Stem cells of aquatic invertebrates as an advanced tool for assessing ecotoxicological impacts

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Review

Stem cells of aquatic invertebrates as an advanced tool for assessing ecotoxicological impacts

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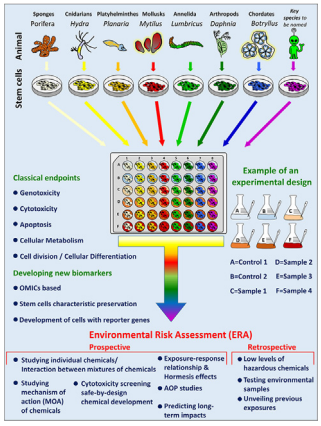
HIGHLIGHTS

- Aquatic invertebrates are key organisms in ecotoxicological studies.
- Aquatic invertebrates Adult Stem Cells (ASCs) present distinctive features.
- ASCs may be harnessed to develop *in vitro* tests for ecotoxicology risk assessment.
- Aquatic invertebrate ASCs-based tools test impacts unique to aquatic invertebrates.
- Tests with ASCs contribute to prospective and retrospective risk assessment.

GRAPHICAL ABSTRACT



Abbreviations: AOP, adverse outcome pathways; ASC, adult stem cell; GMP, Germline Multipotency Program; HTS, high-throughput screening; WBR, whole body regeneration.

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ABSTRACT

Environmental stressors are assessed through methods that quantify their impacts on a wide range of metrics including species density, growth rates, reproduction, behaviour and physiology, as on host-pathogen interactions and immunocompetence. Environmental stress may induce additional sublethal effects, like mutations and epigenetic signatures affecting offspring *via* germline mediated transgenerational inheritance, shaping phenotypic plasticity, increasing disease susceptibility, tissue pathologies, changes in social behaviour and biological invasions.

The growing diversity of pollutants released into aquatic environments requires the development of a reliable, standardised and 3R (replacement, reduction and refinement of animals in research) compliant *in vitro* toolbox. The tools have to be in line with REACH regulation 1907/2006/EC, aiming to improve strategies for potential ecotoxicological risks assessment and monitoring of chemicals threatening human health and aquatic environments. Aquatic invertebrates' adult stem cells (ASCs) are numerous and can be pluripotent, as illustrated by high regeneration ability documented in many of these taxa. This is of further importance as in many aquatic invertebrate taxa, ASCs are able to differentiate into germ cells. Here we propose that ASCs from key aquatic invertebrates may be harnessed for applicable and standardised new tests in ecotoxicology. As part of this approach, a battery of modern techniques and endpoints are proposed to be tested for their ability to correctly identify environmental stresses posed by emerging contaminants in aquatic environments.

Consequently, we briefly describe the current status of the available toxicity testing and biota-based monitoring strategies in aquatic environmental ecotoxicology and highlight some of the associated open issues such as replicability, consistency and reliability in the outcomes, for understanding and assessing the impacts of various chemicals on organisms and on the entire aquatic environment. Following this, we describe the benefits of aquatic invertebrate ASC-based tools for better addressing ecotoxicological questions, along with the current obstacles and possible overhaul approaches.

Contents

1. Introduction

The term ecotoxicology, coined by Truhaut (1977), reflects an interdisciplinary field of science that draws knowledge and techniques from the fields of ecology and toxicology and is sometimes used synonymously with environmental toxicology, although the latter also encompasses the effects of chemicals on human beings (Rand, 1995). Ecotoxicological analyses cover a broad range of organisms and populations at all scales of biological organisation, from the molecular, cellular, physiological, and behavioural to the population levels (Batel et al., 1993; Lyubenova and Boteva, 2016). In recent years, ecotoxicological approaches have been recognised as useful tools that complement and improve the assessment of overall environmental quality affected by the continuous release of a wide range of chemicals from various anthropogenic sources into the environment ultimately reaching all freshwater and marine ecosystems, creating a growing problem worldwide as they affect the aquatic biota (reviews: Johnston and Roberts, 2009; Van Dam et al., 2011; Carlson et al., 2019). With ~1 million multicellular species, whereof ~250,000 have already been fully described (www.coml.org), the aquatic and especially marine environments may be particularly sensitive sinks for different types of pollutants like heavy metals (Yılmaz, 2010), chlorinated solvents and polycyclic aromatic hydrocarbons (Srogi, 2007), fertilizers (Spalding and Exner, 1993), detergents (Lewis, 1991), pesticides (Pinto et al., 2016), endocrine disruptors and chemical compounds from cosmetics (Snyder et al., 2003; Sánchez-Quiles and Tovar-Sánchez, 2015), new nano-hybrid-smart-materials (Nowack and Bucheli, 2007; Zhu et al., 2019), plastic debris (Derraik, 2002), pharmaceuticals (Prichard and Granek, 2016), and microplastics (Cole et al., 2011; Eriksen et al., 2014; Haegerbaeumer et al., 2019). Water quality of diverse aquatic bodies has been traditionally monitored by the use of analytical methods and parameters that provide information

about the presence and the concentrations of specific chemicals in the sediment, water column and biota. In parallel, there was a need for selecting biologically meaningful indicators and biological assays for analysing the actual impacts of pollutants on the aquatic biota. Bioassays, combined with chemical analyses, have provided further data on availability and impacts of specific pollutants on various aquatic organisms (*e.g.*, Müller et al., 1995).

Ecotoxicological drivers may induce impacts on organisms that are immediate and visible, such as viability or reproduction, or inflict more subtle effects such as changes in behavioural, physiological or immunocompetence traits, altogether leading to populations' decline (Weis et al., 2001; Relyea and Hoverman, 2006). Furthermore, chemicals that are toxic to individuals of a given species might not have effects limited to only those specific organisms, but may also affect the entire food chain (Weis et al., 2001; Fleeger et al., 2003; Relyea and Hoverman, 2006).

When employing ecotoxicological risk assessments of aquatic environments, focusing on sensitive aquatic organisms should be the key point in determining environmental indicators (Fossi and Panti, 2017). Since the marine environment is the largest ultimate sink for pollutants, earliest attempts at monitoring of pollutants at a global scale, through the employment of marine invertebrates, date back four decades when Goldberg (1975) proposed the 'Mussel Watch' as the first such monitoring approach for US coastal water pollutants. Similar monitoring programmes later spread to other regions of the world (Farrington et al., 2016; Beyer et al., 2017). Nowadays, a large number of aquatic invertebrate taxa are routinely used in laboratory and in large scale environmental pollution monitoring programmes due to their abundance, large biodiversity and lower ethical concerns in comparison to vertebrates. Many sessile species (from sponges to ascidians) continuously filter large volumes of water which can lead to accumulation of pollutants. Therefore, these taxa can be considered as perfect models for assessing effects of pollutants at low concentrations, below the detection limits of other assays (*e.g.*, pharmaceuticals and nano-sized plastics; Kos et al., 2016; Prichard and Granek, 2016; Haegerbaeumer et al., 2019). However, the ongoing development of industrial production and continuous demand for new chemicals and new advanced materials (such as pharmaceutical products and engineered nanomaterials) increase the need for high-throughput screening (HTS) tests that provide relevant toxicological information and can be integrated into monitoring programmes (Zhu et al., 2014). At the same time, these HTS tests should be pragmatic and science-based to provide reliable aquatic environmental risk assessment tools (Artigas et al., 2012) that can be integrated as part of international pollution testing recommendations and legislation.

It is problematic to identify hazards for all old or emerging pollutants being released into the environment and therefore there is a need for innovative approaches for translating scientific knowledge and methods to support fast and reliable risk assessment and to provide policy relevant information. This requires developing new methods or modifying existing ones for identifying biological fate and effects of pollutants and elucidating similarities or disparities in biological pathways across a variety of key species. One way to achieve such a goal is the development of new bioassays based on freshwater and marine invertebrates that take into account existing legislation, *e.g.*, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation (1907/2006/EC), aiming to ensure a high level of protection of human and environment health. According to Burden et al. (2015a, 2015b) several initiatives are currently underway aiming to improve confidence in alternative methods.

Here we briefly describe the current status of the available testing/monitoring tools in aquatic ecotoxicology and highlight some of the open issues related to them, such as replicability, consistency and reliability in investigating, understanding and assessing the impacts of various chemicals on freshwater and marine organisms and ecosystems. In line with the current need for reliable *in vitro* HTS monitoring tools in the field of ecotoxicology, we propose herein the development and application of advanced technology based on the harnessing of aquatic invertebrate adult stem cells (ASCs) for the assessment of ecotoxicological impacts. The enormous potential inbuilt in the proposed technology is based on recurring reports on the successful implementation of mammalian stem cell technology in pharmaceutics and toxicology tests (Liu et al., 2017; Luz and Tokar, 2018; Liu and Zheng, 2019) and on the distinctive features of ASCs in many aquatic biota, especially in marine invertebrates. Issues like recovery potential following exposure to pollutants, identification of non-lethal epigenetic impacts, and their transgenerational inheritance to unexposed offspring are only a few of the key challenges for which ASC-based technology may provide fundamental solutions for high throughput screening.

2. Freshwater and marine invertebrates in ecotoxicology

In terms of biomass and number of species, invertebrates represent the overwhelming majority of living animals in freshwater and marine ecosystems where they often play fundamental ecological roles. According to phylogenetic relationships, invertebrates may be clustered into three major groups: (i) metazoan early-divergent lineages, such as Porifera and Cnidaria; (ii) the protostome: Spiralia (Platyhelminthes, Mollusca, Annelida) and Ecdysozoa (Nematoda, Arthropoda); and (iii) the deuterostome clades comprising Echinodermata, Hemichordata, and Chordata. The latter include cephalochordates and tunicates, the closest living relatives of vertebrates (Delsuc et al., 2006; Fig. 1), and the vertebrates themselves. In terms of number of species, the most abundant are the Arthropoda and second far behind are the Mollusca.

