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A broad-taxa approach as an important concept in ecotoxicological studies and pollution monitoring

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ABSTRACT

Aquatic invertebrates play a pivotal role in (eco)toxicological assessments because they offer ethical, cost-effective and repeatable testing options. Additionally, their significance in the food chain and their ability to represent diverse aquatic ecosystems make them valuable subjects for (eco)toxicological studies. To ensure consistency and comparability across

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studies, international (eco)toxicology guidelines have been used to establish standardised methods and protocols for data collection, analysis and interpretation. However, the current standardised protocols primarily focus on a limited number of aquatic invertebrate species, mainly from Arthropoda, Mollusca and Annelida. These protocols are suitable for basic toxicity screening, effectively assessing the immediate and severe effects of toxic substances on organisms. For more comprehensive and ecologically relevant assessments, particularly those addressing long-term effects and ecosystem-wide impacts, we recommended the use of a broader diversity of species, since the present choice of taxa exacerbates the limited scope of basic ecotoxicological studies.

This review provides a comprehensive overview of (eco)toxicological studies, focusing on major aquatic invertebrate taxa and how they are used to assess the impact of chemicals in diverse aquatic environments. The present work supports the use of a broad-taxa approach in basic environmental assessments, as it better represents the natural populations inhabiting various ecosystems. Advances in omics and other biochemical and computational techniques make the broad-taxa approach more feasible, enabling mechanistic studies on non-model organisms. By combining these approaches with *in vitro* techniques together with the broad-taxa approach, researchers can gain insights into less-explored impacts of pollution, such as changes in population diversity, the development of tolerance and transgenerational inheritance of pollution responses, the impact on organism phenotypic plasticity, biological invasion outcomes, social behaviour changes, metabolome changes, regeneration phenomena, disease susceptibility and tissue pathologies. This review also emphasises the need for harmonised data-reporting standards and minimum annotation checklists to ensure that research results are findable, accessible, interoperable and reusable (FAIR), maximising the use and reusability of data. The ultimate goal is to encourage integrated and holistic problem-focused collaboration between diverse scientific disciplines, international standardisation organisations and decision-making bodies, with a focus on transdisciplinary knowledge co-production for the One-Health approach.

Key words: animal model, ecotoxicology, environmental risk assessment, freshwater and marine invertebrates, innovative methods.

CONTENTS

I. INTRODUCTION

Invertebrates account for the largest number of species in marine and freshwater ecosystems. According to current estimates, of the ~6.7 million invertebrate species, 16% (1.1 million) live in marine and 2% (150,000) in freshwater environments, where they have colonised a large spectrum of ecological niches (Collier, Probert & Jeffries, 2016). This colonising ability is closely linked to their vast diversity, accompanied by unique biological characteristics, including possession of large numbers of pluripotent adult stem cells (ASCs; Rinkevich *et al.*, 2022) and the ability to regenerate lost body parts, even up to whole-body regeneration in some cases (Rinkevich *et al.*, 2007*b*). Molluscs and crustaceans are the richest taxa in terms of species numbers in the marine environment (Appeltans *et al.*, 2012), while insects are the richest invertebrate taxon in freshwater habitats. Aquatic invertebrates perform critical ecological functions in all aquatic ecosystems (Palumbi *et al.*, 2009; Macadam & Stockan, 2015). They provide a range of ecosystem services, including filtering water (Ostroumov, 2005), processing organic matter (Bart *et al.*, 2020), ecosystem engineering (Howell *et al.*, 2016; Angiolillo & Canese, 2018), recycling nutrients (Lohrer, Thrush & Gibbs, 2004; Atkinson *et al.*, 2017), participating in the carbon cycle (Tue *et al.*, 2017), and playing key roles in mitigating natural risks [e.g. dissipation of wave energy that might impact shorelines (Ferrario *et al.*, 2014; Wiberg *et al.*, 2019)]. In addition, they are sources of 'blue food' (edible aquatic organisms), biomolecules and biomaterials of economic relevance, especially in the pharmaceutical industry (Datta, Talapatra & Swarnakar, 2015).

