Chapter

A Well-Established Method for the Rapid Assessment of Toxicity Using *Artemia* spp. Model

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Abstract

Rapidly, relevantly, and efficiently toxicity assessment is the basis of continuous investigation and control of environmental contaminants. *Artemia* sp. is usually used as a biological model in cost-efficient bioassays under laboratory conditions to determine toxicity based on its advantageous properties of rapid hatching, easy accessibility, and sensitivity to toxic substances. The three sensitive endpoints of acute mortality, acute cyst hatchability, as well as behavioral response (such as swimming speed) are commonly used as evaluation criteria. The establishment of international standards for toxicity assessment of *Artemia* spp. is necessary. Further research is needed to obtain valuable insights from a biological perspective and for bio-conservation purposes.

Keywords: *Artemia*, toxicity assessment, mortality, hatchability, swimming speed

1. Introduction

Toxicology is the science of researching on the negative effects that chemical or physical agents may exert on living organisms under particular exposure conditions. It is a science that attempts to evaluate all the hazards, such as molecular toxicity, cytotoxicity, organ toxicity, etc., that are associated with a substance, as well as to quantitatively determine the exposure conditions under which these hazards or toxicities are induced [1, 2]. Additionally, toxicology is the science that studies the occurrence, character, frequency, mechanism, and risk elements associated with the adverse effects of toxic substances [2].

Many biological models can be applied for toxicity evaluation. Cell culture system is often used in vitro because it is economical and time-saving. But it is very difficult to infer the health of the whole organism, including humans, only from the results of in vitro cell tests. On the contrary, in vivo studies may provide improved prediction of biological reactions in intact systems (whole animal) but are generally expensive, time-consuming, and often elaborate, requiring extensive facilities and infrastructure [3]. Zebrafish (*Danio rerio*), as a classical model vertebrate organism, offers many practical advantages that can overcome these limitations to be highly suitable for application in toxicologically relevant research. Zebrafish can be employed as an outstanding in vivo model system to evaluate biological reactions and is a powerful platform to analyze in detail the mechanisms by which substances induce specific biological responses. Further, conditions in high-order vertebrates can be inferred from the results obtained using zebrafish because there

is a remarkable similarity in cellular structure, signaling processes, anatomy, and physiology, particularly in the early stages of development [4–8]. Current estimates show that more than 90% of the human open reading frames are homologous to those in the genes of this fish [9]. Thus, investigations using this model system can reveal subtle interactions that are likely to be conserved across species.

2. Toxicity assessment with *Artemia* spp. and its advantages

The predominant EU Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation with the aim of sound management of the eco-environment and protection of human societies promoted the decrease in the use of vertebrates and encouraged the use of invertebrates and plants, as well as organ, tissue, and cell cultures, as alternative study materials for toxicity and ecotoxicity testing [10]. Among various invertebrates screened and assessed to investigate their sensitivity to several physical and chemical substances, brine shrimps, *Artemia* spp., which are extremely sensitive to toxicity, stand out as one of the most frequently used species for toxicity testing [11] and are recognized and listed by the US Environmental Protection Agency [12] as the model organism for toxicity testing and emission monitoring.

Artemia sp. is a crustacean adapted to harsh conditions such as those in hypersaline lakes [13], living mainly on phytoplankton [14, 15]. It is closely related to other zooplankton such as copepods and daphnia (**Figure 1**) [16]. Normally, it is routinely employed as a test organism for ecotoxicological studies. The molecular, cellular, and physiological states of *Artemia* spp. change dramatically when they are under contamination stress [17]. At present, a variety of toxicity tests with *Artemia* spp. have been carried out covering both short-term acute and long-term chronic methods (**Table 1**), with the former being the more frequently used. Acute toxicity tests, which are highlighted in this paper, mainly assess the effect exposure to relatively high concentrations (at a mg/L level) for no more than 4 days (96 h). Toxicity under normal conditions is expressed as the lethal concentration causing the death of half of the tested animals (LC_{50}) and is also manifested in impeded hatching and swimming behavior. Chronic toxicity tests mainly have to do with the long-term exposure to relatively low concentrations (at a μg/L level) ranging from a few weeks up to the entire life cycle of *Artemia* spp. [18].

