., 2013; Pickford et al., 2003). Intraperitoneal injections, on the other hand, are not the most relevant mode of exposure for environmental toxicology, although interesting if assessing new biomarkers or specific mechanisms of action (Costa et al., 2010). The appropriate exposure route of a certain compound, thus, depends on the intended study goals, and should be evaluated and

selected during the experimental design. According to Groh et al. (2015) contaminant exposure routes significantly influence subsequent toxicokinetic processes, which may, in turn, differ considerably depending on the contaminant point of entry in the organism.

2.2. Bioindicators

Biological characteristics are also key in determining organism responses (Ashauer et al., 2006; Diamond et al., 2006; Landrum et al., 2012; Segner et al., 2003). These include age or development stage (e.g.: eggs, embryos, juveniles, adults, all life stages); reproduction regime (sexual, asexual) and sex status or rate; seasonal events (e.g.: breeding periods, molting cycle); feeding habits; natural habitat conditions, life strategy and mobility (e.g.: benthic or pelagic, sessile or free-living species) (Ahmed et al., 2015; Canesi and Fabbri, 2015; Costa et al., 2010; Hallgren et al., 2012; Reinert et al., 2002; Segner et al., 2003; Trudel and Rasmussen, 2006). All these characteristics are directly associated to organism physiology, including xenobiotic uptake processes and metabolism, which comprise accumulation, depuration, recovery and elimination (toxicokinetics/toxicodynamics), and post-exposure effects (latency or delaying effects) (Ashauer et al., 2007, 2006; Diamond et al., 2006; Landrum et al., 2012; Rainbow, 2007; Reinert et al., 2002). Historical conditions are also important to determine or explain biological responses (Breitholtz et al., 2006; Hallgren et al., 2012; McCahon and Pascoe, 1990; Segner et al., 2003), as previous contaminant exposure or non-deal health conditions, such as infections, diseases or parasite attacks can lead to higher (resistance) or lower (sensitivity) tolerance to the targeted contaminant (McCahon and Pascoe, 1990). Some of these aspects are discussed below.

2.2.1. Bioindicator size/age

Bioindicators, living organisms applied as proxies in screening environmental health through molecular, cellular, physiological and behavioral alteration responses, which, in turn, are termed biomarkers (Van der Oost et al., 2003). Bioindicator size and molting are often related to life stage (hatching, larval development, juvenile and adult), where, in some cases early life stages are more sensitive than more mature stages (Canli and Atli, 2003). A negative correlation between contaminant concentrations in the aquatic ecosystem and the size of the target species is often assumed, as larger specimens comprise higher biomass for contaminant distributions, leading to the growth dilution effect (Madenjian et al., 2010; Rainbow, 2007; Trudel and Rasmussen, 2006; Ward et al., 2010). Therefore, it is likely that a lower contaminant mass or fewer doses will be sufficient to cause effects in early stages compared to later stages of development. Therefore, isolating one variable (body biomass, growth dilution) from another (a more efficient metabolism concerning contaminant elimination), particularly in field studies, is not simple. In addition, females are usually larger than males due to reproduction events, which may also influence xenobiotic contamination or accumulation rates (Gewurtz et al., 2011; Olsson et al., 2000). However, females may also accumulate higher contaminant amounts, due to greater food consumption and fat content, in the form of metabolic reserves necessary for spawning (Reinert et al., 2002; Rypel et al., 2007). On the other hand, contaminant losses during spawning have also been reported (Olsson et al., 2000; Rypel et al., 2007), due to maternal contaminant transfer to eggs. Thus, the choice of size, age or gender for the tests should take into account what was previously reported but should also consider the objectives of each survey and what answers are being sought. However, pairing the characteristics of individuals in the control and exposed group is always desirable. The adoption of standard characteristics of size, age and sex ratio (or gender) in the study will allow a more accurate comparison among further studies.