Anthropogenic pollutants ultimately reach the aquatic environment and cause a deleterious impact on freshwater and marine ecosystems (Häder *et al.*, 2020), leading to large-scale biological impacts that may culminate in the extinction of some species (Baines *et al.*, 2021). Eventually, these pollutants may reach human populations through the food chain (Adeel *et al.*, 2017; Lecomte *et al.*, 2017). The effects of legacy pollutants [e.g. polycyclic aromatic hydrocarbons (PAHs), dioxins, polychlorinated biphenyls (PCBs), pesticides, and heavy metals], and newly emerging contaminants [e.g. pharmaceuticals, and microplastics and nanoplastics (MNPs)] are usually investigated with *in vitro* and *in vivo* toxicity tests, which may be performed on single-species model organisms or at community levels. These tests are necessary for setting accurate toxicity thresholds and identifying toxicity mechanisms or pathways. They form part of the prospective and retrospective assessment tools required for the market authorisation of new compounds or characterisation of the causes of adverse effects. Commonly used *in vitro* tests have recently been reviewed by Rosner *et al.* (2021).

The immense diversity and abundance of aquatic invertebrates, their relatively simple body organisation, small size, reduced genetic complexity, and high sensitivity to a broad spectrum of chemical compounds have resulted in their widespread use in ecotoxicological studies (Lagadic & Caquet, 1998) and their inclusion in international guidelines for ecotoxicity testing. In addition, apart from cephalopods, they are not included in EU legislation on animal research, such as Directive 2010/63/EU on the protection of animals used for scientific purposes. Furthermore, the ease of rearing many aquatic invertebrates under controlled laboratory conditions, their high fecundity, and their short lifespans enable rapid *in vivo* testing and the potential for multi-generational tests (e.g. to investigate epigenetic effects on unexposed offspring). Furthermore, the rearing of these animals in laboratories reduces the use of natural populations (Collier *et al.*, 2016). Therefore, numerous biomarkers based on aquatic invertebrates have been developed for (eco)toxicity testing (Tosti & Gallo, 2012; López-Pedrouso *et al.*, 2020; Trestrail, Nugegoda & Shimeta, 2020). Aquatic invertebrates also can successfully replace vertebrates in various tests, as demonstrated by their integration into national and international guidelines. Short-term toxicity tests on invertebrates are mandatory under regulation 1907/2006/EC, better known as the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation, and in long-term toxicity studies for substances for which production exceeds 100 tons/year (Tarazona *et al.*, 2014).

There are, however, important shortcomings in terms of environmental relevance associated with the use of laboratory-bred aquatic invertebrates. Specifically, extrapolation from controlled laboratory conditions to natural environments is often difficult as many parameters are not taken into account. These include: (*i*) inter-species variation in toxicity sensitivity associated with substance movement through food webs and potential biomagnification and biotransformation; (*ii*) synergistic impacts, which might also be affected by the alteration of physical attributes of the experimental animals; (*iii*) life traits associated with previous exposure or the transgenerational inheritance of epigenetic signatures due to exposure of previous generations. Additional doubts can be raised regarding which species should be considered 'gate-keepers' (and thus used for different trophic-level ecotoxicity tests) due to species-specific sensitivity to pollutants (Chaumot *et al.*, 2014). These considerations, together with legislation that minimises animal use [e.g. REACH regulations, 2006; bans on animal testing, 2013; the '3 Rs' principle of Replacement, Reduction and Refinement of animal use in research (Burden *et al.*, 2015)], encourage the development of alternatives to *in vivo* toxicity testing based on multiple model organisms and endpoints (Rosner *et al.*, 2021). In addition, the use of omics and high-throughput techniques that allow extrapolation from responses at the cellular level to effects on whole organisms, populations, ecosystems or among species, must be accompanied by computational models (Brockmeier *et al.*, 2017; Vinken *et al.*, 2017; Sakuratani, Horie & Leinala, 2018). Approaches that predict endpoint information for one substance using data from another structurally similar substance (source substance[s]), termed 'read-across', are also increasingly available and accepted for regulatory purposes. Most model organisms for both *in vivo* and *in vitro* tests are chosen to represent the many phyla and ecosystems in the aquatic environment. The aquatic invertebrate models used most widely in the various regulatory test guidelines and reported in ecotoxicological studies include molluscs, arthropods, cnidarians, and annelids. Other taxa such as sponges, flatworms, nematodes and tunicates are poorly represented (Fig. 1). In this review, we describe the common aquatic invertebrate taxa used in ecotoxicological studies and pollution monitoring, emphasising the value that underrepresented taxa could add to provide a more comprehensive understanding of the impact of pollution on the aquatic environment.