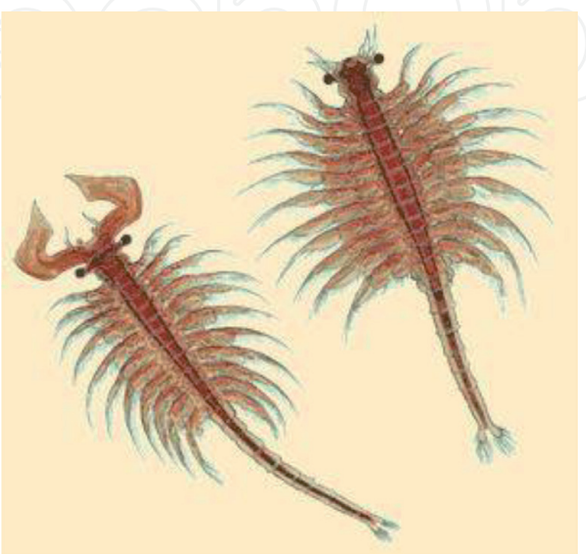

Figure 1.
An adult of Artemia spp.: male (left) and female (right).

Test type	Method	Parameter index
Short-term	Biomarker	AChE
		HSP
		Fluotox
		LP, TBARS, and TRed
		GRed, GPx, and GST
		ALDH and ATPases
	Hatching	Dry biomass
		Morphological disorder
		Size
		Teratogenicity
	Swimming	Speed
		Path length
	Immobilization	Mortality
Long-term	Growth	Body size
		Weight
		Morphological disorder
	Reproduction	Mating
		Reproductive rate
		Offspring
	Immobilization	Mortality

PS: AChE = acetylcholinesterase; HSP = heat stress proteins; LP = lipid peroxidation; TBARS = thiobarbituric acid reactive substances; TRed = thioredoxin reductase; GPx = glutathione peroxidase; GST = glutathione S-transferase; GRed = glutathione reductase; ALDH = aldehyde dehydrogenase; and ATPases = adenyltriphosphatase

Table 1.
Summary of Artemia short- and long-term toxicity tests [19].

Considering the environmental aspect, *Artemia* spp. nauplii were employed to assess the toxicity of various hazardous metal substances such as As, Cr, Sn, etc. [19–22]; organic compounds including pharmaceuticals, agrichemicals, etc. [23–26]; and environmental media such as wastewater [27], seawater [28], and marine discharges [29].

The principal advantages of using *Artemia* spp. in toxicity testing are as follows: (1) rapidity in hatching, (2) cost-efficiency, and (3) commercial availability of nauplii hatched from durable cysts, which dispenses with the need for self-culturing [30, 31]. Moreover, other significant factors that have been taken into consideration include good cognition of its biological and ecological features, small size allowing for easy laboratory operation, as well as its well-developed adaptability to diversified testing conditions [30, 32]. It is noteworthy that the complex adaptive response evolved by *Artemia* to live through and thrive in critical conditions not only explains why it is a favorable candidate for toxicity testing but to some extent also offers insights with regard to biological and environmental perspectives, which in turn might contribute to toxicity testing itself and eventually the well-being of human populations. With that being said, the response mechanism developed by *Artemia* to deal with harsh conditions [13] is worth mentioning (see **Figures 2** and **3**). The harsh living condition is exemplified in hypersaline lakes (salty lakes) where *Artemia* is often the only macroplanktonic inhabitant [13]. The survival and reproduction of