2.2.2. Diet and feeding habits

Different diet habits, as well as different feeding habits (food specialists or generalists) within different trophic levels (Olsson et al., 2000), can be a major source of contamination (Costa et al., 2010; Hallgren et al., 2012; Lazarus et al., 2015). Some species belonging to higher levels of the food chain, for example, can accumulate contaminants due to biomagnification processes (Allert et al., 2013; Lazarus et al., 2015), potentially leading to toxic effects. Thus, feeding aspects should always be taken into account during the experimental design and should include decisions on water flow rate (filter-feeding and organisms with gills); exposure route (spiked water, contaminated sediments or food); type of sediment (benthic or pelagic and sessile or free-living) and dose mode (Breitholtz et al., 2006; Canli and Atli, 2003; Costa et al., 2010; Dang and Wang, 2012; Hallgren et al., 2012; Knudsen et al., 2011; Olsson et al., 2000; Rainbow, 2007). Therefore, according to Breitholtz et al. (2006), although methodologies for testing chemicals with significant partitioning onto food (e.g. fasting or excess food and detritus removal) have been reported, most ecotoxicological assays may not give reliable results.

Nutritional status during ecotoxicological assays is quite controversial and may, in some cases, modify organism responses to contaminants (Ashauer et al., 2006; Davis et al., 2009; Lanno et al., 1989; Wicks and Randall, 2002). Thus, depending on the experimental setup, the organism can either be fed or fasted (Lanno et al., 1989; Naderi et al., 2012). However, fasting or poor diets adopted in some assays to avoid excessive organic matter or other medium interferences (Madsen et al., 2013; Naderi et al., 2012; Ng et al., 2001) may lead to stress, weakness and increased disease risks (McCahon and Pascoe, 1990). In order to reduce the feed effect in ecotoxicology assays concerning vertebrate test organisms (e.g. fish), food should be administered in amounts enough to guarantee that all food is consumed immediately and without leftovers. In the case of invertebrates (e.g. microcrustaceans, such as *Daphnia magna*), according to Handy et al. (2012), feeding with unicellular algae or other particulate food materials should be performed a few minutes before a water change, and renewal of the test media should be carried out immediately after the animals have fed.

2.2.3. Population density

Associations between exposure duration, concentration and organism density in the experimental unit is determinant, as these factors directly influence organism responses (Allert et al., 2013; Hallgren et al., 2012; Salomão and Marques, 2014; Sijm et al., 1998; Vardy et al., 2011). For example, one individual exposed to a certain contaminant concentration in a certain water volume in one experimental unit will uptake different contaminant concentrations compared to several individuals in another experimental unit, resulting in different organism responses (Reinert et al., 2002; Salomão and Marques, 2014).

2.3. Dose mode

The dose mode, defined as the way a certain contaminant enters an aquatic system or experimental unit (i.e. continuous or intermittent/pulsed discharges), is an important variable to take into account in experimental assays (Ashauer and Brown, 2013; Panter et al., 2000; Tennekes and Sánchez-Bayo, 2013; Zafar et al., 2011). Most dose modes applied in ecotoxicity assays are continuous (Amachree et al., 2013; Angel et al., 2010; Diamond et al., 2006; Thorpe et al., 2003; Vardy et al., 2011), intermittent or pulsed (Amachree et al., 2013; Angel et al., 2010; Ashauer et al., 2007; Diamond et al., 2006; Hallgren et al., 2012; McCahon and Pascoe, 1990; Reinert et al., 2002; Souza et al., 2013) and episodic, with a high initial peak or load simulating an acute accident (Bejarano and Farr, 2013; Ondarza et al., 2012).

The most appropriate dose mode for a certain toxicity assay simulating actual contamination conditions must be decided on a case-by-case basis after considering other variables (Landrum et al., 2012; Reinert et al., 2002), such as simulated pollution source, discharge frequency, water flow and renovation in the recipient water body (lentic or lotic system) and hydrological dilution (Bejarano and Farr, 2013; Boxall

et al., 2013; Handy, 1994; Reinert et al., 2002).

In an intermittent exposure scenario, pulse magnitude; number of pulses; time interval between multiple pulsed exposures (related to the recovery period); exposure duration; peak concentration and mean concentration may be relevant and should be taken into account (Angel et al., 2010; Boxall et al., 2013; Diamond et al., 2006; Landrum et al., 2012; Reinert et al., 2002).

