RESEARCH ARTICLE

Artemia spp. Model - A Well-Established Method for Rapidly Assessing the Toxicity on an Environmental Perspective

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ABSTRACT

The toxicity testing bioassay using *Artemia* spp. as a biological model is used widely due to its advantages of rapid hatching and easy accessibility of nauplii hatched from durable cysts in a cost-efficient way, and high adaptabilities to harsh conditions thus ensuring easy handling under laboratory conditions. Rapidly assessing the toxicity of environmental contaminants which is of high significance will be further applied in the future, and three sensitive endpoints commonly used in this regard are acute mortality, acute cyst hatchability as well as behavioral response (swimming speed). The establishment of an international standard using *Artemia* spp. is necessary and that requires joint efforts of various stakeholders. Besides toxicity testing itself, *Artemia* spp. can be investigated to offer some of the valuable insights on a biological perspective and for bio-conservative purposes.

Keywords: *Artemia*; Toxicity Assessment; Mortality; Hatchability; Swimming Speed

INTRODUCTION

Toxicology is the science that deals with the study of adverse effects chemicals or physical agents may produce in living organisms under specific conditions of exposure. It is a science that attempts to investigate qualitatively all the hazards, for example the organ toxicities that are associated with a substance as well as to quantitatively determine the exposure conditions under which those hazard or toxicities are induced [1, 2]. Toxicology is the science that experimentally investigates the occurrence, nature, incidence, mechanism and risk factors for the adverse effects of toxic substances [2].

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Numerous biological models can be employed for toxicity evaluations. In vitro techniques, such as cell culture systems, are often preferred because they are both cost- and time-efficient. While these studies are useful, direct translation to whole organisms and human health is often difficult to infer. In vivo studies can provide improved prediction of biological response in intact systems but often require extensive facilities and infrastructure [3]. Zebrafish (Danio rerio) offer a number of practical advantages as a model organism that overcomes these limitations, making these vertebrates highly amenable for toxicologically relevant research. Zebrafish can be employed as a powerful in vivo model system to assess biological interactions and are an outstanding platform to detail the mechanisms by which substances elicit specific biological responses. A remarkable similarity in cellular structure, signaling processes, anatomy and physiology exists among zebrafish and other high-order vertebrates, particularly early in development [4-8]. Current estimates indicate that over 90% of the human open reading frames are homologous to genes in fish [9]. Thus, investigations using this model system can reveal subtle interactions that are likely to be conserved across species.

THE TECHNOLOGY OF TOXICITY ASSESSMENT WITH *ARTEMIA* SPP. AND ITS ADVANTAGES

The predominant EU REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) legislation with the aim of sound management of the eco-environment and protection of human societies promoted the decrease of vertebrates and encouraged the use of invertebrates, plants as well as organ, tissue, and cell cultures as alternative articles during the process of toxicity and eco-toxicity testing [10]. Among various invertebrates screened and assessed to investigate their sensitivity to lots of physical and chemical substances, *Artemia* spp. brine shrimps, extremely sensitive to toxicity, stood out as one of the most frequently used species for toxicity testing [11], and it was recognized and listed as the toxicity testing organism used for emission monitoring by the United States Environmental Protection Agency [12].

Artemia spp. is a crustacean creature having adapted to harsh life conditions like those found in hypersaline lakes [13], and living mainly on phytoplankton and being an important major consumer [14, 15]. It is closely related with 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 levels 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 (Figure 2), with the former one being the more frequently used. Acute toxicity which is highlighted in this paper mainly assesses the effects on account of relatively high concentrations (mg/L) of exposure for no more than 4 days (96 h). And toxicity is in normal conditions expressed as lethal concentration causing the death of half of the tested animals (LC_{50}), but hatching and swimming behavior impediment as well. Chronic toxicity tests mainly have to do with the long-term exposure starting from weeks up to the entire life cycle of *Artemia* spp. at relatively low concentrations (μg/L) [18].