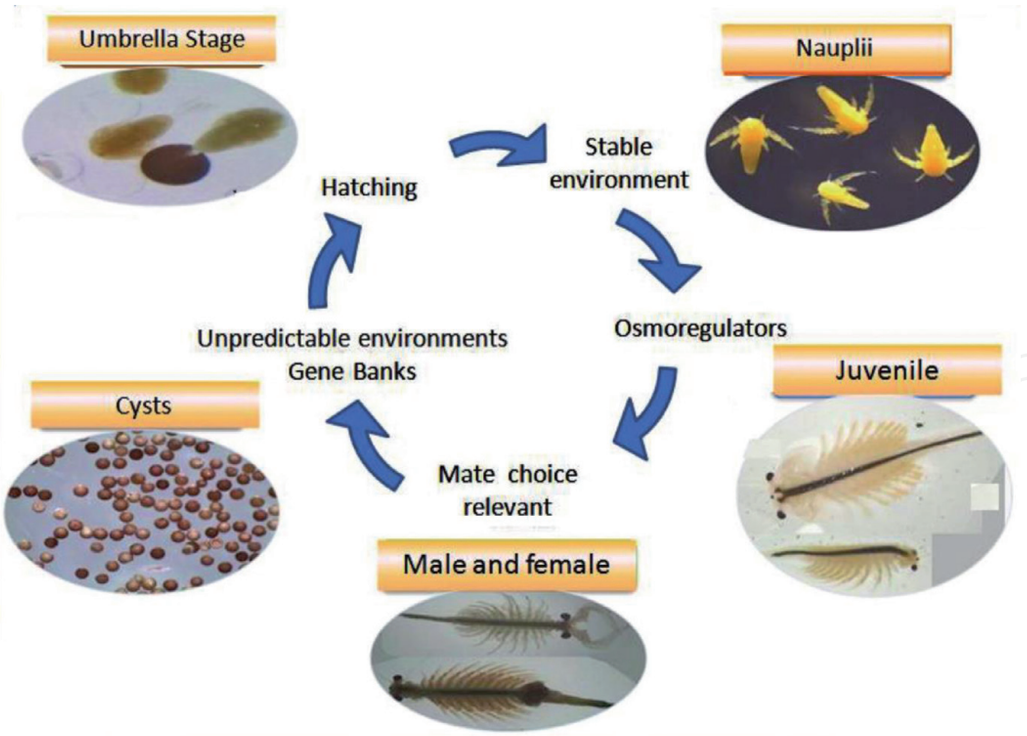

Figure 2.
The life cycle and different stages of Artemia as a salty survivor.

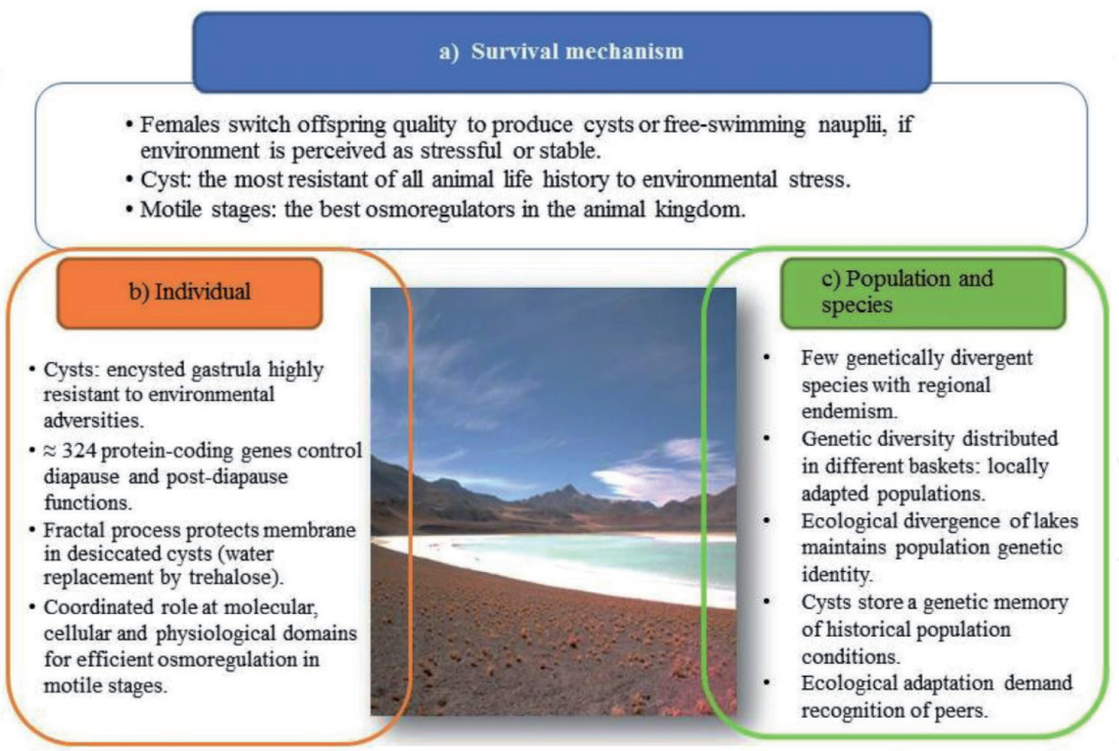

Figure 3.
The reproduction of Artemia brine shrimp (individuals, populations, and species) subject to critical life conditions imposed by salty lakes.

the brine shrimp *Artemia* (individuals, populations, and species) subject to critical life conditions imposed by salty lakes, as schemed in **Figures 2** and **3**, may be summarized as follows: (1) Females are able to cope with the forthcoming environmental conditions by switching the type of offspring to produce either cysts under stressful conditions or free-swimming nauplii under stable conditions, and (2) cysts are

the most environmental stress-resistant among all animal life history forms, while motile stages are the best osmoregulators in the animal kingdom [33]. Cysts are gene banks that store a genetic memory of historical population conditions. They play a role aiding in the dispersal of *Artemia* and serve as reservoirs of genetic variability [34] and the source of evolutionary change and resilience.