Standard lab-scale toxicity assays are usually carried out under fixed exposure times and continuous flow (Angel et al., 2010; Ashauer et al., 2007; Bejarano and Farr, 2013; Boxall et al., 2013; Landrum et al., 2012; Panter et al., 2000; Seim et al., 1984), which does not usually reflect actual real-life scenarios. Standard continuous flows setups usually miss time-varying toxicity effects due to pulsed or repeated exposures (Ashauer et al., 2006; Reinert et al., 2002). For some authors (Angel et al., 2010; Ashauer et al., 2010; Diamond et al., 2006; Handy, 1994; Panter et al., 2000; Reinert et al., 2002), the investigation of intermittent or repeated exposures followed by seldomly applied post-exposure analyses of latent or delay toxicity effects provides a better basis for toxicity evaluations than continuous flow exposure experiments.

Differences in exposure implications (intermittent or continuous) are not usually discussed, and disagreements are noted in the literature concerning their effects (Amachree et al., 2013; Boxall et al., 2013; Handy, 1994; Panter et al., 2000). For example, according to Panter et al. (2000) and Knudsen et al. (2011), response magnification and quicker response induction during intermittent regimes after repeated pulses, suggest a "memory-effect" resulting from previous exposure. In addition, continuous exposure conditions are not usually representative of natural conditions (Panter et al., 2000), as aquatic animals are not usually exposed to continuous contaminant concentrations (Angel et al., 2010; Ashauer et al., 2010; Boxall et al., 2013; Hallgren et al., 2012; Reinert et al., 2002). However, several authors (i.e. Amachree et al. (2013), Ahmed et al. (2015), Benzer (2017), and Handy (1994), report that continuous exposure assays are useful to predict bioaccumulation processes of certain pollutants, like metals, while intermittent doses are useful in simulating vertical and horizontal daily movements observed in fish and other organisms, leading to exposure in different contaminant concentrations gradient zones, which may, in turn, result in intermittent exposure scenarios (Panter et al., 2000).

Based on different assessments concerning responses by different species, both pulsed or intermittent doses and recovery period duration between successive pulses are considered important variables that should be accounted for when assessing toxic endpoints (Angel et al., 2010; Ashauer et al., 2007, 2006; Boxall et al., 2013; Diamond et al., 2006; Panter et al., 2000; Reinert et al., 2002). However, only some contaminants and aquatic bioindicators have been assessed in this regard (Angel et al., 2010) (Fig. 1).

Exposure duration (long or short term exposure) (Fig. 1a and g), frequency (time-varying of repeated exposures) (Fig. 1e and f) and magnitude (peak concentration or total mass applied) (Fig. 1i and j) can be used to simulated diverse real environmental scenarios, like point-source industrial discharges or high-peak accidents (Fig. 1g and i) (Ashauer et al., 2006; Bejarano and Farr, 2013; Boxall et al., 2013; Diamond et al., 2006; Landrum et al., 2012; Reinert et al., 2002).

Intermittent discharge effects may vary compared to continuous discharges at the same contaminant concentration, depending on the physicochemical properties of the investigated contaminant (Boxall et al., 2013; Handy, 1994) (Fig. 1i and j), due to (i) gradual selection of more resistant/tolerant bioindicators after each pulse (Angel et al., 2010; Boxall et al., 2013; McCahon and Pascoe, 1990); (ii) organism acclimatization/adaptation due to the inter-pulse intervals (recovery periods) (Ashauer et al., 2010, 2007; Boxall et al., 2013; Diamond et al., 2006; Reinert et al., 2002); (iii) time required for sufficient uptake of metabolically available contaminants (Angel et al., 2010; Boxall et al., 2013; Landrum et al., 2012); (iv) slow contaminant processing or not enough to prevent cumulative doses (dependent on type of the contaminant and time between pulses) (Boxall et al., 2013; Diamond et al., 2006; Landrum et al., 2012).

Potential latent or delayed effects may occur even after completion of exposure assessments (Handy, 1994; Reinert et al., 2002), although this is seldom discussed in standard laboratory assays. In fact, delayed effects may result in lower threshold concentrations or more conspicuous responses (Handy, 1994; Knudsen et al., 2011), which should be accounted for in toxicity evaluations and risk assessment interpretations (Pan et al., 2016; Reinert et al., 2002). It has been reported that shorter toxicity tests may underestimate toxicity responses or effects, as well as real mortality rates (Angel et al., 2010; Handy, 1994; Knudsen et al., 2011; Reinert et al., 2002). For instance, some data are based on delayed responses, such as protein synthesis that lead to plasma vitellogenin alterations in male fish (Knudsen et al., 2011; Panter et al., 2000) or organ effects, such as liver modifications (Costa et al., 2010; Handy, 1994).