2.1. The use of invertebrates in ecotoxicological studies

The great diversity of invertebrates and their widespread distribution routinely expose them to various levels of pollutants, providing the rationale for using them as biological models in ecotoxicological studies. This tenet is further backed by the variety of adaptations these organisms exhibit towards the presence of chemical compounds (*e.g.* insecticides and endocrine disruptors; Dixon et al., 2002; Robles-Vargas, 2015). While a significant body of literature over the past several decades employed *ad hoc* chosen organisms, ranging from ciliates and rotifers to crustaceans and polychaetes, gradual standardisation of invertebrate toxicity tests with internationally accepted guidelines and standards has enabled their rapid and widespread application in toxicity assessment of chemicals. The primary guidelines for (eco)toxicity tests and (eco)toxicity testing for freshwater and marine environments are listed in Supplementary Table 1, together with the various recommended parameters and endpoints. Interestingly, a search in Scopus (on June 2020) for publications in the last twenty years with the keyword "ecotoxicology" for the aquatic environment, combined with different invertebrate phyla revealed a high discrepancy regarding their use in ecotoxicology (Fig. 1), with 1913 studies noted for Arthropoda, the most commonly studied phylum (Cladocera, Anostraca, Decapoda and Copepoda; Nebeker and Puglisi, 1974; Verslycke et al., 2007; Ji et al., 2008; Pérez and Beiras, 2010; Sánchez-Bayo, 2011; Leignel et al., 2014; Okamoto et al., 2014; Mesarič et al., 2015; Andreï et al., 2016; Herrmann et al., 2016; Georgantzopoulou et al., 2016; Mehennaoui et al., 2016, 2018; OECD 202 and 211 using *Daphnia magna* and *Daphnia pulex*). Other common invertebrate species used in bioassays (Fig. 1, Supplementary Table 2) are found among Mollusca with 965 entries (*e.g.*, Nogueira et al., 2017; Świacka et al., 2019; Khan et al., 2020), Annelida with 162 entries (*e.g.*, Magesky and Pelletier, 2018; Wallin et al., 2018; Nunes, 2019) and Echinodermata 161 entries (*e.g.*, Nacci et al., 2002; Manzo, 2004; Sugni et al., 2007, 2008, 2010; Pinsino et al., 2008, 2010; Warming et al., 2009; Falugi et al., 2012; Pieterek and Pietrock, 2012; Della Torre et al., 2014; Nobre et al., 2015; Przeslawski et al., 2015; Morroni et al., 2016; Pagano et al.,

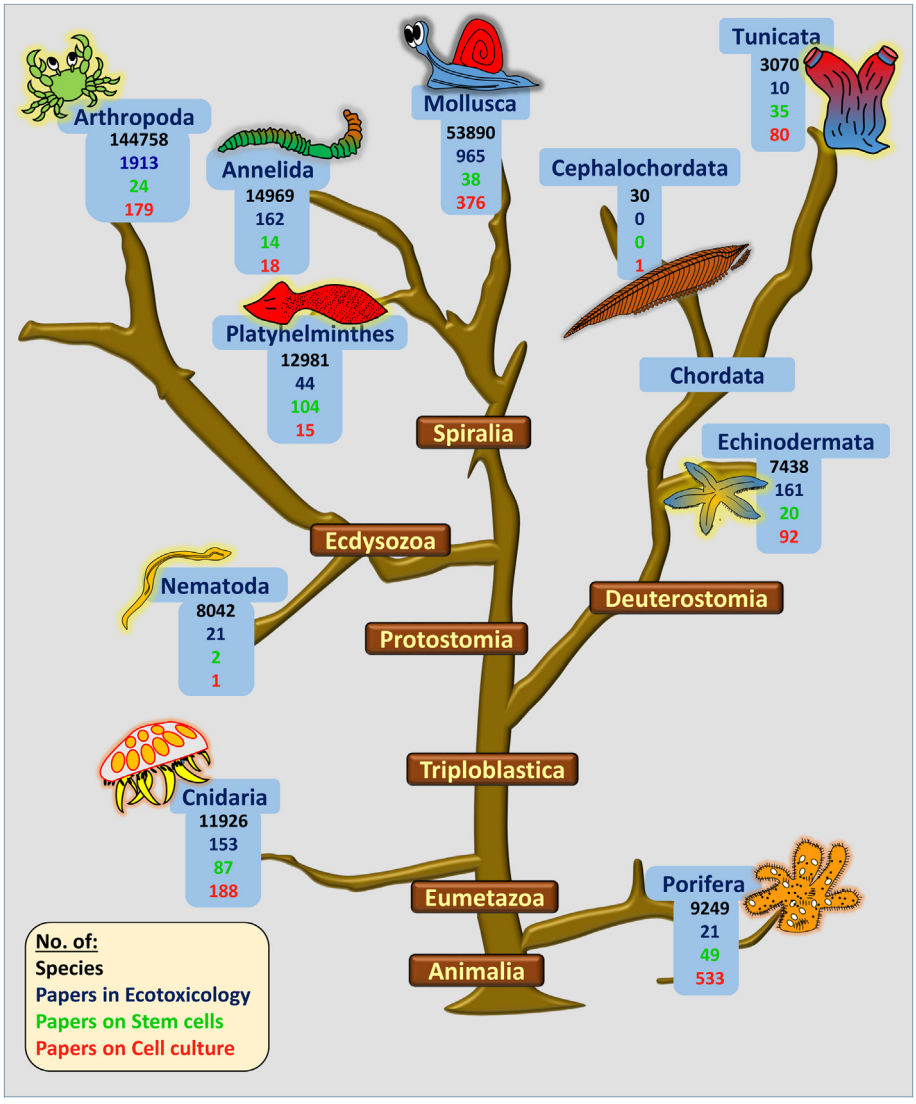

Fig. 1. Phylogenetic relationship between the main invertebrate phyla living in freshwater and marine environments. Number of species in the various phyla or subphyla were mined on June 2020 from WORMS (http://www.marinespecies.org/aphia.php?p=stats) and Balian et al. (2008). Number of articles containing the key words 'ecotoxicology', 'stem cells' or 'cell culture' were mined from Scopus on June 2020, for each of the aquatic/marine invertebrate phyla or subphyla.

2017; Messinetti et al., 2018; Trifuoggi et al., 2019; Parolini et al., 2020; Thomas et al., 2020).

Of the four major invertebrate taxa with species having large pluripotent stem cell populations (*i.e.* Porifera, Cnidaria, Platyhelminthes and Urochordata), cnidarians are the most intensively used in ecotoxicology (153 publications; *e.g.*, Quinn et al., 2008, 2012; Ambrosone and Tortiglione, 2013; Zeeshan et al., 2017; Ballarin et al., 2018). Cnidarians have important ecological roles as predators and prey in planktonic and benthic aquatic ecosystems, and corals also act as reef builders. Much of the ecotoxicological testing with species from this phylum is on hydroids, anemones and corals. *Hydra* is known to be sensitive to various pollutants (Quinn et al., 2012). Also, corals were mostly employed for monitoring the impacts of different pollutants (Flores et al., 2020) as well as the impacts of environmental and anthropogenic drivers on symbiosis (Negri et al., 2005; Rinkevich et al., 2005; Shafir et al., 2009; Cima et al., 2013; Shafir et al., 2014; Svanfeldt et al., 2014; Corinaldesi et al., 2018) due to rising concern for increasingly frequent coral-bleaching episodes.

The most prominent Platyhelminthes species studied in ecotoxicology (44 publications) are the planarians (Wu and Li, 2018). They have three germ layers, simple organ systems and cephalic control of reproduction and behaviour. Planarians are secondary consumers, relatively easily acquired and\or cultivated at low cost. They exhibit a variety of sub-lethal responses and altered biological responses to many mammalian-affecting chemicals, therefore they are recommended as model systems for *in vivo* testing in neuro-, behavioural, reproductive, developmental, cytotoxic, mutagenic and teratogenesis studies (Knakievicz, 2014; Stevens et al., 2014). Planarians also show remarkable regeneration capacity and are used in many tests for comparison of regeneration ability in toxicant-exposed animals *versus* control animals (Ding et al., 2019; Leynen et al., 2019; Rodrigues Macêdo et al., 2019; Gambino et al., 2020). Differences in the range of phenotypic outcomes might be observed between different species of planarians exposed to the same toxicants (Van Roten et al., 2018); however, similar molecular mechanisms might be activated in all the species (such as Tumor Suppressor Genes, TGSs; Van Roten et al., 2018).

Filter feeder sponges have been the object of 21 publications (Fig. 1). Sponges are used for biomonitoring various pollutants like hydrocarbons, organochlorinated compounds, heavy metals, pesticides, and more (Mukherjee et al., 2015).

Among marine invertebrates, ascidians (tunicates; sea squirts) are recognised as evolutionarily significant because their tadpole larva represents a simplified body plan of chordates. Thanks to a number of advantages, ascidians such as *Ciona intestinalis* (currently *Ciona robusta*)

or *Phallusia mammillata* are increasingly used (10 publications) as suitable model systems for toxicological assessments by exploiting their different developmental stages such as embryos, larvae and metamorphosing juveniles (Supplementary Table 2; Bellas et al., 2004, 2005; Mansueto et al., 2011, 2012; Gallo and Tosti, 2013; Lettieri et al., 2015; Navon et al., 2020).

2.2. Toxicological and ecotoxicological endpoints

Currently, the most established sets of environmental assessment methods rely on whole-animal exposures and their survival rates (Supplementary Table 2). Other endpoints such as biochemical and molecular biomarkers have become widely adopted (McCarthy and Shugart, 1990; Forbes et al., 2006; Thomas et al., 2010; Paniagua-Michel and Olmos-Soto, 2016). Commonly tested biochemical biomarkers include: antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase; Ferro et al., 2013, 2018), stress response proteins (*e.g.*, glutathione *S*-transferase, glutathione reductase, metallothioneins, heat shock proteins, multixenobiotic resistance proteins; Franchi et al., 2011, 2012), and markers of oxidative stress damages (*e.g.* lipid peroxidation, protein carbonylation; reviewed in Handy and Depledge, 1999; Jemec et al., 2010; Amiard-Triquet et al., 2012). Furthermore, with the rapid increase of sequenced genomes, aquatic ecotoxicology has moved towards the 'omics' era, opening the new field of ecotoxicogenomics, providing valuable tools for monitoring pollution and understanding the toxicity pathways as well as the adaptive responses of organisms. Transcriptomics, proteomics, metabolomics, and epigenomics are complementary approaches in environmental toxicology, delivering an integrated view of the mechanisms of action of pollutants and changing hydrographical conditions. Transcriptomics is widely used to evaluate the effects of major threats to marine life, like chemical pollution, hypoxia, microplastic pollution, global warming and ocean acidification. In these studies, whole transcriptome profiling of model organisms such as mussel or sea urchin larvae were explored (Jenny et al., 2016; Runcie et al., 2016; Evans et al., 2017; Détrée and Gallardo-Escárate, 2018; Wang et al., 2019). Examples for whole proteome studies are the observed responses to acidification of the cell-free coelomic fluid of the sea urchin *Paracentrotus lividus* (Migliaccio et al., 2019) or of the starfish *Asterias rubens* (Varela-Coelho et al., unpublished results) which enabled identification of 31 impacted proteins, two of which were shown to accumulate at acidic pH: alpha-tubulin involved in cytoskeleton structure, and vitellogenin involved in lipid storage in oocytes. This effect combined with a decrease in the biosynthesis of asterosaponin spawning inhibitors, appears to contribute to an enhanced reproductive ability at acidic pH of starfish *Crossaster papposus* (Dupont et al., 2010). The combined proteogenomic approach is notably of high interest to investigate responses in aquatic invertebrates exposed to stress. A protein sequence database, using a draft genome sequence or RNAseq reads as starting material, can be constructed by a simple *in silico* translation in the six (or three for oriented stranded RNAseq) open reading frames (Armengaud et al., 2014). Although this approach gives many aberrant polypeptide sequences, it can still be used for interpreting shotgun proteomics data resulting in the identification of the different proteins. The potential of this proteogenomic approach is illustrated by a study using the amphipod *Gammarus fossarum* as sentinel species to monitor the quality of freshwater (Trapp et al., 2014, 2015; Gouveia et al., 2017) as well as by other studies (Tomanek, 2011, 2015; Migliaccio et al., 2019).

Epigenetic biomarkers are new emerging tools that incorporate environmental cues affecting gene expression in individual cell types (Williams et al., 2014). Successful implementation of epigenetic tools in the study of environmental impacts on a range of terrestrial animal models and in diagnostics of various human diseases (Berdasco and Esteller, 2019; Jeremias et al., 2020) highlights the potential for development of similar toolkits for aquatic invertebrates. Indeed, epigenetic studies that have been conducted in bryozoans, polychaetes, mollusks and copepods (Suarez-Ulloa et al., 2015), demonstrating that exposure to toxins and other environmental stressors may cause specific alterations of their epigenetic signature as in other model animals (Eirin-Lopez and Putnam, 2019). Such studies imply that identification and designing of aquatic invertebrate epigenetic biomarkers are within reach.