3. Application status of the toxicity assessment with *Artemia* spp.

Ecotoxicological studies employing *Artemia* spp. as testing species have been extensively performed, and among the endpoints that were mainly investigated, acute mortality, acute cyst hatchability, as well as behavioral response, as a result of their relatively high sensitivity, are commonly used.

3.1 Acute mortality test

Acute mortality is one of the most commonly used endpoints for toxicity testing, though there is no standardized protocol based on OECD and ISO regulations. Since the establishment of the *Artemia* Reference Center (ARC-test) and the issuance of the first short-term acute mortality (24 h static test) protocol with *Artemia* larvae [35–38], extensive toxicity assessment research using this bioassay has been carried out via calculating the median effectiveness concentration on mortality (24 h LC_{50}). Besides observation of lethal endpoints for *Artemia* exposed to reference toxicants including $CuSO_4$, $K_2Cr_2O_7$, and SDS [39, 40], many are related with toxicity monitoring of environmental pollutants such as heavy metals, pesticides, oil drilling fluids, organic compounds of ecotoxicological concern, and others [41–44]. Indeed, in the wake of various environmental issues challenging humans and living surroundings, the importance of toxicity assessment using *Artemia* has been gradually recognized and more frequently employed. The following are two examples in recent years.

The "Brine Shrimp Lethality" study is one of the biological assays to determine the safe exposure limit of naturally occurring agents extracted from plants before being used as pesticides for crops and for other botanical protections [16]. Crop protection is one of the important food safety-related issues and is thus vital to human populations worldwide. As crop protection nowadays rely heavily on synthetic pesticides [45], the massive use of these pesticides for the purpose of killing pests and preventing diseases in plants has inevitably led to several side effects such as pest resistance resulting in the use of increased application rates [46], harm to nontarget organisms, and environmental contaminations with the potential influence on the food chain [47] that might cause pesticide poisoning of humans directly. Botanically derived natural products therefore have attracted attention among phytochemists. "Brine Shrimp Lethality," a rapid general bioassay, offers a unique advantage in the standardization and quality control of those bioactive compounds that are usually undetectable using traditional physical analytical methods. The objective of carrying out the biological assay focuses on establishing a cause-effect relationship (**Figure 4**) between exposure to a hazardous substance and an appeared effect expressed by dose-response curve to determine a safe exposure limit [48]. The threshold level as well as the toxicity features obtained from the dose-response curves can help determine the safe levels of chemicals in botanical extracts and chemical exposure [49]. The threshold information ($ThD_{0.0}$) measured in mg/kg/day and based on the assumption that human beings are as sensitive as the tested animals; in this case the brine shrimp *Artemia* sp. is of paramount importance in generalizing animal data to humans and interpolating what might be considered a safe human dose for a given chemical.