II. USE OF STANDARDISED TESTING IN CHEMICAL RISK ASSESSMENT AND WATER QUALITY CONTROL

Work on the safety of pesticides and chemicals began in 1971, following establishment of the Organisation for Economic Cooperation and Development (OECD) Environment Committee. The OECD Mutual Acceptance of Data (MAD) agreement on the assessment of chemicals is an essential component of the worldwide standardisation of methodologies for chemical safety (OECD, 1981). The OECD test guidelines (TG) programme develops guidelines, which are published online (Rasmussen *et al.*, 2019). An advantage of MAD has been a reduction of unnecessary animal testing because substances only need to be tested once rather than in every country of notification. The 3Rs principles for animal experimental usage (Russell & Burch, 1959) was influential on EU legislation for regulations to protect animals used in research and other scientific purposes in 1986. The 3Rs subsequently was made a legal requirement in Directive 2010/63/EU on the protection of animals used for research purposes. A key aspect of the resulting replacement of vertebrates with invertebrates has been a dramatic increase in the use of the latter in research and chemical safety assessments (see Table 1 for the OECD TGs related to using invertebrates in the testing and assessment of chemicals). There are few ethical guidelines governing the use of invertebrates in science (Drinkwater, Robinson & Hart, 2019), other than their ability to feel pain or demonstration of advanced cognition. Scientific organisations like the Association for the Society of Animal Behaviour publish some rules for using invertebrates in research (ASAB, 2018). However, there remain differences among countries in the legal protection