In the context of post-exposure toxicity or when repeated sub-lethal

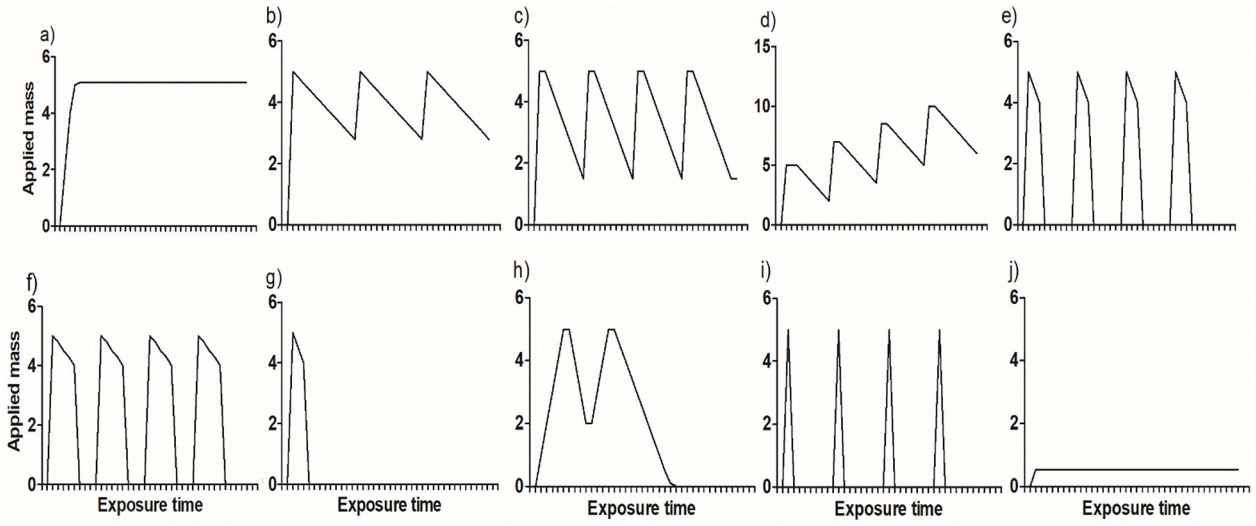

Fig. 1. Differential dose modes which may be applied in ecotoxicity assays (exposure time and applied mass). (a) continuous; (b) regular intermittent; (c) intermittent (rivers); (d) intermittent (lakes); (e) intermittent doses with longer recovery periods; (f) intermittent doses with shorter recovery periods; (g) single dose or acute pulse with short duration (accidents); (h) double dose; (i) high peak dose; (j) mean concentration of high peak dose, same mass in a continuous dose. (Sources: McCahon and Pascoe 1990; Ashauer et al., 2007; Angel et al., 2010).

episodes occur, chronic effects may also be important (Handy, 1994). According to Panter et al. (2000), this theory can be further supported by the mechanism for the synthesis of vitellogenin. The normal circulating concentrations of E2 in male fish are too low to trigger the synthesis of vitellogenin. Nevertheless, after exposure to E2, vitellogenin synthesis is initiated with the mRNA synthesis, translation and post-translational modifications, in a relatively slow process, in a kind of "lag phase" before vitellogenin appears in the male fish circulation in concentrations that allow its detection and/or quantification in the applied methodology. This is also supported by the investigation by Knudsen et al. (2011) which determines a period for "lag phase" to induced vitellogenin levels in the fish plasma of the exposed group (370 ng E2 L^{-1}) was one day post exposure, and four days after the second exposure to 6 h pulses of 206 ng E2 L^{-1} with a 48 h interval.