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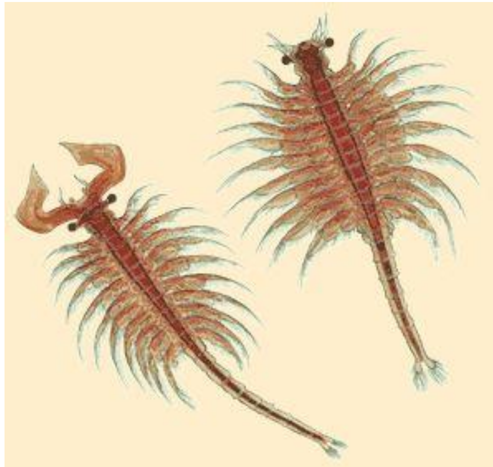

FIGURE 1 | An adult of *Artemia* spp.: male (left) and female (right)

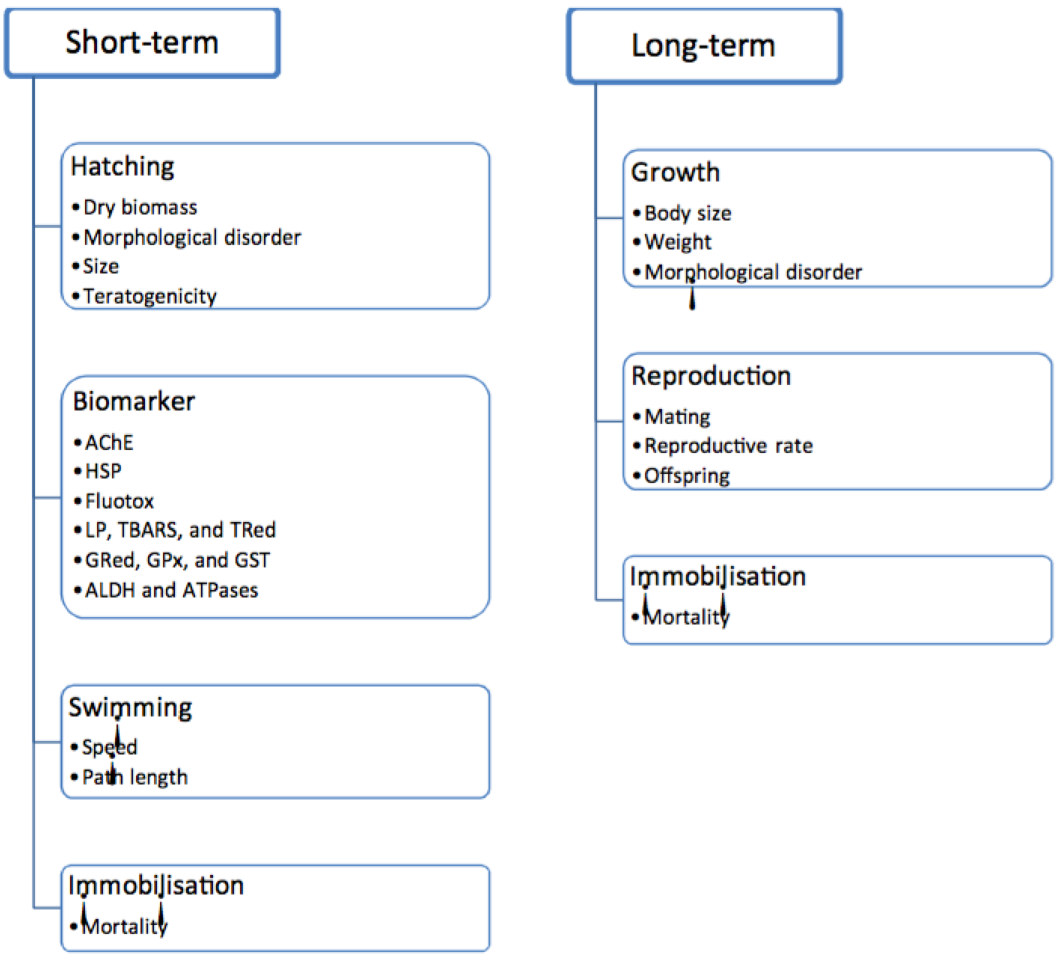

FIGURE 2 | Summary of *Artemia* short- and long-term toxicity tests.

(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). [19]

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On an environmental aspect, *Artemia* spp. nauplii, at the larval stage, were employed to assess the toxicity of various hazardous metal substances such as As, Cr, Sn, *etc*. [20-22], organic compounds including pharmaceuticals, agrichemicals and others [23-26], as well as environmental media like wastewater [27], seawater [28] and marine discharges [29].