A significant drawback of existing ecotoxicity approaches is that biological models show distinct inter-phyla and intra-phylum sensitivities to pollutants (Supplementary Table 2), thus posing the question about which of these should be considered 'gate-keeper' species (Chaumot et al., 2014). This variability highlights the importance and necessity of conducting a battery of tests across species and endpoints to consolidate the toxicity profile of various substances. Moreover, in order to become suitable for field biomonitoring, the selected models should ideally be simple, robust and sufficiently sensitive to contaminant exposure (Hook et al., 2014). Bearing in mind these last points, new approaches based on *in vitro* systems should enable read across by simultaneously testing cells from several representative species as alternative/additional methods to study the effects of environmental stressors.

3. State-of-the-art on *in vitro* approaches in aquatic invertebrate ecotoxicology

3.1. Aquatic invertebrate in vitro systems

In vertebrates, insects and plants, cell cultures are routinely used as important tools in a variety of scientific practices including ecotoxicology, encouraging the same rationale to be implied for marine invertebrates' ecotoxicology (Rinkevich et al., 1994). *In vitro*, *in silico* and microfluidics-based "organ on-chip" alternatives to *in vivo* toxicity testing are being promoted (Burden et al., 2014, 2015a, 2015b; Scholz et al., 2013), for minimizing the sampling of whole organisms for testing (*e.g.*, Downs et al., 2016). *In vitro* approaches not only satisfy ethics requirements, but may also be cheaper, less time consuming, less prone to inter-individual variability and allow simultaneous cross-species assessments, particularly if standardised protocols can be developed for selected endpoints and can be transferred across laboratories. In addition, *in vitro* cell cultures represent simplified biological models in controlled conditions, allowing testing of the effects of specific chemicals without the impact of other environmental influences, or life history traits. This particular point is important in situations where exposure to low concentrations of pollutants might have beneficial-biostimulatory effects or might induce resistance to those pollutants in future encounters while high concentration of same stressor have harmful or even life-threatening effects (known as Arndt-Schulz law; Stebbing, 1982). Indeed, these biphasic dose-responses known also as hormesis effects have been recorded in several invertebrates (Calabrese and Mattson, 2011; Saggese et al., 2016), and have been successfully tested using human cell lines in various *in vitro* studies (Iavicoli et al., 2018). This last point also suggests that the hormesis effects might be tested with aquatic invertebrates' cell cultures to compare different organisms without *in vivo* experiments. Indeed, those cell cultures allow acute (high doses, over short-term: hours) and chronic (low doses, over long-term: days or weeks) exposures for toxicity testing of chemicals that can be performed before executing *in vivo* dose-effect validation steps which are still required (OECD, 2018). The timeframe of possible exposure periods depends however on the stability of cell culture parameters which have to be evaluated over time and across subcultures (successive rounds of cultures). This stability requirement is best met by continuously proliferating cell lines, and thus represents a technical bottleneck for marine/freshwater invertebrate cell cultures for which only primary cell cultures (of low proliferation and limited lifespan) are currently available (see below). However, those miniaturised approaches have the potential to cope with the needs of aquatic toxicity assessment of tens of thousands

Table 1
List of biomarkers and bioassays recommended or incorporated in national environmental monitoring programmes worldwide based on marine invertebrates (Davies and Vethaak, 2012; HELCOM COMBINE, 2014; Lehtonen et al., 2014; OSPAR Commission, 2013; Viarengo et al., 2007).

	Organisational level	Bioassay name/biomarker name		Measured response/endpoint	Responsive tissue and species	Evaluation criteria and guidelines	Pollutant	Status and regional sea convention	Ecological relevance
Subcellular response	Nucleic acids	Comet assay	Genotoxicity	DNA damage (DNA strand brakes)	Haemocytes, gill and digestive gland cells – mussels	Provisional, harmonisation needed	Genotoxins (carcinogens and mutagens)	Additional OSPAR 2nd tier UNEP MAP	
		DNA adducts	Genotoxicity	DNA damage (adducts)	Gill and digestive gland cells – mussels	Not available	Carcinogens	Optional OSPAR	
		Micronuclei	Genotoxicity	DNA damage	Haemocytes, gills - mussels	Yes (region specific)	Aneugenic/clastogenic (genotox.)	Implemented – core - OSPAR 2nd tier UNEP MAP Suggested as core - HELCOM	
Cellular response	Lysosomal responses	Lysosomal membrane stability NRR/cryostat sections	General stress	Lysosomal alterations	Haemocytes/digestive gland - mussels	Yes	Not specific	Implemented – core - OSPAR, UNEP MAP Suggested - core - HELCOM	
		Lipofuscin	General stress	Lysosomal alterations	Digestive gland mussels		Not specific	2nd tier UNEP MAP	
		Neutral lipids	General stress	Lysosomal alterations	Digestive gland mussels		Organic chemicals	2nd tier UNEP MAP	
		Lysosomes/cytoplasm ratio	General stress	Lysosomal alterations	Digestive gland mussels			2nd tier UNEP MAP	
		Lysosomal enlargement	General stress	Lysosomal alterations	Mussels	YES		Implemented - core – OSPAR	
		Peroxisome proliferation	General stress	Peroxisome proliferation	Digestive gland mussels			2nd tier UNEP MAP	
		Total Oxyradical Scavenging Capacity - TOSC	Resistance to oxidative stress	Absorbance capacity of oxyradicals	Digestive gland mussels			2nd tier UNEP MAP	
		Lipid peroxidation/MDA	Oxidative stress	MDA level increase	Gills, digestive gland			2nd tier UNEP MAP	
		Metallothioneins	Metal exposure	MTs content increase	Digestive gland -mussels	Provisional (region specific)	Metal exposure	Additional OSPAR 2nd tier UNEP MAP	

	Enzymatic activity	AChE	Neurotoxicity/general stress	Enzyme inhibition	Gills	Provisional (region specific) intercalibration needed	Organo phosphorous pesticides, carbamate pesticides, heavy metals	Implemented – core - OSPAR 2nd tier UNEP MAP Candidate-HELCOM	
		Catalase, SOD, GPx	Oxidative stress	Enzyme activity increase	Gills, digestive gland, haemolymph		Not specific	2nd tier UNEP MAP	
		GST	Biotransformation of organic xenobiotic/oxidative stress	Enzyme activity increase	Gills, digestive gland		Organic chemicals/Not specific	2nd tier UNEP MAP	
Tissue response	Tissue pathology	Digestive tubule thickness & atrophy	General stress	Tubule thickness, atrophy, cell types	Digestive gland - mussels	Yes		Implemented - core – OSPAR	
		Haemocytes infiltration	General stress	Inflammation	Mussels - haemocytes			Implemented - core – OSPAR	
		Cell aggregates	General stress	Inflammation	Mussels – brown cells, granulocytes	Yes		Implemented - core – OSPAR	
Organism	Reproduction	Reproductive success	General stress		Amphipods			Suggested - core - HELCOM	
		Imposex	General stress		Marine gastropods *Nucella lapillus*, *Litorrina littorea*		Organotin compounds	Implemented – core – HELCOM, OSPAR	
	Physiology	Stress on stress (SoS)	General stress	Mortality	Bivalves		Not specific	Implemented - core – OSPAR, UNEP MAP	
		Scope for growth	General stress	Energy status (energy for growth)	Bivalves			Additional OSPAR	
In vivo bioassays: Whole organism response	Early life stages (embryo, larvae) tests	Sediment, seawater elutriate and pore-water bioassays	Toxicity of env. matrices	% normal larvae, size increase	Bivalve D-larva; Sea-urchin pluteus larva	No	Not specific	Optional OSPAR	High
				Mortality	Copepods (*Tisbe*, *Acartia*), mysids (*Siriella*, *Praunus*), and decapod larvae (*Palaemon*)	Yes (for Tisbe)		Optional OSPAR Suggested - core - HELCOM	High
		Whole sediment bioassays	Toxicity of sediment	Mortality	Amphipods (*Corophium* spp.) and *Arenicola marina*	Yes	Not specific	Optional OSPAR	High
		Water	Toxicity of sea water	Mortality, % normal development, % net response, larval length	*Tisbe battagliai* larvae, bivalve embryo, sea urchin embryo, *Nicotra*, *Dinophilus*	Yes	Not specific	Optional OSPAR	

HELCOM: Helsinki Convention: Cooperative Monitoring in the Baltic Marine Environment.
UNEP MAP: United Nations Environment Program: Mediterranean Action Plan.
OSPAR: Oslo and Paris Conventions: Convention for the Protection of the Marine Environment of the North-East Atlantic.

of emerging synthetic chemicals and residues of anthropogenic compounds (sunscreens, microplastics, nanoparticles, industry byproducts, municipal effluents and agriculture runoff *etc.*; Slotkin et al., 2016, 2017; Bernhard et al., 2017; Tan and Schirmer, 2017) and are proposed to be employed for HTS systems. Cell culture from aquatic invertebrates (reviewed by Rinkevich, 1999, 2005, 2011) would indeed offer a large number of opportunities for *in vitro* toxicity tests by: (i) pre-validating cell-based toxicity tests with multiple biological endpoints (Liu et al., 2017); and (ii) identifying signal transduction pathways affected by the chemicals. *In vitro* approaches can thus be used as a first phase of a standard strategy to reveal the potential impacts on a target organism (*e.g.* heavy metals; Kamer et al., 2003). In other cases, cell culture-based bio-sensing techniques have been used for real-time monitoring aiming to detect toxicity of different classes of substances in water (reviewed in Tan and Schirmer, 2017). For instance, established fish cell lines were used for assessment of genotoxicity while screening water effluents and sediment extracts (Kamer and Rinkevich, 2002; Avishai et al., 2002; Castaño et al., 2003; Bols et al., 2005; Rakers et al., 2014; Rehberger et al., 2018). Additionally, new chip-based technologies to cope with aquatic ecotoxicological issues are currently being designed (Campana and Wlodkowic, 2018).

While *in vitro* approaches support the implementation of new regulations (*e.g.*, bans on animal testing, 2013; REACH regulations, 2006; the 3Rs principle; Burden et al., 2015c), replacing of animal tests with just a single *in vitro* alternative may not provide the foreseen outcomes, thus, the use of several *in vitro* models is encouraged to overcome this limitation while reducing the number of *in vivo* tests (Scholz et al., 2013). Replacement of *in vivo* by *in vitro* tests may be validated only if the results are correlated (Rodrigues et al., 2019). The promising concept of "adverse outcome pathways (AOP)" links mechanistic responses at cellular level with the effects at whole organism, population, community and potentially ecosystem levels. Practical application of AOPs will require the identification of key links between responses, as well as key indicators, at different levels of biological organization, ecosystem functioning and ecosystem services (Connon et al., 2012). International networks like SEURAT-1, Euroecotox and AXLR8 have been established in order to coordinate the standardisation of such alternative approaches. As a result, many *in vitro* bioassays based on vertebrate cells or bacteria (Calux, Microtox, *etc.*) are currently used in biomonitoring of aquatic pollutants, attesting to the potential for integration of invertebrate *in vitro* systems alongside whole organism tests (Table 1).