Contaminant uptake, depuration and excretion (either passive or active by diffusion and biotransformation/excretion, respectively) or accumulation processes (Handy, 1994; Landrum et al., 2012; Olsson et al., 2000; Rainbow, 2007) comprise other variables associated to dose mode and bioindicator interactions, which also affect ecotoxicity assay designs and require consideration. In this regard, toxicokinetics (uptake and elimination rates) and toxicodynamics (dynamics of injury and recovery by the organism) (Ashauer et al., 2010, 2007; 2006; Calafat et al., 2015; Landrum et al., 2012; Reinert et al., 2002) are utilized to assess these processes, and depend on metabolic activity and specific bioindicator characteristics (Calafat et al., 2015; Canli and Atli, 2003; Knudsen et al., 2011).

Previous knowledge regarding contaminant effect recovery time is also essential, determining not only the contaminant load that will in fact produce effects, but also the pulse frequency that will lead to organism responses (Cedergreen et al., 2005). This is clear in studies that apply the same contaminant mass and exposure time, while altering dose regime and recovery period (or not) (Fig. 1i and j) (Angel et al., 2010; Ashauer et al., 2010, 2006; Diamond et al., 2006; Handy, 1994; Knudsen et al., 2011; Naddy and Klaine, 2001; Reinert et al., 2002; Seim et al., 1984). For example, according to Reinert et al. (2002) in some cases, double-pulse exposures with short pulse intervals (Fig. 1h) were shown to be as or even more toxic than continuous exposure under the same mass and duration conditions.

Certain symptoms determine pre-exposure conditions and emphasize the need to include a recovery phase in assessments simulating accidents (i.e. absence of abnormal behavior; post-dosing mortality cessation; resumed feeding activity; pre-copula re-establishment; and population growth rate re-establishment (Hallgren et al., 2012; McCahon and Pascoe, 1990)).

2.4. Concentration vs. load associated to the size of the experimental unit

The most widely applied variable utilized to aquatic environment contamination is contaminant concentration. Therefore, many countries still determine discharge threshold limits based on this parameter (Diamond et al., 2006; Landrum et al., 2012). Initial pollutant concentrations provide an estimation of maximum exposure levels in one-dose mode simulations, according to Landrum et al. (2012). It is important to note, however, this is not always the most appropriate way to compare laboratory and field exposures under different dynamic conditions.

In real-life scenarios, differences in receiving water bodies may be observed (i.e. lotic or lentic, with different flow rates, widths and depths, among others) (Bejarano and Farr, 2013). In addition, differential sunlight penetration rates and temperatures may also occur, directly affecting pollutant degradation.

Total water volume in experimental assessments, as well as concentration, are required, in order to inform contaminant mass. Two experimental units presenting different volumes and surface light exposure can contain the same initial contaminant concentration, but test subjects will be exposed to different masses per body mass unit that may, in turn, lead to differential responses (Salomão and Marques, 2014).

Concerning bioindicators displaying certain characteristics, such as high water mass (the presence of several individuals in a certain water mass (area), for example, fish species that aggregate in shoals) and high column mobility (species that migrate vertically throughout the water column), filter habits and the presence of gills, among others, pollutant loads in aquaria may be the most appropriate way to assess toxicity, based on uptake and depuration rates and the maximum contaminant accumulation (critical body threshold) (Angel et al., 2010; Ashauer et al., 2007; Boxall et al., 2013; Diamond et al., 2006; Reinert et al., 2002; Salomão and Marques, 2014; Seim et al., 1984).

Total mass calculations require more complex assessments compared to concentration calculations, especially for short lived contaminants: (i) the size of the experimental units (e.g. aquarium volume); (ii) dose modes: single dose, continuous or intermittent dose; (iii) how many organisms are simultaneously exposed in the same experimental unit. Due to different volumes may have similar concentration, but the mass of the contaminant in the water can vary substantially (Salomão and Marques, 2014). In addition, daily contaminant loads or total discharged mass may be useful in toxicity studies in cases of reduced flow and/or limited water mass exchanges in field cases. Surprisingly, experimental unit volume, required to estimate contaminant mass, is frequently missing in this type of evaluation, or not clear or only presented as a range (Bjerregaard et al., 2008; Knudsen et al., 2011). In this regard, Salomão and Marques (2014), when performing comparisons of different-sized experimental units considering water renewal and number of organisms per unit (total contaminant mass/number of organism or organism weight), report that total mass better explains several indicators, leading to more consistent results (Knudsen et al., 2011; Salomão and Marques, 2014). These include assessments on sub lethality (chronic assays) with the endpoint of no observed effect concentration (NOEC), the lowest observed effect concentrations (LOEC). However, concentration *versus* applied mass and specimen life stage are seldom addressed in toxicity studies (Christen et al., 2010; Knudsen et al., 2011; Salomão and Marques, 2014).