The principal advantages of using *Artemia* spp. in toxicity testing are described as follows: (1) rapidity in hatching; (2) being cost-efficient; (3) accessibility of nauplii hatched from durable cysts that can be purchased commercially, which dispenses with the needs of 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 life conditions not only deciphers why it's a favorable candidate for toxicity testing but also to some extent offers insights with regard to biological and environmental perspectives which in return might contribute to toxicity testing itself and the well-being of human populations eventually. With that being said, the responsive mechanism *Artemia* has developed to deal with harsh conditions [13] is worth mentioning (see Figures 3 and 4). The harsh living condition is exemplified in hypersaline lakes (salty lakes) where *Artemia* is the unshared macro-planktonic inhabitant [13]. The survival and reproduction of *Artemia* brine shrimp (individuals, populations and species) subject to critical life conditions imposed by salty lakes as schemed in Figure 3 and 4 can be comprehended and described as follows. (1) Females are able to cope with the forthcoming environmental conditions by switching offspring quality to produce either cysts under stressful conditions or free-swimming nauplii under stable conditions; (2) Cysts are the most resistant of all animal life history forms to environmental stress, 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 in aiding in the dispersal of *Artemia*, and serving as reservoirs of genetic variability [34], the power for evolutionary change and resilience.

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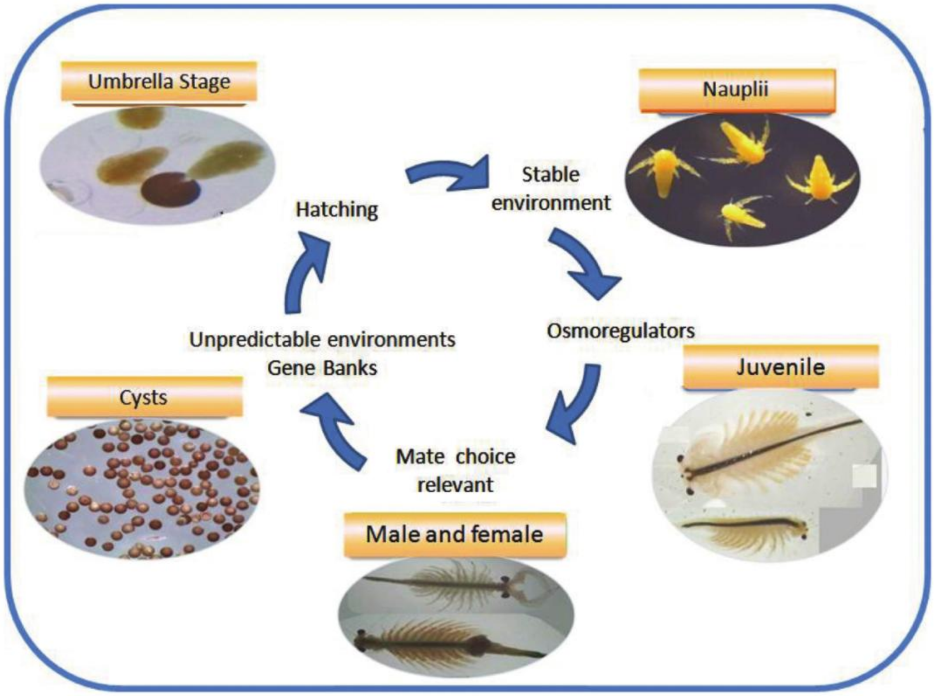

FIGURE 3 |The life cycle of a salty survivor: the extremophile *Artemia*. Different stages of the cycle are actively involved in survival and reproduction under critical environmental conditions [35].