3.2. Aquatic invertebrate primary cell cultures

Aquatic invertebrate primary cell cultures have been established from different tissues of various organisms (reviewed by Rinkevich, 1999, 2005, 2011) such as: (i) regenerating and differentiated tissues of cnidarians (from sea anemone tentacles, Barnay-Verdier et al., 2013, Ventura et al., 2018; from ectodermal monolayers, Rabinowitz et al., 2016; from polyp tissue fragments of scleractinians, Domart-Coulon et al., 2004, Vizel et al., 2011, Lecointe et al., 2013; from apical fragments of octocoral colonies, Huete-Stauffer et al., 2015; from scyphozoans mesoglea, Frank and Rinkevich, 1999); (ii) tissue explants or dissociated cells from sponges (Pomponi et al., 1998; Rinkevich et al., 1998; Sun et al., 2007; Müller and Müller, 2018; Conkling et al., 2019), ctenophores (Vandepas et al., 2017) and corals (Frank et al., 1994; Helman et al., 2008; Mass et al., 2012; Drake et al., 2017); (iii) cultures from embryonic/larval stages and different organs from marine and freshwater bivalves and gastropods (Nogueira et al., 2013; Yoshino et al., 2013); (iv) various shrimp (Decapoda, Arthropoda) cell types (Jayesh et al., 2012); (v) regenerating organs of echinoderms (Odintsova et al., 2005); (vi) tunicate buds (Rabinowitz and Rinkevich, 2005, 2011; Rinkevich and Rabinowitz, 1997) and zooids; and (vii) nervous system cells from ascidian larva (Zanetti et al., 2007). Fig. 2 highlights *B. schlosseri* primary cultures established by several methods (dissociated cells, bud/zooid explants or blood cells) that were used and studied by various techniques.

Circulating cells from aquatic invertebrates (haemocytes or coelomocytes and their differentiating precursors) (Ladhar-Chaabouni et al., 2017) are frequently used in *in vitro* toxicity tests based on several parameters (*e.g.* viability, phagocytosis, ROS production and lysosomal membrane stability) (Cima et al., 1998; Cima and Ballarin, 1999; Matozzo et al., 2002a, 2002b, 2003, 2012, 2014; Matozzo and Marin, 2005; Matozzo and Ballarin, 2011; Söderhäll et al., 2003; Hartenstein, 2006; Franchi and Ballarin, 2013; Franchi et al., 2017; Munari et al., 2014; Marisa et al., 2015; Ladhar-Chaabouni and Hamza-Chaffai, 2016), as they are easily sampled and adhere to plastic and glass culture dishes. Haemocytes can be used alone or in the form of a feeder layer for polarised epithelial cells, such as in the case of molluscan cephalopod haemocytes used as a feeder layer for glandular cell types from the sepiolid nidamental gland (Domart-Coulon, unpublished data). Examples for typical toxicity tests based on haemocytes include: (i) *in vitro* assay based on haemocytes primary cells from the freshwater mussel *Dreissena polymorpha* used to test the effects of ecotoxin-exposure and other stressors on innate immune function (Galloway and Depledge, 2001); (ii) ascidian haemocytes used to assess the toxic effects of various antifouling compounds and define their mechanism of action at the cellular level (Cima and Ballarin, 2004, 2012, 2015; Cima et al., 1995, 2008); (iii) ecotoxicological tests performed on haemocytes and gill cells of the molluscan *Haliotis tubeculata* to evaluate triclosan, an antibacterial agent commonly detected in natural waters and sediments (Gaume et al., 2012); (iv) tests on *Crassostrea gigas* oyster cells to study short-term acute stress (<24 h) of a mixture of 14 pesticides (Moreau et al., 2014); (v) use of *Mytilus galloprovincialis* haemocytes to demonstrate synergistic interactions between toxic chemicals (Moore et al., 2018) or the impacts of seawater acidification and emerging contaminants (Munari et al., 2019); and (vi) use of *M. galloprovincialis* haemocytes to evaluate the effects of nanoplastics on immunity and the microbiota (Auguste et al., 2020). Primary cultures of sea urchin coelomocytes were also used to test the effects of $CdCl_2$ and UV-B on HSP70 expression (Matranga et al., 2005).

In spite of all these efforts to establish cell cultures, immortalized cell lines of aquatic invertebrates still do not exist (Rinkevich, 2011; Grasela et al., 2012). However, several biomarkers in ecotoxicology have been promoted based on the use of primary cultures (not cell lines). One of the earliest *in vitro* assays used snail (terrestrial) tissue culture and was developed almost 40 years ago (Bayne et al., 1980a, 1980b); it consists of an *in vitro* cell-mediated cytotoxicity (CMC) assay monitoring parasite-host interaction using co-cultures of sporocyst and haemocytes from snails. This assay was extensively used to investigate basic cellular and molecular mechanisms of immune recognition, and haemocyte effector function in a host-parasite system (Bayne, 2009; Yoshino and Coustau, 2011; reviewed in Yoshino et al., 2013). Cell viability in cell culture (monitored by neutral red assay, MTT assay or trypan blue exclusion assay) is another approach used to evaluate cytotoxicity of various compounds (Domart-Coulon et al., 2000; Mamaca et al., 2005; Katsumiti et al., 2018; Downs et al., 2014, 2016). As environmental contaminants can interfere with lysosomal integrity and reactions, initiating or amplifying features preceding cell death, loss of lysosomal membrane integrity and other lysosomal-related tests are also used as early indicators for pollutant impacts in various taxa of invertebrates like annelids, mollusks and crustaceans (Moore et al., 2006). Two additional innovative assays of cell toxicity were set by Downs et al. (2010) and used in corals. The first assay is based on 3, 3′ dimethyl naphthoxacarbocyanine iodide (JC9), and its sibling dye JC1, which are used to monitor the condition of mitochondrial membrane potential. This assay is an indirect biomarker for mitochondrial ATP production. The second assay comprises the use of Acridine orange 10-nonyl bromide (NAO) for quantification of mitochondria per cell. Genotoxicity is another ecotoxicological field that is commonly studied through *in vitro* ecotoxicology approaches (primarily the use of the comet assay, Mitchelmore and Hyatt, 2004; Mamaca et al., 2005, Akpiri et al., 2017; Sahlmann et al., 2017; or the micronucleus test, Bolognesi and

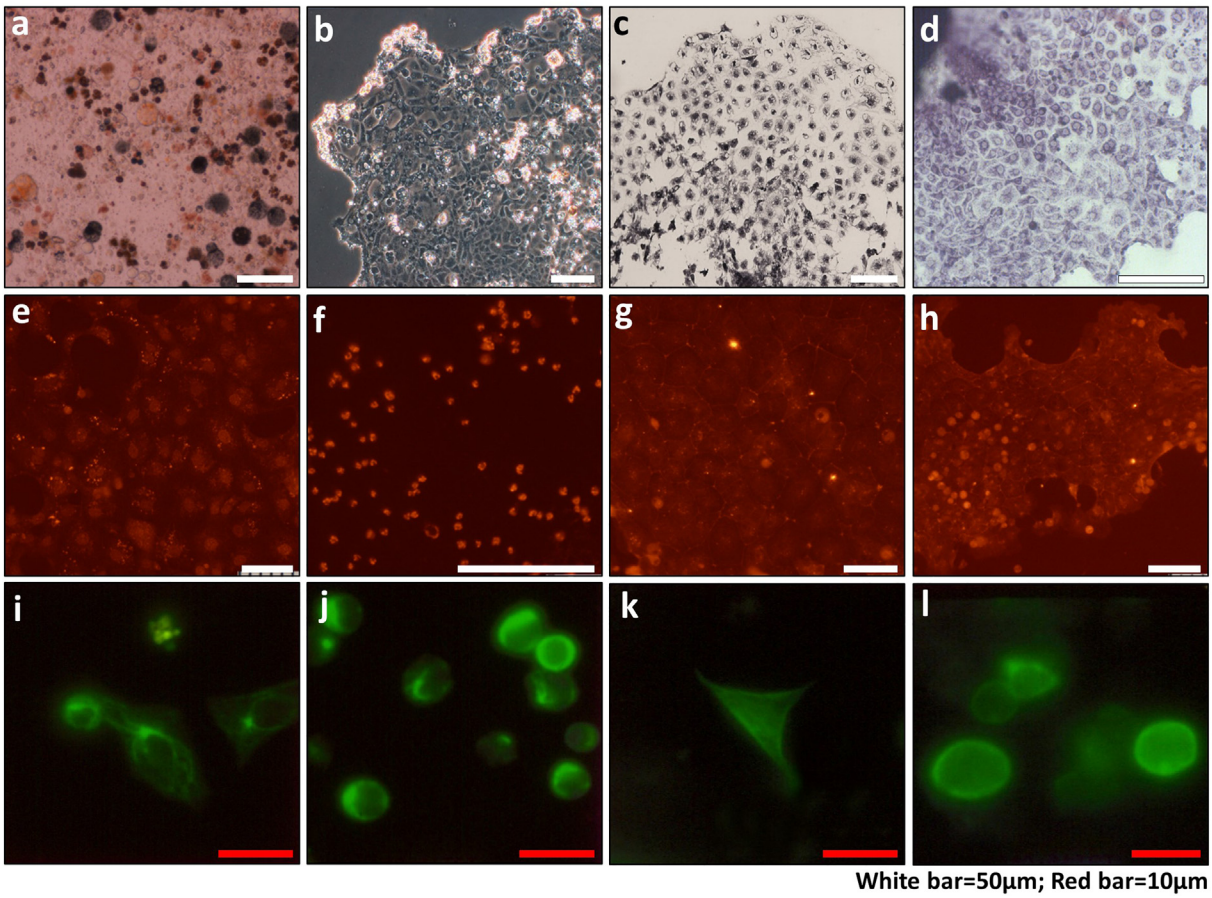

Fig. 2. Primary cell cultures established from *Botryllus schlosseri:* (a) dissociated cells from whole colony after 2 h of incubation in culture media; (b) 9 day old culture epithelial tissue established from bud explant observed with Olympus IX70 under phase contrast conditions; (c) expression of Mortalin mRNA in 9 day old bud epithelial monolayer; (d–g) Expression of proteins in epithelial monolayers originating from 9- to 14-day-old cultured blastogenic stage D buds explants; (d) staining with AP-conjugated anti actin antibody; (e) staining with cy3-conjugated anti-phospho Smad1/Smad5/Smad8 antibody; (f) staining with cy3-conjugated anti-beta catenin antibody; (g) staining with cy3- conjugated anti-phospho-Mek antibody; (h) cells established from a zooid explant stained with cy3-conjugated anti-phospho-Mek antibody; (i-l) impacts of *Botryllus schlosseri* haemocytes exposure to tributyltin (TBT) as revealed by immunohistochemical staining with cytoskeleton specific antibodies (i) untreated haemocytes stained with Alexa Fluor dye conjugated anti-tubulin antibodies; (j) haemocytes exposed *in vitro* to 10 µM TBT for 60 min and stained with Alexa Fluor dye conjugated anti-tubulin antibodies; (k) untreated haemocytes stained with Alexa Fluor dye conjugates of phalloidin (specific for actin); (l) haemocytes exposed *in vitro* to 10 µM TBT for 60 min and stained with Alexa Fluor dye conjugates of phalloidin.

Hayashi, 2011) in various cell types from different tissues (haemoblasts, gills, digestive gland, sperm and embryonic cells) and a wide range of invertebrates (cnidarians, platyheminthes, bivalve, mollusks, annelids, arthropods and echinoderms). Further research in the field focuses on DNA repair after induced genotoxicity (Gajski et al., 2019; Svanfeldt et al., 2014).