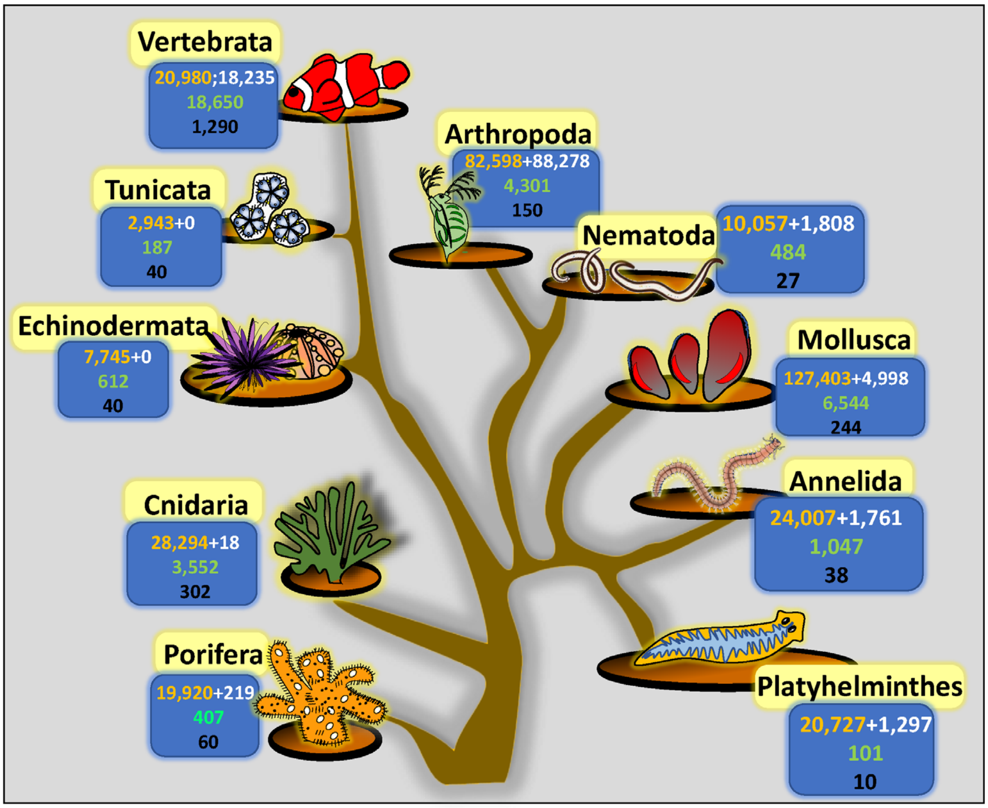


Fig. 1. Phylogenetic tree showing the number of marine (yellow numbers) and freshwater (white numbers) species in the various taxa retrieved, from WoRMS (2022) and Balian *et al.* (2008), respectively; the total number of publications in the domains of ecotoxicology, toxicology and pollution (green numbers) in the last 10 years (see Q1 in Table S1 for search string); and the number of publications based on omics technologies in the domains of ecotoxicology, toxicology and pollution (black numbers) in the last 10 years (see Q2 in Table S1 for search string). Data were obtained from a search of *Web of Science* for the search terms listed in Q1 and Q2 on 31 December 2021.

Table 1. Standardised regulatory tests utilising aquatic invertebrates.

Phylum	Species	Tests	Guidelines	References
Arthropoda	*Chironomus dilutus*	Acute toxicity test	OECD Test No. 218–219	OECD (2018)
		Sediment-water life-cycle toxicity test	OECD Test No. 233	OECD (2005)
		Bioaccumulation test	ASTM E1688-19	ASTM (2020)
		Multigeneration test for assessment of endocrine-active chemicals	OECD Test No. 218-219	OECD (2018)
	Daphnia magna	Acute toxicity test	EPA OCSPP 850.1010	EPA (2016*a*)
		Immobilisation	OECD Test No. 202	OECD (2004)
		Chronic toxicity test	EPA OPPTS 850.1300	EPA (2016*f*)
		EC50	ASTM E1193-20	ASTM (2016)
	Daphnia magna, *Daphnia pulex*	Chronic toxicity test: reproduction test (male induction)	OECD Test No. 211	OECD (2018)
	Gammarus fasciatus	Acute toxicity test	EPA OCSPP 850.1020	EPA (2016*b*)
	Gammarus pseudolimnaeus	Acute toxicity test	EPA OCSPP 850.1020	EPA (2016*b*)
	Gammarus lacustris	Acute toxicity test	EPA OCSPP 850.1020	EPA (2016*b*)
	Hyalella azteca	Toxicity test	ISO 16303	ISO (2013)
	Leptocheirus plumulosus	Chronic toxicity test	ASTM E1367-03	ASTM (2014)
	Rhepoxynius abronius	Chronic toxicity test	ASTM E1367-03	ASTM (2014)
	Ampelisca abdita	Chronic toxicity test	ASTM E1367-03	ASTM (2014)
	Eohaustorius estuarius	Chronic toxicity test	ASTM E1367-03	ASTM (2014)
	Mysidae	Acute toxicity test	EPA OCSPP 850.1035	EPA (2016*c*)
	Penaeidae	Acute toxicity test	EPA OCSPP 850.1045	EPA (2016*d*)
	Amphiascus tenuiremis (Copepoda)	Acute lethal toxicity	ISO/DIS 14669	ISO (2007)
	Nitocra spinipes (Copepoda)	Acute lethal toxicity	ISO/DIS 14669	ISO (2007)
	Tisbe battagliai (Copepoda)	Acute lethal toxicity	ISO/DIS 14669	ISO (2007)
	Acartia tonsa (Copepoda)	Acute lethal toxicity	ISO/DIS 14669	ISO (2007)
Mollusca	*Crassostrea virginica*	Acute toxicity test (shell deposition)	EPA-OPPTS 850.1025	EPA (1996)
		Acute toxicity test (embryo-larval)	EPA OCSPP 850.1055	EPA (2016*e*)
		Bioconcentration factors	EPA OCSPP 850.1710	EPA (2016*g*)
		Acute toxicity test (embryo)	ASTM E724-98	ASTM (1998)
		Bioconcentration test	ASTM E1022-94	ASTM (1994)
	Crassostrea gigas	Acute toxicity test (embryo-larval)	EPA OCSPP 850.1055	EPA (2016*e*)
		Acute toxicity test (embryo)	ASTM E724-98	ASTM (1998)
		Bioconcentration test	ASTM E1022-94	ASTM (1994)
	Mercenaria mercenaria	Acute toxicity test (embryo-larval)	EPA OCSPP 850.1055	EPA (2016*e*)
		Acute toxicity test (embryo)	ASTM E724-98	ASTM (1998)
	Mytilus edulis	Acute toxicity test (embryo-larval)	EPA OCSPP 850.1055	EPA (2016*e*)
	Mytilus edulis	Acute toxicity test (embryo)	ASTM E724-98	ASTM (1998)
	Mytilus edulis	Bioconcentration test	ASTM E1022-94	ASTM (1994)
	Pecten sp.	Bioconcentration test	ASTM E1022-94	ASTM (1994)
	Unionidae	Toxicity test (glochidia and juvenile)	ASTM E2455-05	ASTM (2005)
	Bivalvia	*In-situ* field bioassays	ASTM E2122-01	ASTM (2001)
	Potamopirgus antipodarum	Reproduction test	OECD, Test No. 242	OECD (2016*a*)
	Lymnaea stagnalis	Reproduction test	OECD, Test No. 243	OECD (2016*b*)
Annelida	*Lumbriculus variegatus*	Toxicity test using spiked sediment	OECD, Test No. 225	OECD (2007*a*)