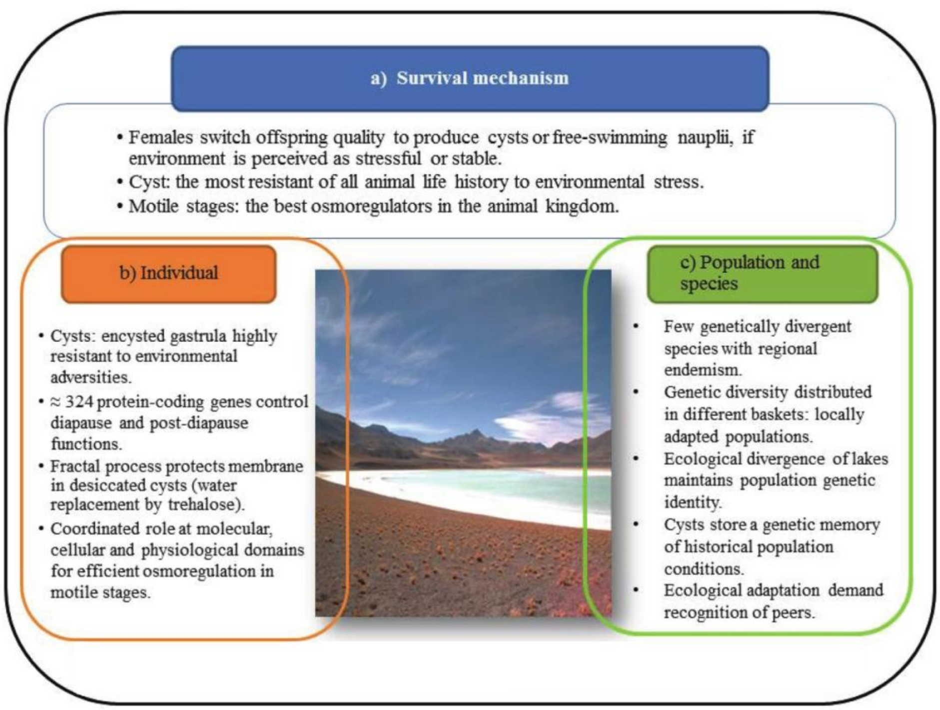

FIGURE 4 | Salty lakes impose critical conditions on reproduction of individuals, populations, and species. Adaptation to such conditions has evolved a network of different functional and hierarchical domains [35].

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APPLICATION STATUS OF THE TOXICITY ASSESSMENT WITH *ARTEMIA* SPP.

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

(1) Acute mortality test

Acute mortality is one of the most commonly used endpoints for toxicity testing, though currently no internationally standardized toxicity testing protocols exist according to OECD and ISO. Since the emergence of *Artemia* Reference Center (ARC-test) and the issuance of the first short-term acute mortality (24h static test) protocol with *Artemia* Larvae [36-38], extensive toxicity assessment researches using this bioassay have been carried out via calculating the median effectiveness concentration on mortality (24h LC_{50}). Besides lethal endpoints observation 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, ecotoxicological concern organic compounds and others [41-44]. Indeed, in the wake of various environmental issues affecting humans and living surroundings, the importance of toxicity assessment using *Armetia* has been gradually recognized and more frequently employed, the following are two examples in recent years.

"Brine Shrimp Lethality" study is one of the biological assays to determine the safe exposure limit of natural occurring agents extracted from plants before being used as pesticides for crop and other botanical protection [16]. Crop protection is one of the important food safety related issues and thus is vital to human populations worldwide. As crop protection nowadays relies heavily on synthetic pesticides [45], the massive use of these pesticides for the purpose of killing pests and preventing the plants from diseases has inevitably led to several side effects for the likes of pest resistance resulting in the use of increased application rates [46], harm to non-target organisms and environmental contaminations with the potential influence on the food chain [47] which might cause pesticide poisoning of human bodies directly. Botanical 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 bio-active compounds to which traditional physical analytical methods are usually insensitive. The object of carrying out the biological assay focuses on establishing a cause effect relationship expressed by dose-response curves (Figure 5) between exposure to a hazardous substance and an appeared effect 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 botanical extracted chemicals 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 those tested animals, in this case the brine shrimp *Arthemia* spp., is of paramount importance in generalizing animal data to humans and interpolating what might be considered safe human dose regarding a given chemical.

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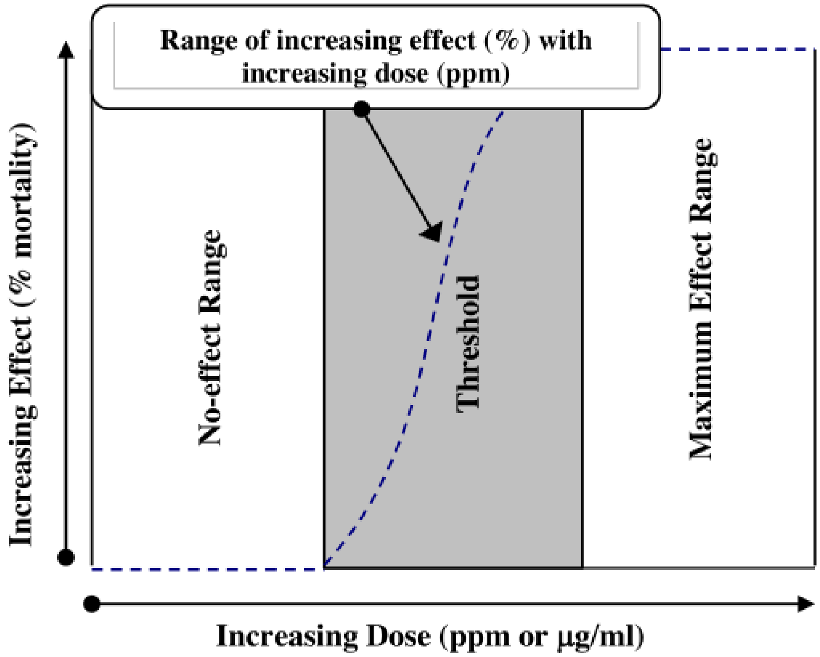