The weight of exposed animals should be also informed, as several differences in laboratory results may be observed for different fish weights and biomass, leading to differential conclusions concerning effects and contaminant toxicity thresholds. For example, Knudsen et al. (2011) evaluated the effect of fish presence, number of fish and total fish biomass in an exposure tank and decreases in 17β-estradiol (E2) water concentrations by fish incorporation. The findings indicate a correlation between total fish biomass increases in the aquarium and E2 removal ($r2 = 0.74$, $p < 0.001$). Furthermore, fish (24–74 g) E2 removal rate from the water increases linearly with increasing E2 water concentrations (E2 uptake = 0.0202 ng g/h × [E2]water (ng L^{-1}) + 0.376, $r^2 = 0.96$, $p < 0.001$; and 20.2 h^{-1} concentration ratio rate between water and organism), whereas a considerably higher concentration rate of 123 h^{-1} was verified for smaller fish (1.8 g). In another assessment, different LOEC for male fish in terms of vitellogenin production due to exposure to 17α-ethinylestradiol (EE2) have been reported for different species displaying different weights and biomasses in laboratory exposure conditions. However, erroneous data interpretations may occur when not taking fish weight and biomass into account, as one may believe it is possible to rank the assessed species from the lowest LOEC to the highest LOEC independently of both factors. When these results are converted to applied load per gram of body mass using the volume of the experimental units, dosing mode, experimental duration and number and total weight of exposed fishes, different sensitivities are noted. Table 1 exhibits four examples of different fish species and their respective converted and non-converted applied load per gram of body mass LOECs.

Thus, many authors simply compare sensitivities and simply generalize and comparing different life stages, sizes and exposure volumes, which, in turn, influence reports on actual organism sensitivity and their applications in risk assessments.

Table 1
Some fish species and their respective converted and non-converted LOECs of applied load per gram of body mass, considering aquaria volume.

Species	Non-converted LOEC (ng/L)	Converted LOEC (ng/g)	Aquaria volume (L)	Reference
Platichthys flesus	8.1	2.18	960	Madsen et al. (2013)
Oreochromis niloticus	16.0	35.5	130	Salomão and Marques (2014)
Oncorhynchus mykiss	100.0	375	200	Verslycke et al. (2002)
Oreochromis niloticus	120.0	21.1	2	Salomão and Marques (2014)

2.5. Single vs. multiple contaminant exposure

Exposure to contaminant combinations (multiple exposure) is seldom addressed in toxicity assays (Almeida et al., 2018; Dietrich et al., 2010; Garreta-Lara et al., 2018; Heberer, 2002; Landrum et al., 2012; Lazartigues et al., 2013; Monosson, 2005; Rossier et al., 2016; Thorpe et al., 2003; Toumi et al., 2018; Wang et al., 2018). Multi-exposure of aquatic organisms to combined contaminants (cocktail) may lead to additive, synergistic or antagonistic effects and are more environmentally relevant (Juhel et al., 2017; Landrum et al., 2012; McCahon and Pascoe, 1990; Monosson, 2005; Rossier et al., 2016; Thorpe et al., 2003; Toumi et al., 2018; Wang et al., 2018), sometimes at concentrations below their individual LOEC (Thorpe et al., 2003). A recent example is a study carried out by Gomes et al. (2018), who assessed the effects of two psychotropic drugs commonly found in the environment, carbamazepine (CBZ) and clonazepam (CZP), both isolated and co-administrated, on oxidative stress biomarkers and essential metal homeostasis in *Danio rerio* specimens. Isolated exposures led to varied decreasing and increasing responses, while positive synergistic effects were observed for co-exposure regarding metallothionein levels in liver, as well as in brain, decreasing basal metal and metalloids in this organ. This clearly demonstrates the importance of assessing multi-exposures in order to develop adequate risk analyses.