(Continues on next page)

Table 1. *(Cont.)*

Phylum	Species	Tests	Guidelines	References
		Acute toxicity tests	ASTM E729-80 279–280	ASTM (1996)
		Bioaccumulation tests	EPA-823-R-00-001 (v. 1); EPA-823-R-00-002 (v2)	EPA (2000)
	Tubifex tubifex	Bioaccumulation tests	EPA-823-R-00-001 (v.1); EPA-823-R-00-002 (v.2)	EPA (2000)
	Polychaeta spp.	Acute, chronic, and lifecycle aquatic toxicity tests	ASTM E1562-00	ASTM (2000)
	Polychaeta spp.	Sediment toxicity tests	ASTM E1611-07	ASTM (2007)
	Nereis spp.	Bioaccumulation tests	EPA-823-R-00-001 (v. 1); EPA-823-R-00-002 (v.2)	EPA (2000)
Echinodermata	Sea urchin	Fertilisation test	EPA-821-R-02-014	EPA (2002)
	Sand dollar	Fertilization test	EPS 1/RM/27	Environment Canada (2011)
	Sea urchin	Short-term toxicity test	ASTM E1563-21	ASTM (2021)
Nematoda	*Caenorhabditis elegans*	Growth, fertility and reproduction	ISO 10872:2020	ISO (2020)

afforded to invertebrates used in research. For example, the UK does not have the same laws regarding the euthanasia of crustaceans as New Zealand (Ministry for Primary Industry, 2017).

In parallel with chemical legislation, pollution regulation and environmental protection legislation were also developed. In October 2000, the Directive 2000/60/EC, also known as the Water Framework Directive (WFD; https://ec.europa.eu/environment/water/water-framework/info/intro_en.htm), was adopted, forming a framework for community action in the field of water policy. The WFD also places various international obligations on member states regarding the protection of sea waters from pollution, including compliance with: (*i*) the Conventions on the Protection of the Baltic Sea Environment (signed in Helsinki on 9 April 1992 and confirmed by Council resolution 94/157/EEC); (*ii*) the Convention on the Protection of the Northeast Atlantic Marine Environment, signed in Paris on 22 September 1992 and confirmed by Council Decision 98/249/EEC; (*iii*) the Convention on the Protection of the Mediterranean Sea against Pollution, approved by Council Decision 77/585/EEC signed in Barcelona on 16 February 1976, and the Convention against pollution from land-based sources signed in Athens on 17 May 1980 [83/101/EEC (WFD 2000)].