FIGURE 5 | Dose-response curve [50]

Another example in relation to *Artemia* acute toxicity test [51] is for the purpose of prevention and reduction of red-tide. Red tide induced by algae is quite disastrous and may pose a threat to inshore fishery. The poisonous *Chattonella marina* that produces ROS (reactive oxygen species) [52] and hemolytic toxins [53] is one kind of red tide related algae and has caused a great number of 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.

(2) Acute cyst hatching test

Analogous to 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 24h~96h in static conditions was investigated to assess the effect of environmentally deleterious agents for the likes of 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 chemicals' effect [62], it is a variable of great interest to be considered while carrying out the hatching test, and the use of full-temperature-range might help increase of ecotoxicological data in a drastic manner.

(3) Acute behavioral test (swimming speed)

Regarding the acute behavioral test, motion behavior changes as responses to exposure to pollutants have been investigated for a

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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 turned out to be more sensitive than mortality and with a sensitivity similar to and sometimes higher than 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 ecotoxicological concern organic substance and observed a decline of the swimming speed under the toxicant concentration of 40-160 g/l after 24h exposure and 10-160 g/l after 48h exposure, respectively. Another example is related with marine pollution for the likes of oil spilling, oil mining and oily water discharge that can greatly threaten human health as contaminants can be accumulated into the human body through the food chain. In this regard, *Artemia* spp., as one of the toxicity monitoring species, is of great importance to 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 12h exposure in average, and in the same trend the motion angle of *Artemia* increased from 25° to 37°. Contrarily, when subjected 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 of exposure on average, and in the same trend the motion angle of *Artemia* decreased from 30° to 21°.

PROSPECTS FOR THE DEVELOPMENT OF TOXICITY ASSESSMENT WITH ARTEMIA SPP. IN THE FIELD OF ENVIRONMENTAL SCIENCE

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 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 no internationally standardized method for the time being. Hence, intercalibration exercises as well as international standardization activities are rather necessary [71]. Among the three mostly used endpoints involving acute mortality, acute cyst hatchability as well as behavioral response, acute mortality was intercalibrated based on the available standard [40] at both the Italian [69] and European [72] levels, 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 in relevant stakeholders including government, NGOs, experts from academia, industry, consumer association and others.

Swimming speed as the most popular behavioral endpoint has a great potential in

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the coming future, this is because results can be obtained via video-camera analysis at ease and also because the swimming speed is of great ecological significance as the behaviour 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, how the contaminants interact on *Artemia* spp., namely, the toxic effect mechanisms need to be illuminated.

One is to believe that due 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, on a bio-conservative point of view, the unique biological characteristics of brine shrimp *Artemia* make it a model organism to evaluate management policies in protection of aquatic resources [74]. The brine shrimp *Artemia* is such a versatile creature that it is a paradigmatic model having not only scientific research values but also values to satisfy human needs, owing to its amazing life traits including well-developed adaptability to high salinity living 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 larval stages. However, the booming marine fish farming activity worldwide is likely to give rise to some risks in correlation with the high genetic divergence between different *Artemia* species. Exploitation of new *Artemia* cysts harvesting sites and the 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 pools monitoring, therefore become quite necessary in order to maintain biodiversity. Gene banks establishment from cysts collected from various sites guarantees population persistence while proceeding with management affairs. Taking into account the simple constitution of hypersaline habitats, 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 resources utilization, and more importantly, biodiversity preservation.

ACKNOWLEDGMENTS

The authors of this study express their gratitude to the National Natural Science Foundation of China (No. 31600257), Public Projects of Zhejiang Province (No. 2016C32022), Academic Climbing Project for Young and Middle-aged Leads in the Universities of Zhejiang Province (pd2013339), and the Project of Zhejiang Provincial Department of Education (Y201738582) for the financial support of this study.

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