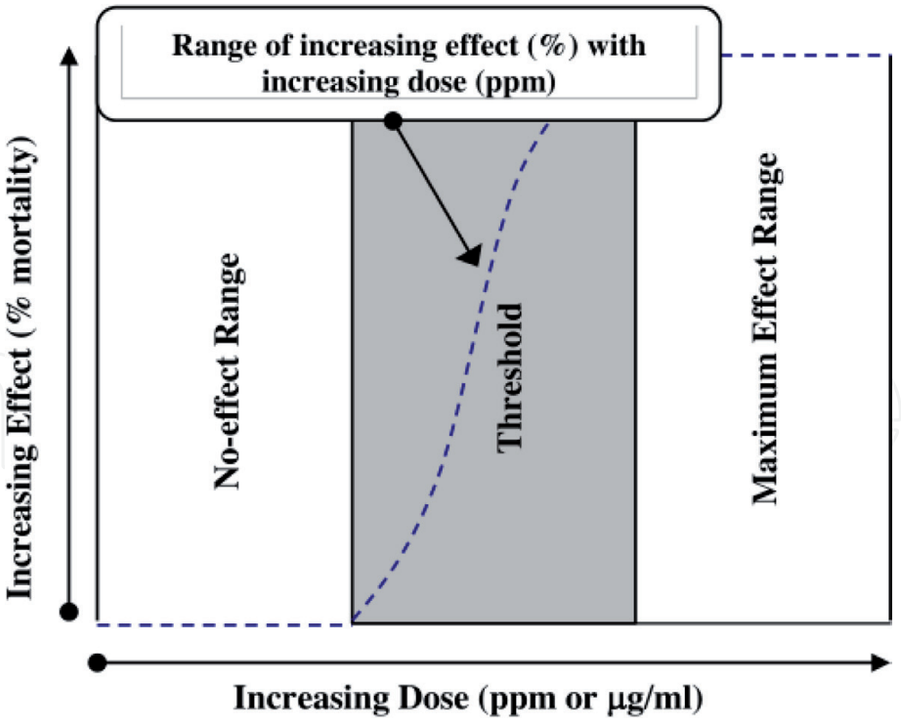

Figure 4.
Dose-response curve.

Another example in relation to the *Artemia* acute toxicity test [50, 51] is for the purpose of prevention and reduction of red tides. The red tide induced by algae is quite disastrous and may pose a threat to inshore fishery. The poisonous *Chattonella marina* that produces reactive oxygen species (ROS) [52] and hemolytic toxins [53] is one kind of red tide-related algae and has caused massive fish death and a considerable amount of economic loss in many places around the world. The "Brine Shrimp Lethality" study in this regard can help reveal the toxic characteristics of *Chattonella marina*, offer some valuable red tide prevention evidences, and further benefit the offshore fishery industry.

3.2 Acute cyst hatching test

Analogous to the acute mortality test, acute cyst hatching testing, which observes the retarded emergence of nauplii from cysts [54] or the morphological disorders and size of hatched nauplii [55] when exposed to toxic agents, is another frequently used assay for toxicity assessment. The hatching toxicity test lasting between 24 and 96 h in static conditions was investigated to assess the effect of environmentally deleterious agents such as heavy metals [54, 56, 57], organic compounds [58, 59], antibiotic drugs [60], and others. As temperature profoundly influences the hatching percentage of cysts [61] and significantly affects the chemicals' effect [62], it is a variable of great interest to be considered while carrying out the hatching test, and the use of a full temperature range might help increase the ecotoxicological data in an extensive manner.

3.3 Acute behavioral test (swimming speed)

Regarding the acute behavioral test, motion behavior changes in response to pollutant exposure have been investigated for a range of aquatic organisms [63–67]. In particular, swimming speed as a sublethal behavioral endpoint can be detected by employing a video camera tracking system developed by Faimali et al. [63],

also known as the Swimming Speed Alteration (SSA) recording system, which has already been used on the brine shrimp, *Artemia* [68]. Moreover, the research results of Garaventa et al. [68] and Manfra et al. [69] showed that swimming speed was more sensitive than mortality and had a sensitivity similar to and sometimes higher than that of the hatching rate endpoint. Therefore, it is a well-defined behavioral response and an adaptable endpoint that can be used for ecotoxicity testing. For instance, Manfra et al. [69] recorded the swimming speed alteration of *Artemia* exposed to diethylene glycol (DEG), an organic substance ecotoxicological concern, and observed a decline in the swimming speed under the toxicant concentration of 40–160 g/L after 24 h exposure and 10–160 g/L after 48 h exposure. Another example is related with marine pollution such as oil spilling, oil mining, and oily water discharge that can greatly threaten human health as contaminants can be accumulated in the human body through the food chain. In this regard, *Artemia* spp., as one of the toxicity-monitoring species, is of great importance in the evaluation of the health of the marine ecosystem. Pan [70] investigated the swimming speed and motion angle alteration of *Artemia* exposed to diesel oil. For comparison purposes, when experiments were carried out under normal conditions, namely, seawater, the swimming speed of *Artemia* increased by 51%, from 2.47 mm/s at the start time to 3.72 mm/s after 12 h exposure on average, and in a similar trend, the motion angle of *Artemia* increased from 25 to 37°. In contrast, when subject to diesel oil, the swimming speed of *Artemia* decreased by 40%, from 2.37 mm/s at the start time to 1.42 mm/s after 12 h exposure on average, and in a similar trend, the motion angle of *Artemia* decreased from 30 to 21°.