In addition, the maritime policy of the EU is included in the EC Marine Strategy Framework Directive (Directive 2008/56/EC, June 2008), which established a framework for the domain of community action in maritime environmental policy (Marine Strategy Framework Directive, 2008). The United Nations 2030 Agenda for Sustainable Development also addresses water quality and pollution *via* Goal 14, which aims to establish the conservation and sustainable use of the oceans, seas, and marine resources (https://sdgs.un.org/goals/goal14). Goal 14.1 aims 'by 2025, [to] prevent and significantly reduce marine pollution of all kinds, in particular from land-based activities, including marine debris and nutrient pollution'.

To fulfil the goals of both chemical legislation and the WFD, there is an urgent need for tools for the assessment of environmental hazards and risks to support responsible decisions that enable sustainable development. Invertebrate tests, both those standardised through OECD TGs (see Table 1), and those using non-standard species, are required to capture the breadth of potential effects and impact of pollutants, and to span the diversity of species and ecosystems impacted by the >85,000 chemicals currently available in commercial markets, only a small fraction of which has been evaluated by US regulators (Gross & Birnbaum, 2017). Similarly, in the EU, many of the 27,000 registered chemicals (i.e. those produced or imported at volumes >1 tonne/annum) have yet to be evaluated. By 2027, the European Chemicals Agency's (ECHA) Integrated Regulatory Strategy aims to clarify which registered compounds are low priority for additional regulatory action and which are high priority for regulatory risk management or data collection (ECHA, 2021).

III. KEY AQUATIC INVERTEBRATES IN STANDARDISED AQUATIC ECOTOXICOLOGY/MONITORING

(1) Arthropoda

Over 80% of known animal species on Earth are arthropods, occupying crucial positions in aquatic ecosystems as detritivores, herbivores, omnivores, and carnivores (Ødegaard, 2000). They are vital components of healthy ecosystems and are key indicators of environmental change and pollution (Chakravarthy & Sridhara, 2016). Arthropods are highly recommended for aquatic pollution studies due to their short life cycle, high reproductive potential, wide distribution, and representativeness of plankton and benthic fauna in the littoral and intertidal zones where most chemical spills occur (Lee, 1977). Their exoskeleton-moulting process, which is hormone dependent, is an important endpoint for ecotoxicological studies, serving as a good indicator of endocrine-disrupting/reproductive disorders induced by chemicals such as industrial chemicals and pesticides (Peterson, Kashian & Dodson, 2001; Zou, 2005; Mensah, Muller & Palmer, 2012; OECD, 2018).

Among the arthropods, insects (around 76,000 species; Balian *et al.*, 2008) and crustaceans (67,000 species; Ahyong & Huang, 2020) are the most prominent groups in aquatic environments. Freshwater aquatic insects live all or part of their lives in lentic (still) or lotic (running) water systems (Starr & Wallace, 2021). The EPT (Ephemeroptera, Trichoptera, Plecoptera) index, based on pollution-intolerant taxa, is widely used in freshwater environmental studies (Lenat & Penrose, 1996) as a common metric for environmental health, water quality, ecosystem integrity, and response to pollution (Blöcher *et al.*, 2020) Additionally, acute toxicity is also frequently tested on the early developmental stages of sensitive aquatic insects (Kreutzweiser *et al.*, 2008; Beketov *et al.*, 2013; Camp & Buchwalter, 2016). Many crustacean species are widely employed in both freshwater and marine ecotoxicology [American Public Health Association (APHA), American Water Works Association (AWWA), World Economic Forum (WEF), 1995-Standard Methods for the Examination of Water and Wastewater; Pane *et al.*, 2012], as they are strongly affected by environmental stressors such as pollution by light, nutrients, and toxins (Wacker & Harzsch, 2021). Among the crustaceans, Cladocera, Copepoda, and Amphipoda are the most extensively used taxa in bioassays.