2.5.1. Environmentally relevant concentrations

According to the US EPA (U.S. Environmental Protection Agency, 2011), environmentally relevant concentrations are those expected to occur in the environment. However, the definition of what an environmentally relevant concentration is for a certain contaminant seldom takes into account important variables, such as contaminant half-life and persistence in a specific aquatic system; dose mode (discharge regime) and source. For contaminants displaying short half-lives, mass content variations may occur, depending on the discharge or dose mode. Therefore, continuous or frequent verification of contaminant concentrations in the water medium should carried out, especially for intermittent and discontinuous discharges.

Additionally, analytical methods limitations must be observed when evaluating contaminant concentrations in the assessed bioindicators, their pathways and potential degradation products (as several degradation products from different classes may be as toxic or even more than the original compounds (Li et al., 2016; Mboula et al., 2015; Qin et al., 2014), since suitable analytical methods play a crucial role in data precision and accuracy, especially when investigating novel contaminants and their metabolites.

2.6. Single species vs. multiple species exposed to the same contaminated water

In general, knowledge gaps still remain concerning the links between suborganizational changes and their consequences for individual organisms and populations, as well as the influence of interspecific interactions and environmental factors (Groh et al., 2015). Bioassays with a single species are one of the main aquatic ecotoxicology tools currently applied and cover a wide range of modes of action of toxic substances, being a useful tool in prognostic effect assessment. However, the extrapolation of the results obtained in bioassays with a single species to ecosystems may be limited, since important theoretical ecology aspects are not considered (Schmitt-Jansen et al., 2008).

Understanding such complex interactions in investigated ecosystems requires a systematically examining processes to better understand the mechanisms underlying the various levels of biological organization - within organisms (molecule to organism), between organisms (reproduction rates, genetic diversity and susceptibility of the various species) and within ecosystems (competition and interaction in the food chain) (Boxall et al., 2013; Eggen et al., 2004; Gupta et al., 2017; Hallgren et al., 2012; Mattsson et al., 2017; McCahon and Pascoe, 1990; Salomão et al., 2014; Schmitt-Jansen et al., 2008; Segner et al., 2003; Slootweg et al., 2015).

The identification of new toxicants assessment strategies with greater ecological relevance, as well as the development of integrated approaches that combine chemical analytical, ecological and ecotoxicological tools, is a challenging task (Schmitt-Jansen et al., 2008). In this context, the Ecological Risk Assessment (ERA) can be applied as an important tool in decision-making processes applied in assessing ecosystems sustainability. The integration of the three aforementioned lines of evidence (LoE) (chemical, ecotoxicological and ecological) significantly increase the accuracy of analyses concerning the real risks to potentially affected ecological receptors (Mendes et al., 2017).

3. Time affecting all variables

Time significantly affects toxicity responses in laboratory assays, although defined in several ways. For example, time may refer to life stage/age, uptake, recovery, and depuration rate in certain bioindicators, while referring to contaminant half-life and is active period in organisms before excretion or biodegradation when assessing contaminant and environmentally relevant concentrations. Regarding dose mode, time is associated to intermittent discharge frequency occur, exposure duration, and inter-pulse intervals. Concerning contaminant concentration vs applied load, time refers to the exposure period required to define mass resulting in effects on selected bioindicator species. Concerning single *vs.* multiple exposures, in addition to intrinsic contaminant characteristics, such as half-life, interactions and ultimate effects on the target organism may differ depending on synchronism, as different contaminants enters the aquatic ecosystem (simultaneously or in sequence). Finally, when assessing single species *vs.* several species simultaneously exposed to the same contamination source, time refers to how long bioaccumulation may take and how the pollutants may affect species according to their food web positions. Thus, understanding which time definition is required is paramount to obtain consistent results. As stated previously for other parameters, this depends on the intended study goals and should be evaluated and selected by the authors during the experimental design.

4. Conclusions

The complexity of the interactions addressed in this review indicate that, although conventional ecotoxicity assays are extremely useful and relevant tools, results should be interpreted cautiously and conclusions should express exactly what parameters were assessed, clearly indicating exposure period, dose regime and experimental conditions, thus avoiding generalizations and the simplification of complex issues posed by a significant and increasing number of compounds available in modern society.

Declaration of competing interest

The authors declare no competing interests.

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