Other biomarkers often used in *in vitro* studies are those that address biological/physiological processes, *e.g.*, gene expression (Pfeifer et al., 1993), coral calcification (Domart-Coulon et al., 2001; Mass et al., 2012), cell proliferation and developmental biology (Lecointe et al., 2013; Rabinowitz et al., 2009, 2016), cellular stemness (Rabinowitz and Rinkevich, 2011), and cellular and protein damage (Ventura et al., 2018). Additional ecotoxicological approaches are based on neurotoxicity assessed by acetylcholinesterase activity (Brown et al., 2004), metabolic impairment measured by total haemolymph protein (Auffret et al., 2006) or upregulation of biotransformations and enzymatic detoxification pathways (*e.g.*, CYP450) in cells isolated from invertebrates. An example for the latter studies includes the scallop *in vitro* cell culture model which was validated for pollution monitoring by studying the presence and induction of phase II detoxification enzymes such as glutathione *S*-transferase (Le Pennec and Le Pennec, 2003). Some of these biomarkers are recommended by various environmental programs (OSPAR, HELCOM, MEDPOL) for assessment of damage to cellular, genetic and subcellular components and used simultaneously for assessment of various mechanisms of toxicity (Katsumiti et al., 2018).

Additional relevant tests for applying aquatic cell cultures in ecotoxicology might be adapted from the current practices with mammalian cell lines. An example of such a test might be the scrape/loading dye transfer bioassay (Babica et al., 2016), measuring changes in cell-cell communication and now used in environmental toxicology (Gingrich et al., 2021). However, such an assay needs confluent monolayers of epithelial cells which is a technical barrier as most aquatic invertebrate cells established in primary culture do not grow enough to reach confluence. Such an assay also needs a better characterisation of the nature of cell-cell junctions in each taxon of aquatic invertebrates. Therefore, adaptation of such methods for aquatic invertebrates needs additional preliminary experiments as obtaining confluent monolayer or finding other suitable alternatives in case of the scrape/loading dye transfer bioassay.

3.3. Drawbacks on the use of primary cell cultures from aquatic invertebrates in ecotoxicology

Among the thousands of different cell lines that are available from 150 species, the most abundant are from insects, fish, mice and humans (http://www.attc.org/). In spite of intensive ongoing efforts, stable and well characterised cell lines from aquatic invertebrates have not yet been established (Rinkevich, 1999, 2005, 2011), and this gloomy status

is further highlighted by clustering the different cell repository types (Table 2). Major limitations in establishing cell lines from marine invertebrates are associated with the common contamination states of primary cultures with associated and symbiotic bacteria and protists (Rinkevich, 1999; Mo et al., 2002; Rabinowitz et al., 2006; Grasela et al., 2012; Clerissi et al., 2018). The lack of detailed knowledge on *in vitro* requirements for most aquatic invertebrate cell types and the failure of most invertebrate primary cultures to continue division 24–72 h post cell isolation from initial organism has become a bottleneck. Nonetheless, some encouraging advances have been recently achieved for a sponge cellular model (Conkling et al., 2019), and cells derived from sea anemone regenerating tentacles (Ventura et al., 2018), cell cultures for which cryopreservation has been also successfully performed (Munroe et al., 2018; Fricano et al., 2020). Five reviews on marine invertebrate cell cultures (Rinkevich, 1999, 2005, 2011; Mothersill and Austin, 2000; Cai and Zhang, 2014) and the data presented in Fig. 1 assessed >1000 peer-reviewed publications (Fig. 1; Porifera 533, Mollusca 376), revealing the need to focus on cell culture methodologies in lieu of applied studies. Indeed, we still lack vital information regarding aquatic invertebrate cell requirements *in vivo* before we turn to *in vitro* approaches, and detailed knowledge on *in vitro* requirements for cell types of specific taxon of marine invertebrates is fragmented, requiring much guesswork (Grasela et al., 2012). This emphasizes the needs for interdisciplinary approaches to elucidate the conditions for long-lasting *in vitro* methodologies for marine invertebrate cells.

Clearly, a major requirement is standardisation in aquatic invertebrate experimental systems, and this is achievable by employing tests on fewer selected model organisms and by standardisation of *in vitro* protocols across laboratories (Piazza et al., 2012; Hudspith et al., 2017; Knapik and Ramsdorf, 2020).

4. Mammalian stem cells as a promising tool in (eco)toxicology - what can we learn from mammalian stem cells and how to translate this knowledge to aquatic invertebrate ASCs

The use of mammalian stem cells in toxicology is already an established field. Stem cell-based toxicity tests combine the advantages of an *in vitro* system with conservation of *in vivo* characteristics, and the ability to differentiate into any type of cell (Liu and Zheng, 2019). Stem cells are derived from healthy individuals and retain phenotypically and physiologically normal features during numerous subcultures. Furthermore, they support genome editing, including integration of a fluorescent protein, knock-down of specific genes and introduction of tags that are passed to all the cells that differentiate from them (Drubin and Hyman, 2017). The capacity for self-renewal and pluripotency as the main characteristics of stem cells (Slack, 2018) and additional traits like lower apoptotic threshold, enhanced DNA repair activity, and efficient antioxidant defence (Stevens et al., 2018) makes their behaviour different from other cell lines in various toxicological tests (Nagaria et al., 2013). Three kinds of stem cells are used in toxicology tests: pluripotent embryonic stem cells (ESC; Wnorowski et al., 2018), induced pluripotent stem cells (iPSC; Yamanaka and Blau, 2010; Yu et al., 2007) and adult stem cells (ASC; Slack, 2018). iPSCs and ESCs isolated from similar genetic background have similar traits like gene transcription levels, surface markers and morphology (Narsinh et al., 2011) and can differentiate into all types of cells including gametes (Takahashi and Yamanaka, 2006). ASCs of mammals are multipotent or unipotent cells that exist *in vivo* in postnatal organisms in niches located within various organs. Besides their lower differentiation potency, ASCs' disadvantage in toxicology is that they are isolated from diverse tissues by different protocols, which may lead to inconsistent responses (Wnorowski et al., 2018).

Stem cell-based toxicity assessment tools need accompanying technologies for obtaining and maintaining stem cells and to validate their stemness. Innovations like stirred suspension bioreactors that facilitate large-scale production of cells, and hydrogel-microencapsulation that promotes cell expansion while remaining in a pluripotent state, have advanced stem cells exploitation as model systems. Detailed and validated protocols to induce differentiation of stem cells into the various precursors and lineages are being established (Liang et al., 2019) and are indispensable parts of the various tests. The most widely accepted *in vitro* stem cell-based cytotoxicity test is the embryonic stem cell test (EST) that uses D3 mouse ESCs (mESCs) and mouse 3T3 fibroblasts cell lines. Toxicity is established by calculating IC50 (median inhibitory concentration) using the MTT assay and ID50 (the inhibition of differentiation of half ES cells into cardiomyocytes) of cardiogenic differentiation of the D3 mESC line (Spielmann et al., 1997). Additional EST based protocols have been validated by the European Union Reference Laboratory (EURL-ECVAM; https://eurl-ecvam.jrc.ec.europa.eu/aboutecvam/archivpublications/publication/Embryotoxicity_statements.PDF/view) as alternatives to animal testing (Seiler and Spielmann, 2011; Liu et al., 2017). The Adherent Cell Differentiation and Cytotoxicity test (ACDC) based on mESC cells differentiating into cardiomyocytes was adopted by the US Environmental protection agency (EPA) for screening environmental pollutants and ESNATS (Embryonic Stem-cell-based Novel Alternative Testing Strategies; www.esnats.eu) funded by the European Commission validated hESCs (human embryonic stem cell) based assays for predicting toxicity. Additional national programs, like the US EPA's ToxCast, encouraged the identification of metabolic and regulatory pathways of hESCs affected by chemicals (Kleinstreuer et al., 2011). Altogether, mammalian stem cells were successfully implemented in ecotoxicological assessments (Dong et al., 2018; Gliga et al., 2017; Hodjat et al., 2015; Sirenko et al., 2017; Yin et al., 2015; Worley and Parker, 2019). These assays showed that environmental chemicals may affect various stem cell features like the capacity for self-renewal, differentiation and transformation. They may also induce cellular senescence by various mechanisms at either cellular or molecular levels through the induction of oxidative, genomic, proteomic or epigenetic changes.

The latest advances in the field of stem-cell based toxicity assessment include engineering of stem cell-based 3D constructs to mimic internal organs. Thus, 3D constructs that mimic the development of the brain or reproductive system have been successfully used to test neurotoxicity or endocrine disruptors, respectively (Colleoni et al., 2011; Schwartz et al., 2015; West et al., 2012). State of the art techniques like 3D bio-printing (Gu et al., 2018) and stem cell-based organ–on-a-chip (OOC) that enable interstitial fluid flow that mimics physiological conditions, and simulates human internal organs like the kidney, liver or lungs that are involved in bio-activation and filtration of various environmental toxins, have been developed. Such devices can be interconnected and serve as a model for an entire body (Cho and Yoon, 2017) and have been referred to as a body-on-a-chip device (Wnorowski et al., 2018). However, these methods are not yet broadly used since they still require much more study and field-testing.

Table 2
Overview of commercially available differentiated cell lines[a].

Suppliers[b]	Human	Terrestrial vertebrates	Aquatic vertebrates	Invertebrates (terrestrial)
ATCC	886	370	9	3
DSMZ	654	170	5	14
ECACC	808	624	26	17
IZSBS	123	183	13	2
RIKEN	1540	797	28	18
Total	2859	2050	82	50

[a] No invertebrates stem cells are available among the different repository of cells.
[b] ATCC: American Type Culture Collection, DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen, ECACC: European Collection of Cell Culture, IZSBS: Istituto Zooprofilattico Sperimentale, RIKEN: Riken Cell Bank.

5. Unique properties and reservoirs of ASCs from aquatic invertebrates

In aquatic invertebrates, ASCs participate in a wide range of biological processes including asexual reproduction, regeneration/whole body regeneration, torpor, induction of rejuvenation, and delayed senescence

(Rinkevich et al., 2010; Lehoczky et al., 2011; Wagner et al., 2011; Rinkevich and Rinkevich, 2013; Hyams et al., 2017; Fields and Levin, 2018). In many of these organisms, two types of ASCs should be considered (Rinkevich, 2009). The first are the cells of the germline that act in the somatic environment, independent of the somatic traits that possess the ability to deliver the genetic blueprint of the organism to proceeding generations of stem (or germ) cell lineages. The second reflects the somatic ASC lineages that are capable of tissue homeostasis, repair and regeneration of tissues and organs. In various taxa (*e.g.*, sponges, cnidarians), the boundaries between germ- and somatic- stem cells are blurred as the germ-line is sequestered from somatic cells either late in ontogeny or not completed at all during the lifespan of an organism (Rinkevich, 2009).

While much is known about ASCs and their properties in vertebrates (especially mammals) and some model terrestrial invertebrates (*i.e. Drosophila*), very little has been learned about the nature and properties of ASCs in marine and freshwater invertebrates. It is thus understood that their use in ecotoxicology assays needs consideration of the stemness nature of the chosen ASC type, and for other properties to be elucidated.