Cladocera (Diplostraca), commonly known as water fleas, are small crustaceans widely used as bioassay organisms when evaluating the impact of toxic substances (Sarma & Nandini, 2006). Cladocera reproduce by cyclic parthenogenesis when conditions are suitable. When conditions worsen, males and resting eggs are produced. Ernest Warren introduced *Daphnia magna* as a model organism for toxicity studies, laying the groundwork for the field of ecotoxicology (Warren, 1900). Since then, *D. magna* has become the most widely used crustacean in ecotoxicity tests and is considered the standard bioassay organism by many academic institutions and governmental organisations (Siciliano *et al.*, 2015). Various chemicals have been examined on cladocerans, including heavy metals (Sadeq & Beckerman, 2019), pesticides (Toumi *et al.*, 2015), nanomaterials (Ellis *et al.*, 2021*a*), microplastics (MPs) (Frydkjær, Iversen & Roslev, 2017), and natural toxic substances such as the cyanobacterial toxin microcystin (Herrera, Echeverri & Ferrao-Filho, 2015). In early life stages, *Daphnia* is sensitive to virulence traits and is therefore used to study interaction with bacterial pathogens (Ebert, 2008). The genome of *D. pulex* was sequenced in 2007 by the *Daphnia* Genomics Consortium (DGC), revealing that it has the highest similarity to human genes among arthropods (http://wfleabase.org/). The use of *Daphnia* (Fig. 2A) in genetic screening could facilitate an understanding of the intricate control of genes and of the cellular and molecular processes that respond to environmental challenges (Siciliano *et al.*, 2015). Since *D. magna* naturally occurs only in temperate regions, toxicity testing with this species conducted in tropical regions has drawn criticism, as *D. magna* experiences low reproduction and high mortality rates at tropical temperatures (Mark & Solbé, 1998).

Copepoda form the largest crustacean group with over 13,000 known species (Longhurst, 1985). Most copepod species

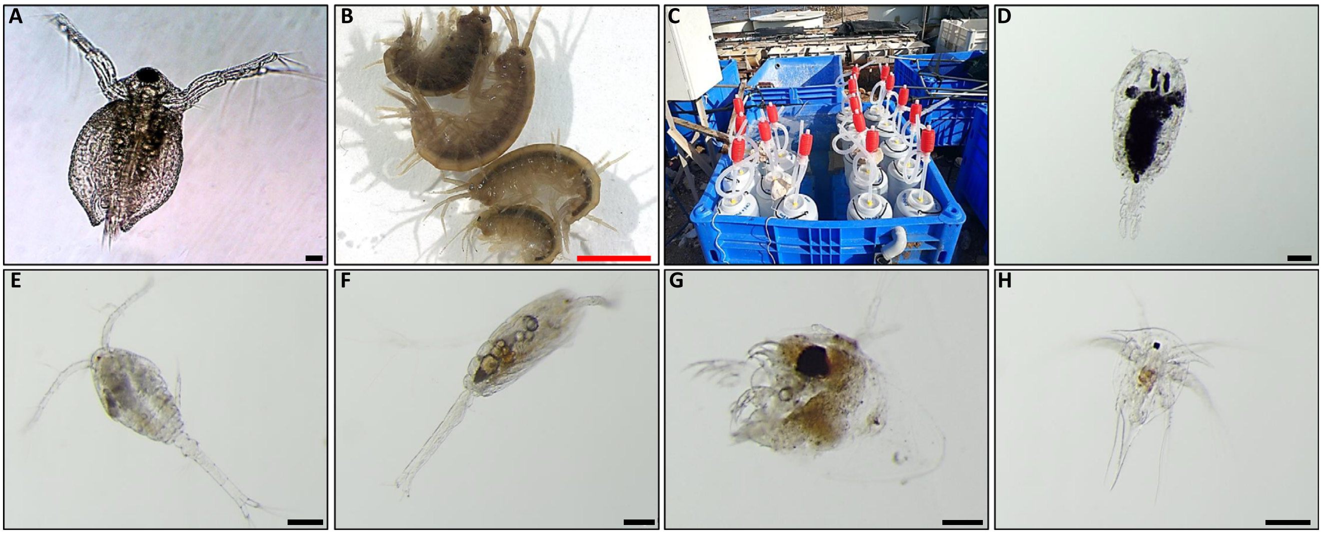


Fig. 2. (A) *Daphnia* sp. (B) *Gammarus fossarum*. (C–H) Whole zooplankton community crude-oil acute toxicity (LC50) test using experimental microcosms. (C) Experimental setup. (D–H) Zooplankton species exposed to crude oil in concentrations of 50–1000 ppm (v/v) for 48 h. (D) *Sapphirina* sp. (E) *Oithona nana*. (F) *Oithona plumifera*. (G) *Pseudoevadne tergestina*. (H) *Cirripedia nauplius*. Black scale bar = 100 μm; red scale bar = 5 mm.