4. Prospects for development of toxicity assessment with *Artemia* spp.

To rapidly figure out the deleterious effects brought about by environmental toxicants, acute toxicity assessment with *Artemia* spp. is of paramount importance as it shows a decent ability in pre-screening of toxic substances [10] and, thus, will be further developed in the future.

Despite the widespread application of this bioassay, there is currently no internationally standardized method. Hence, intercalibration exercises as well as international standardization activities are rather necessary [71]. Among the three frequently used endpoints involving acute mortality, acute cyst hatchability, as well as behavioral response, acute mortality was intercalibrated based on the available standards [40, 69, 72], while acute hatchability was intercalibrated at the Italian level [69]. To make *Artemia* spp. an international standard model in ecotoxicity testing calls for joint efforts engaging all relevant stakeholders including the government, NGOs, researchers, industry, consumer associations, and others.

Swimming speed as the most popular behavioral endpoint promises to be of great potential. This is because results can be obtained via easy video camera analysis at ease and also because the swimming speed is of great ecological significance as the behavior alteration means an integral whole body response that can connect the physiological and ecological features of an organism with its environment [73]. Nevertheless, to better employ this endpoint, the interaction of *Artemia* spp. with contaminants, particularly the mechanisms of response to toxic effect, needs to be illuminated.

One is to believe that owing to the advantages of using *Artemia* spp. as the biological model described in the previous section of this paper, besides toxic testing application itself, application into other environmentally related fields such as applied biology might also be put into practice. For example, from a bio-conservation point of view, the unique biological characteristics of brine shrimp *Artemia* make it a model organism to evaluate management policies for the protection of aquatic

resources [74]. *Artemia* is such a versatile creature that it is a paradigmatic model having not only scientific research values but also the ability to satisfy human needs, owing to its unique life traits including a well-developed adaptability to high salinity conditions as well as easy handling under laboratory conditions, which have been successfully applied to marine fish farming that uses *Artemia* nauplii as food for fish larvae. However, the booming marine fish farming activity worldwide is likely to give rise to some risks in terms of the high genetic divergence between different *Artemia* species. Exploitation of new *Artemia* cyst harvesting sites and introduction of an exotic species linked to traits relevant to aquaculture can drive other local genotypes to extinction. Risk assessment and evaluation of management decisions in exploited resources, for instance, the availability of genetic information as well as molecular tools for follow-up gene pool monitoring, therefore, become quite necessary in order to maintain biodiversity. Gene banks established from cysts collected from various sites guarantee population persistence while proceeding with management affairs. Taking into account the simple constitution of hypersaline habitats, the evaluation of population/species persistence with *Artemia* can be modeled in laboratories and further extrapolated to other species, offering some of the aspects regarding rational aquatic resource utilization and, more importantly, biodiversity preservation.

5. Conclusions

After more than five decades of use in ecotoxicology, *Artemia* spp. have demonstrated its suitability for use in pre-screening of toxic agents [10]; thus, it seems that *Artemia* sp. endpoints may be used as a toxicity testing method to meet market demand, even though there are no internationally standardized toxicity testing protocols at present according to the ISO and OECD.

Biomarkers and teratogenicity are the less popular endpoints used in short-term toxicity tests because of their limited sensitivity. However, behavioral endpoints, especially swimming inhibition, seem to have a wider application potential in the future with the development of computer technology. Both continuous and intermittent observations of single or groups of living organisms can be studied by image and video analysis. Hatching rate and acute mortality are the most commonly used endpoints in the standardization process at a different level. Usually, hatching rate (48 h static test) was intercalibrated at the Italian level [69], while acute mortality (24 h static test) was intercalibrated based on the available standard [40] at the Italian [69] as well as the European level [72]. Both provided data on $CuSO_4$ as a reference toxicant. Among the long-term toxicity tests, the 14-d static renewal mortality test was intercalibrated at the Italian level [69] with SDS according to the UNICHIM protocol (2012).

Further concentrated efforts are necessary to make *Artemia* sp. an official internationally recognized standard biological model in ecotoxicology evaluation. It involves (I) a national member (who then contacts the ISO) upon a request by an industry sector or group for a standard; (II) scope, main definitions, and contents of standards which are scientifically assessed by experts in relevant fields; and (III) multi-stakeholder discussion and reviewing process including experts from related industries, consumer associations, academic institutions, nongovernmental organizations, and governments.

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