The mammalian systems have shown high variations between stem cell populations due to interspecies differences, including morphology, surface antigens and sensitivity to various chemicals due to a variability in epigenetic reprograming, DNA repair and expression of genes, including genes involved in drug metabolism (Krtolica et al., 2009). Similar scenarios may develop when comparing ASCs from aquatic organisms that differ significantly from all types of stem cells studied so far, primarily with the mammalian ASCs. Aquatic invertebrates and mammal ASCs share the properties of long-term self-renewal capability and the ability to differentiate into mature cell types having specific morphologies and function(s) (Cable et al., 2020). Yet, they diverge in many characteristics of which the most prominent are: (a) abundancy- aquatic invertebrate ASCs might constitute up to 1/3 of body mass in some organisms (Handberg-Thorsager et al., 2008; Gentile et al., 2011) while mammal ASCs are rare (Cable et al., 2020), *e.g.*, constituting 1 in 10,000 to 15,000 cells in the bone marrow (Weissman, 2000); (b) potency- aquatic invertebrate ASCs reveal the trait of totipotency, capable to differentiate into all cell types as manifested in whole organisms regeneration, *via* asexual reproduction (*e.g.*, budding) or *via* regeneration from minute fragments (Rinkevich et al., 2007; Bely and Nyberg, 2010; Lai and Aboobaker, 2018) while mammal ASCs are multipotent or unipotent capable to differentiate only into cell types of their tissue of origin (Visvader and Clevers, 2016); (c) germ/soma division- ASCs in some aquatic invertebrate may differentiate into both germ and stem lineages as reported for *Hydra* interstitial cells (I-cells; Hwang et al., 2007). In addition, it is documented that germ-stem cells in aquatic invertebrates may trans-differentiate to somatic ASCs, a phenomenon recorded in some regeneration scenarios (Gremigni and Puccinelli, 1977; Gremigni, 1981). In mammals, ASCs are strictly somatic cells. Germ lineage is sequestered at embryonic stage, and transdifferentiation of cells between soma and germ lineages have not been documented *in vivo*; (d) expression of germ cell markers- aquatic invertebrate ASCs are identified in many cases with the expression of germ cells markers known as Germline Multipotency Program genes (GMP; *e.g.*, PIWI, VASA, PL10 and NANOS proteins; Mochizuki et al., 2001; Shukalyuk et al., 2007; Seipel et al., 2004; Rosner et al., 2009; Rinkevich et al., 2010; Rosner and Rinkevich, 2011; Fierro-Constaín et al., 2017). Expression of these genes were not documented in mammal ASCs; (e) morphology- aquatic invertebrate ASCs may have differentiated cell morphologies that were recorded in various phyla, such as archaeocytes in sponges and amoebocytes in anthozoans (Funayama, 2008, 2018; Gold and Jacobs, 2013). This was not reported for mammal ASCs; (f) location- aquatic invertebrate ASCs are usually not associated with distinct specialised niches. Even when ASCs are detected in temporary niches-like sites in botryllid ascidians (Voskoboynik et al., 2008; Rinkevich et al., 2013; Rosner et al., 2013), a considerable part of their lifespan is in the circulatory system instead of homing into specific sites, and ASCs preserve their stemness characteristics out of the niches. On contrary, ASCs in mammals are associated with specialised anatomical defined niches that absolutely control their fate (Ferraro et al., 2010); (g) carcinogenesis-ASCs in aquatic invertebrates rarely develop neoplastic or age-related diseases (Buss, 1982; Rinkevich, 2000, 2009, 2011; Weissman, 2000; Fields and Levin, 2018), while mammalian ASCs have been directly implicated in carcinogenesis (Barker et al., 2009; Cable et al., 2020).

In most of the aquatic invertebrate phyla ASCs have been identified, though ASCs from early-diverging animal lineages, Porifera, Cnidaria and Platyhelminthes, are the most abundantly and extensively studied phyla (Fig. 1). Planarians exhibit outstanding potential for stem-cell based ecotoxicological assessments that will be described in detail below. Sponges contain at least two types of well-characterised PIWI-expressing totipotent ASCs, archaeocytes (25–30% of total cells) and choanocytes (4–5% of total cells), both of which can differentiate into germ cells (Mukherjee et al., 2015; Funayama, 2018; Ereskovsky et al., 2020). Both the archaeocytes, typified by variable morphology and phagocytic activity, and choanocytes, typified by a flagellum, can turn into motile cells. Some cnidarians, mainly hydroids, are characterised by several stem cell populations. *Hydra* contains three types of stem cell: (i) the pluripotent interstitial (i-cells), stem cells that give rise to several types of cell including germ cells; (ii) mitotic unipotent endodermal epithelial cells; and (iii) mitotic unipotent ectodermal epithelial stem cells (Siebert et al., 2019). Elimination of the interstitial cells by treatments with colchicine results in formation of *Hydra* without nerve cells that can survive in the laboratory indefinitely if regularly force-fed and burped (Tran et al., 2017) indicating that although both endodermal and ectodermal epithelial stem cells can support homeostasis of the epithelial tissues, they cannot de- or transdifferentiate to replace i-cells. Transcriptome profiling of archaeocytes of *E. fluviatilis*, the i-cells of the cnidarian *H. vulgaris*, and the neoblasts of the flatworm *Schmidtea mediterranea* revealed 180 genes (orthologues) shared by these cells, encompassing genes coding for cell cycle, DNA replication and repair; moreover, RNA binding proteins were especially abundant (Alié et al., 2015).

In Mollusca, Annelida and Arthropoda the ASC populations are small but characterised. Bivalvia contain several ASCs/progenitors: haemocyte precursors (Jemaà et al., 2014), stem-like cells situated in the mantle, heart and digestive gland (Vogt, 2012), neurogenic stem cells (Deryckere and Seuntjens, 2018) and germ stem cells capable for both self-renewal and production of progenitors (in *Potamopyrgus antipodarum*; Cherif-Feildel et al., 2019). Other mollusks, such as snails, may reveal more complex states of ASCs population, where both, quiescent and proliferating stem cells circulate in the blood (Rodriguez et al., 2020). In the Annelida, GMP expressing pluri-/multi-potent putative stem cells were identified in the 'segment addition zone' (SAZ), in front of the pygidium (Özpolat and Bely, 2016). Furtherly, the Decapoda (*e.g.*, crayfish) have been found to possess several types of ASCs such as the satellite cells of the heart and musculature, hematopoietic stem cells (Benton et al., 2014), and the E-cells - stem cells located in the distal ends of the tubules constituting the hepatopancreas, the organ associated with detoxification mechanisms in response to exposure to environmental toxins (Vogt, 2020).

Among the Nematoda and Echinodermata, two phyla whose members are frequently used as ecotoxicological models, ASCs were neither identified nor characterised (Fig. 1); Nematoda lack somatic stem cells, they do not possess cells that can de-differentiate (Sköld et al., 2009) and do not show regenerative abilities (Bely, 2010; Cary et al., 2019). In contrast, in Echinodermata, the current theory is that their high regenerative capacity is mainly due to morphallaxis, involving de-differentiation or trans-differentiation of specialised cells without direct evidence of the presence of "stocked" undifferentiated stem cells. A possible exception to this "rule" are Crinoids, where regeneration is achieved mainly by pre-existing undifferentiated stem cells (amoebocytes; Ben Khadra et al.,

2018). Nevertheless, stem cell candidates in Echinodermata have been proposed in the coelomic epithelium of sea cucumber (Mashanov et al., 2017) and starfish (Holm et al., 2008; Sharlaimova et al., 2020), among GMP expressing cells located in the adult rudiment of regenerating sea urchin (Bodnar and Coffman, 2016). However, self-renewal and capacity to differentiate into different cell types was not shown, and therefore, the stemness nature of these cells has yet to be confirmed. Recently, pluripotent PIWI expressing cells were detected in the coelomic fluid cell population in the regenerating holothurian *E. fraudatrix* (Zavalnaya et al., 2020).

Conversely, the late-diverging Tunicata (phylum Chordata) have several types of putative stem cells. In colonial botryllids ascidians three types of ASCs populations have been described: hematopoietic stem cells (Rosental et al., 2018), multipotent epithelia (epidermis and peribranchial origin; Ricci et al., 2016) and the pluripotential haemoblasts (soma and germ lineages; Kawamura and Sunanaga, 2010). The haemoblasts have a round shape, relatively small size (5 μm in diameter), high nuclear/cytoplasmic ratio, prominent nucleoli, comprise 1–2% of the coelomic cell population (Kawamura and Sunanaga, 2010). These ASCs migrate between transient niches in the zooids and buds (Voskoboynik et al., 2008; Rinkevich et al., 2013; Rosner et al., 2013). In solitary tunicates, circulatory stem cells were further identified in *S. plicata* haemolymph and intestinal submucosa that has been proposed as their putative niche (Jiménez-Merino et al., 2019), while in *Ciona intestinalis* PIWI and AP positive stem cell niches are located in the transverse vessels of the branchial sac (Jeffery, 2019) providing the progenitor cells, most likely the haemoblasts, for distal regeneration (Jeffery, 2015).

6. State of art on aquatic invertebrate ASC-based expertise currently used in ecotoxicology

Both the presence of ASCs themselves and regenerative processes can be used as endpoints in stem-based studies of environmental toxicology. Toxicological studies testing direct impacts of environmental pollution on aquatic invertebrate stem cells (*e.g.*, effects of washing soda on archaeocytes, Mukherjee et al., 2015; effects of toxins on mitotic activity of E-cells of the hepatopancreas, Vogt, 2020) are limited. On the contrary, regeneration-related endpoints are valid endpoints used in many tests. Regeneration based endpoints, include among others changes in regeneration efficiency and its duration, and the appearance of teratogenic effects.

6.1. Regeneration as a tool in ecotoxicology

Regeneration is defined as "the ability of adult cells to use some combination of proliferation, migration and differentiation for the purpose of ensuring continued biological function in adult animals" (Lai and Aboobaker, 2018). Regeneration can occur naturally, following stress, or be experimentally induced. The regenerating tissues may be formed from preexisting pluripotent stem cells, or by de-differentiation or trans-differentiation of differentiated cells. In some species, both possibilities may occur. The study of regenerative processes in aquatic invertebrates promises to offer new models to understand the effects of pollutants on organisms and become one of the most significant endpoints to test toxicity (Bely and Nyberg, 2010; Tanaka and Reddien, 2011).

Whole body regeneration (WBR) has been described in Porifera, Cnidaria, Ctenophora, Platyhelminthes, Bryozoa, Annelida, Echinodermata and Urochordata (Bosch and David, 1987; Baguñà et al., 1989; Reddien and Sánchez Alvarado, 2004; Henry and Hart, 2005; Bely, 2010; Bely and Nyberg, 2010; Cary et al., 2019; Rosner et al., 2014, 2019). Other species are capable of a more restricted form of regeneration of amputated body parts or following autotomy (shedding of a body part; Fleming et al., 2007). Autotomy was described in over 200 invertebrate species from Cnidaria, Annelida, Mollusca, Arthropoda and Echinodermata (Fleming et al., 2007). In some animals this might be a mechanism to remove accumulated toxins (Vidal and Horne, 2003). Some organisms have less efficient regeneration capacity, like Arthropods that can replace their appendages incrementally at each molt.

The regenerative capacity of various freshwater and marine species has been used successfully to evaluate toxicity of various environmental pollutants. Particular examples include: i) sponge regenerations, tested following exposure to urban pollution (Zahn-Daimler et al., 1975) and detergent (Zahn et al., 1977); ii) *Hydra* regenerative capacity, used successfully to evaluate the potential toxicity of pharmaceuticals (Pascoe et al., 2003), phenolic chemicals (Park and Yeo, 2012), and nanomaterials (Murugadas et al., 2016). *Hydra* regeneration is also at the centre of a new early warning system for environmental teratogenic threats in running waters (Traversetti et al., 2017); iii) polychaete (Annelida) posterior segment regeneration, used to test impacts of microplastics (Leung and Chan, 2018) and graphene oxide (carbon nanomaterial; De Marchi et al., 2017) while *Lumbriculus variegatus* (Oligochaeta) regeneration was studied following exposure to lead (Sardo et al., 2011); iv) crustaceans (Arthropoda) limb regeneration can occur throughout their lifetime and the cell lineages involved in this process have been characterised by live imaging at single-cell resolution (Alwes et al., 2016). Environmental pollutants may cause retardation of regeneration of limbs (heavy metals, chlorophenols, dithiocarbamates), inhibition of regeneration and decrease in the growth increment per molt (hydrocarbons and dioxins), accelerate regeneration and molting (DDT) or morphological alterations in the regenerated limbs (mercury, cadmium, tributyltin, diflubenzuron; Weis et al., 1992).

Regeneration is not always directly associated with stem cells, as it seems to be the case in corals. Indeed, corals possess high regenerative aptitude which is manifested by their ability to regrow a functional colony from relatively small amounts of living tissue whereas no stem cells have been detected to date (nubbins; Shafir et al., 2001, 2006a, 2006b). This enabled use of different coral species to test the impacts of household detergents (Shafir et al., 2014), crude oil (Shafir et al., 2007) and anti-fouling agents (Shafir et al., 2009). Another example are the Echinodermata that represent a phylum with exceptional regenerative capabilities following autotomy or traumatic injury and capable of reconstruction of both external appendages and internal organs (Candia Carnevali, 2006; Reinardy et al., 2015). Echinodermata regeneration has been attributed to processes like trans- and de-differentiation, and not to the presence of stem cells, and various tests have been developed to assess the impacts of the exposure to pollutants on these types of regeneration. Reinardy et al. (2015) have presented a functional assay to investigate the mechanisms of tissue regeneration and bio-mineralisation, by measuring the regrowth of amputated tube feet (sensory and motor appendages) and spines in the sea urchin. The timing and extent of regeneration of brittle stars following exposure to organotin compounds or feather stars following exposure to PCBs and endocrine disrupting compounds have also been described (Sugni et al., 2007, 2008, 2010). Cephalopods (a molluscan class) also have extensive regeneration capacity of various organs including their syphons (Tomiyama and Ito, 2006), muscles, nerves, or entire appendages (Imperadore and Fiorito, 2018). However, inclusion of cephalopods in Directive 2010/63/EU (Di Cristina et al., 2015), prevent their use in regeneration assays and further strengthens the need for *in vitro* alternatives for other invertebrates that may be banned from such assays in the future.

6.2. Aquatic invertebrates with high abundance of ASCs as models to assess toxicology both in vitro & in vivo: the planarian example

Cell lines of aquatic invertebrates are not available; therefore, the closest alternatives for assessing direct impacts of pollutants on stem cells are on basal invertebrate species with large populations of stem cells such planarians. There are thousands of free-living planarian species, which may be terrestrial, marine or fresh-water dwellers (Reddien and Sánchez Alvarado, 2004). Both the freshwater and marine species, like

Pseudostylochus intermedius, contain large populations of stem cells (Sato et al., 2001), although most of the studies nowadays concentrate on 15 freshwater species. The most distinctive trait making planarians excellent model organisms for ecotoxicology is their stem cells, the neoblasts, which give rise to their enormous regenerative ability (Gehrke and Srivastava, 2016; Reddien, 2018). The pluripotent neoblasts (5–10 μm in diameter) situated within the parenchyma constitute about 20%–30% of adult soma cells and are capable of differentiating into the approximately 40 different cell types found in these organisms. Neoblasts are characterised by the capacity of indefinite self-renewal and expression of GMP genes, of which the most prominent and common are the *PIWI* orthologues. Neoblasts have a special morphology marked by the existence of chromatoid bodies, a large nucleus and high nuclear/cytoplasmic ratio. Neoblasts are the only proliferating cell type in asexual planarians and are sensitive to gamma radiation. Studies demonstrated that neoblasts represent several subpopulations which have been characterised at the level of gene expression (Salvetti and Rossi, 2019), of which only the *sigma* population is capable of self-renewal (Aboukhatwa and Aboobaker, 2015).

Many regulatory mechanisms are shared between planarian and human stem cells: hundreds of genes that are differentially expressed in stem cells relative to differentiated cells; different post-translational regulation *via* alternative splicing leading to expression of different isoforms in stem and differentiated cells (*e.g.*, an interplay between MBNL and CELF proteins that are differentially expressed in stem and differentiated cells; Solana et al., 2016); conserved epithelial-mesenchymal transition (EMT) mechanisms that control stem cell migration (Abnave et al., 2017); Tumor suppressor genes (TSGs) activated following exposure to toxicants (Van Roten et al., 2018); bivalent histone modifications (Dattani et al., 2018); and different roles for genes in stem cells and in differentiated cells (*e.g.*, *P53*; Stevens et al., 2018).

Neoblasts are susceptible to environmental stressors; data point to the importance of DNA repair during long term exposure (Stevens et al., 2018), as well as the influence of the particular niche within the animal on the response of the neoblasts to the stressor stimuli. Moreover, variable sensitivity to genotoxic materials was also detected between homeostatic and regenerating animals. Although some existing reports describe *in vitro* neoblasts cultured for prolonged periods, none of them contain functional and molecular tests to prove their identity and potency (Lei et al., 2019). A newly published paper has shown that neoblast- enriched cultured cells (approximately 60% of the cells being *PIWI* expressing neoblast; Lei et al., 2019) can proliferate *in vitro* and rescue lethally irradiated animals within the first 24 h in culture. Further methodology should be developed for long-term culturing of neoblasts.

7. Aquatic invertebrate ASCs - innovative research directions in ecotoxicology

The implementation of mammalian stem cell platforms in toxicology and ecotoxicology assays may inspire similar approaches (albeit with different reasoning) in aquatic ecotoxicology. Dealing with aquatic invertebrates, inter- and intra- phyla differences of ASC types (*e.g.*, the cnidarians i-cells *vs.* the platyhelminth neoblasts *vs.* the tunicates haemoblasts, or i-cells *vs.* the two epithelial cells of the hydrozoans), and variations in sensitivities to pollutants may lead to the development of research on several archetype ASCs from more than a single key aquatic taxon as illustrated in Fig. 3. While in vertebrates the research has been advanced by the development of laboratory induced stem cell like cellular components (iPSCs and ESCs), their lack in the aquatic invertebrates has led to the consideration of just natural ASCs from marine invertebrates as novel tools for ecotoxicological tests. In some aquatic invertebrate taxa ASCs may reveal unique metabolism and epigenome signatures that are vital for developmental biology phenomena (see below) that are not systematically studied by the ecotoxicological assays currently employed.

The bottleneck in development of aquatic invertebrate ASC-based assessment tools as proposed in Fig. 3 is the lack of permanent cell lines. This obstacle should be removed by concentrating resources and developing international research collaborations. It is thus suggested that aquatic invertebrate ASCs may serve as novel, promising tools in ecotoxicology for the following three classes of needs:

1) In a wide range of freshwater and marine invertebrate taxa, ASCs are major participants and play a key role in developmental biology phenomena like senescence, delayed senescence and longevity (Lauzon et al., 2000; Jemaà et al., 2014; Petralia et al., 2014; Rinkevich, 2017), whole body regeneration (Rinkevich et al., 1995, 2007, 2009; Blanchoud et al., 2018), asexual reproduction including budding, fragmentation, gemmule-hatching, indeterminate growth, fission and torpor phenomena (Rinkevich et al., 1995; Lázaro and Riutort, 2013; Vogt, 2012; Özpolat and Bely, 2016; Hyams et al., 2017; Malinowski et al., 2017; Funayama, 2018; Manni et al., 2019). In addition, ASCs are important in shaping and controlling astogenic processes of many colonial organisms, including cnidarians, sponges and ascidians (Rinkevich, 2002; Hughes, 2005; Rosner et al., 2006; Shunatova and Borisenko, 2020). As such, ASCs are essential not only for 'their classical' roles in tissue maintenance and homeostasis (Singh, 2012; Chua et al., 2020), but are important to the above listed phenomena that include a wide range of responses to environmental and biological cues (*e.g.*, regeneration, torpor, senescence) as life history unique properties (*e.g.*, asexual reproduction, budding, indeterminate growth, fission, astogeny). Environmental cues during these processes may lead to epigenetic alterations (Verhoeven and Preite, 2014; Thorson et al., 2017), that can be monitored in stem cells. Moreover, in vertebrates quantitative and qualitative decline in stem cell number and function following exposure to environmental stressors may lead to stem cell exhaustion resulting in organism aging and death (Ren et al., 2017). This may also relate to aquatic invertebrates, where manipulation of ASCs number and activities may impact the above listed major biological phenomena, a topic that current ecotoxicological assays do not evaluate, primarily on the cellular/molecular biology levels.
2) Germ cell sequestering in the Animalia is manifested through either the establishment of a long-lasting germ cell lineage during the embryonic stage, or through somatic embryogenesis modes of development where no true germ line is set aside (Blackstone and Jasker, 2003; Extavour and Akam, 2003; Rosner et al., 2009). Somatic embryogenesis mode of development not only allows a wider (sometimes over the life span of an organism) ontogenic window for germ line sequestering but also enhances the chances for introducing somatic variants into the germ line (Buss, 1983). In the somatic embryogenesis mode of reproduction, organisms are capable of developing germ cells from somatic ASCs at any ontogenic phase, from birth to death. The literature reveals that a wide range of animals belonging to the placozoans, sponges, cnidarians, platyhelminths, nemerteans, entoproctans, ectoproctans, annelids, hemichordates and urochordates are capable of somatic embryogenesis (Buss, 1982, 1983; Blackstone and Jasker, 2003; Juliano and Wessel, 2010; Dannenberg and Seaver, 2018; DuBuc et al., 2020).
During the life span of an organism with somatic embryogenesis, various stressors, including those considered under the broad title of 'ecotoxicology', may affect all somatic cells including ASCs. Studies on vertebrates and some invertebrates have revealed the impacts of chronic, as well as of mild, pollutants on the organism mutational levels (primarily of carcinogens and mutagens) and those impacting epigenome signatures of cells (Hofmann, 2017; Liu et al., 2017; Rodriguez-Casariego et al., 2018; Eirin-Lopez and Putnam, 2019) some of which may be transmitted to subsequent generations *via* germline-mediated transgenerational inheritance (Vandegehuchte et al., 2010;

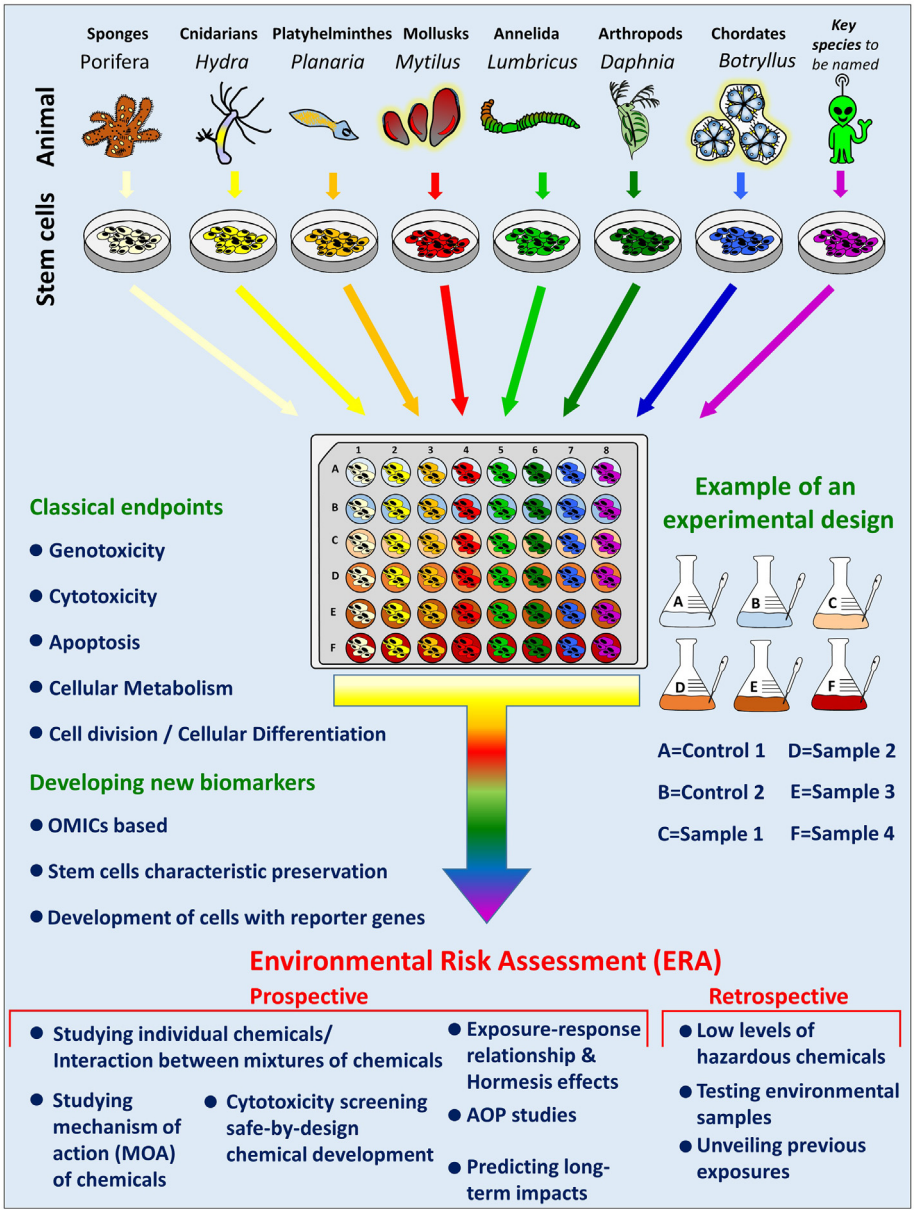

Fig. 3. Schematic representation of a possible HTS test based on marine invertebrates' stem cells. The setup presented in the diagram enables investigation of impacts of test samples on stem cells originating from species from different phyla. Stem cells of different origin might have different sensitivities and responses to pollutants. A battery of various endpoints might be used to analyse the impacts in parallel.

Gapp et al., 2014; Heard and Martienssen, 2014). Epigenetic mechanisms may result in phenotypic plasticity (Thorson et al., 2017) and acquirement of resistance to various toxicants (Rodriguez-Casariego et al., 2018). Epigenetic abnormalities (epimutations) in ASCs may promote phenotypic plasticity resulting in processes affecting not only the organism or the population, but rather the whole ecosystem, such as the impacts on biological invasions (Ardura et al., 2017), an increased disease susceptibility and tissue pathologies (Nilsson and Skinner, 2015), and changes in social behaviour (Wolstenholme et al., 2012). Epimutations can be easily detected in ASCs using 'omics'-based endpoints.

3) As in iPSCs and ESCs cases, the initiation and establishment of ASC lines will provide a toolkit for the establishment of differentiated cells lines which, by advanced protocols, will drive stem cell differentiation to various differentiated lineages. Such differentiated cells lines (currently not available) might be complementary to usage of stem cell lines for ecotoxicological assessments, primarily on pollutants whose impacts are cell/tissue specific (*e.g.*, steroidal oestrogen), as performed by EST tests.

Other innovative research directions in ecotoxicology are associated with environmental risk assessment (ERA), a framework built on successive steps (tiers), aiming to assess the putative adverse effects and set the regulatory acceptable concentrations for chemicals (Queirós et al., 2019; Jeremias et al., 2020). The higher more expensive and time-consuming tiers that involve populations and field tests are used only following lower tiers (laboratory tests) risk assessment. ERA assesses environmental risks of contaminants in a prospective or a retrospective manner. Prospective risk assessment is performed in the context of market authorization of a compound, whereas the retrospective risk assessment is generally aimed to identify the causes of adverse effects that have already occurred (Calow and Forbes, 2003). As prospective assessments are not accurate, the combination of prospective

and retrospective assessments provides an "ecological reality check" (Burton et al., 2012). As a derivative of the unique features of aquatic ASCs, tests based on these cells have a potential to add extra efficiency to both approaches. In prospective RA studies, ASCs based tools may contribute to both low and higher tiers when applied for: (a) predicting long-term impacts (including on offspring); (b) testing individual or mixture of chemicals; (c) testing both acute and chronic toxicities; (d) establishing dose- and time- response curves and hormesis effects; (e) studying of mechanisms of action of chemicals; (f) safe-by-design of chemicals; (g) AOP studies, thus enabling replacing some of whole animal-based experiments. In retrospective RA studies ASCs may also contribute to low and high tiers when used for: (a) monitoring environmental samples contaminated with unknown chemicals; (b) assessing low level of hazardous chemicals; (c) unveiling previous exposures of organisms or their ancestors to chemicals. Successful implementation of these applications necessitates to reinforce the currently available classical endpoints for aquatic *in vitro* studies with additional methods adapted from mammalian *in vitro* studies as well as with new endpoints to be developed (*e.g.*, omics based; Fig. 3). In addition, studies with ASCs should be supplemented with differentiated cell lines, similarly to the mammalian-based EST assays, to assess also pollutant which are cell-type specific (like endocrine-disrupting chemicals).

8. Future prospects and research needs

The use of ASCs is a step forward in (eco)toxicological studies as they represent a promising model in environmental toxicology which supports the AOP concept. "The outcome of research and the resulting philosophy in a scientific discipline is much dependent on the features of the research models" states Vogt (2012) in his paper entitled "Hidden treasures in stem cells of indeterminately growing bilaterian invertebrates". This could be also taken the other way around. Advance of a scientific discipline could generate new research models to better address scientific questions. In particular, this could hold true for ASCs and their potential applicability in environmental toxicology. Once we have appropriate biological model systems, sufficient mode of action data could be generated and we can look for patterns, and from those patterns infer general rules, theory and models.

Due to their lower genetic complexity, aquatic invertebrate ASCs represent a reliable tool for understanding fundamental biological processes, for investigating mechanisms of stress response, toxicity, detoxification, regeneration and adaptive ability. Toxicity assays involving ASCs (*e.g.* epigenome alteration, genotoxicity, immunotoxicity, regeneration impairment and budding capability) can be used to predict the effects of xenobiotics on animals (humans included), especially those used in aquaculture and in fragile ecosystems. In addition, since stressed aquatic ecosystems favour the colonisation by invading alien species (Occhipinti-Ambrogi and Savini, 2003), they can also give valid support for the evaluation of organisms' adaptive capabilities and environmental quality. Invertebrate ASCs can also help in understanding the mechanisms of epigenetic toxicity as they are related to the production of germ cells. As ASCs are often long lived, they must be protected against any damage which means that they possess efficient systems either for damage repair or for damage protection. ASC damage may have serious consequences on an organism, population, community, and ecosystem level. Identifying the effects of environmental stressors, including pollution, on ASCs could yield valuable information on the hazard potential of environmental stressors in environmental toxicology studies. In parallel, there is a need for reporting standards and the proposed Criteria for Reporting and Evaluating Ecotoxicity Data (CRED; Moermond et al., 2016). Reporting standards could be used in ecotoxicity research with aquatic stem cells to improve the reproducibility, transparency and consistency of aquatic ecotoxicity studies in order to facilitate comparisons across different laboratory settings.

Due to methodological problems, the field of stem cell research and its application in marine invertebrates is less developed and the community is scattered. Moreover, another reason is also a great number of taxa (Porifera, Cnidaria, Mollusca, Crustacea, Echinodermata) from which potentially stem cells can originate and must be studied meticulously. There have been symposia oriented towards cell lines and stem cells in marine invertebrates (*e.g.*, Marine Invertebrate Cell Culture Symposium 2012 in Concarneau, France). Some universities have included basic knowledge on stem cells and cell lines into their syllabi (*e.g.*, University of Exeter). Another opportunity for researchers interested for stem cells and cell lines from invertebrates is the Coordinated Research Infrastructures Building Enduring Life-science Services (CORBEL) framework which includes several European Research Infrastructure Consortia (ERIC) offering services on invertebrates, and also includes a database on marine invertebrate models MARIMBA-CORBEL (http://marimba.obs-vlfr.fr/home). A far more developed stem cell community is EuroStemCell (https://www.eurostemcell.org/history-eurostemcell) working on regenerative medicine, representing a consortium of >400 laboratories across Europe. In addition to these, 11 supporting institutions are also included, such as: Karolinska Institute, German Stem Cell Network (GSCN https://www.gscn.org/), Stem Cells Australia (http://www.stemcellsaustralia.edu.au/, DanStem *etc.*). EuroStem covers many aspects of stem cells, and beside researchers it also encompasses ethicists, social scientists and especially science communicators for exact transfer of information to the general public. Currently, the fully dedicated network of researchers to study stem cells in marine invertebrates is the EU COST Action 16,203 MARISTEM (duration from 2017 to 2021, http://maristem.eu/), a network of researchers from 61 institutions from all over the Europe, the Middle East and Russia.

However, further efforts are required to foster collaboration among the various institutions working on aquatic invertebrate stem cells in order to increase the use of invertebrate ASCs in biological (and toxicological) research. In particular, there is a need to overcome the communication and technical problems that up to now have hampered the achievement of stable stem cell cultures from aquatic invertebrates. This is one of the main aims of the EU COST Action 16203 MARISTEM (Ballarin et al., 2018). Research on aquatic invertebrate stem cells requires the identification of more markers, in addition to the classical PIWI, NANOS, VASA *etc.*, in order to distinguish between differentiation levels of the cells in different species so as to allow the identification and isolation/enrichment of totipotent/pluripotent stem cells. This point is directly linked to the problem of de-differentiation/trans-differentiation. In many cases (*e.g.*, starfish regeneration or ascidian palleal budding) we cannot exclude that cells from injured tissues of budding areas de-differentiate and re-acquire a stem cell phenotype able to form a blastema or a bud primordium. In mammals, it has been possible to induce this reprogramming by the addition of the Yamanaka's factors in the culture medium (Okita et al., 2007). In invertebrates, this can occur spontaneously under certain conditions, which requires additional investigation towards a deeper understanding of the phenomenon.

Addressing the above points implies the putting in place of academic politics able to support researchers with a broad education in zoology, marine science and biotechnology and to establish ties with biotechnology and biomedical industries as well as with decision makers, in order to transform research outcomes into guidelines for animal (including human) health and environmental protection. Global problems encompassing diverse aspects such as environmental pollution, changes in global climate, greenhouse effect, ozone depletion, *etc.*, and the awareness of the complexity of ecosystems in environmental policy, clearly indicates the urgent requirement of additional information on biological systems. Consequently, new model systems and new approaches, not only in research but also in the broader paradigm of education, are needed to properly address the challenging environmental problems we face today.

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Declaration of competing interest

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

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