are omnivorous, feeding on a range of macroinvertebrates, protozoa, algae, and bacteria. Copepods go through six naupliar phases and six copepodite stages throughout development. Reproduction is sexual, and most species have resting stages that permit survival during periods of poor environmental conditions. These varied traits of copepods make them excellent model organisms for ecotoxicological studies (Kulkarni *et al*., 2013). The calanoid copepod *Acartia tonsa* and the harpacticoid species *Amphiascus tenuiremis* and *Nitocra spinipes* have been recommended by the International Organisation for Standardisation (ISO, 2007) and OECD (2007*b*) for the evaluation of chronic and acute lethal toxicity of marine contaminants such as herbicides (Noack *et al*., 2016), sediment organic contaminants (Macken *et al*., 2008), discharge waters (Sonmez, Sivri & Dokmeci, 2016) and oestrogens (Andersen, Halling-Sørensen & Kusk, 1999). Other studies have stressed the importance of using local species to ensure the ecological relevance of such tests (Butler *et al*., 2020). Marine copepods have also been utilised as model organisms for the acute and chronic testing of crude oil and chemical dispersants (Almeda *et al*., 2013; Fig. 2C–H), with the regulation of their cytochrome P450 (CYP) genes being one of the best-known tests (Han *et al*., 2017).

Amphipods are ubiquitous crustaceans that include nearly 10,000 marine and freshwater species. They cover a wide trophic range as herbivores, detritivores, and predators, while providing an important food source for many organisms higher in the food chain. Amphipods are very sensitive to a wide range of pollutants. Experiments with amphipods include lethal concentration 50% (LC50) and effect concentration 50% (EC50) assays for the testing of chemicals (Alonso, De Lange & Peeters, 2010). Amphipods are also used in the Polychaeta/Amphipoda (P/A) index, which measures the differential sensitivity of these two taxonomic groups (Dauvin, 2018). The genus *Gammarus* (Fig. 2B) is widely distributed in freshwater and marine ecosystems and has been extensively studied for its responses to various stressors and pollutants (Barnard, 1983). Biomarker analyses on *Gammarus* spp. have been used to assess the effects of antioxidant responses, behaviour, cellular damage, defence mechanisms, energy reserves, endocrine responses, iono/osmoregulation mechanisms, and lysosomal responses (Kunz, Kienle & Gerhardt, 2010; Sroda & Cossu-Leguille, 2011; Arce Funck *et al*., 2013; Gismondi & Thomé, 2014; Trapp *et al*., 2014; Mehennaoui *et al*., 2016; Gouveia *et al*., 2018; Batista *et al*., 2021). In addition, multigenerational studies (Geffard *et al*., 2010; Vigneron *et al*., 2019; Cribiu *et al*., 2020) have led to a more ecologically relevant understanding of the impact of toxicants on ecosystems (Minguez *et al*., 2015). Recent advances in transcriptomics (Caputo *et al*., 2020) and proteomics (Trapp *et al*., 2014, 2016; Gouveia *et al*., 2019) have enabled the identification of new biomarkers, including reference genes for quantitative PCR (qPCR) data normalisation (Mehennaoui *et al*., 2018), and microbiome (Gouveia *et al*., 2020). These studies have contributed to a better understanding of the physiological and molecular responses of non-model species to contaminants and the pathways underlying detoxification (Armstrong *et al*., 2019).

Other crustaceans commonly employed in ecotoxicological studies and environmental biomonitoring include decapods (Reynolds & Souty-Grosset, 2011), barnacles (Da Silva, Ridd & Klumpp, 2009), brine shrimps (Hnamte, Kaviyarasu & Siddhardha, 2020), notostracans (Lahr, 1997), and isopods (Reboleira *et al*., 2013). The use of decapods in toxicological tests is likely to be amended following recent observations of their ability to feel pain and distress (Passantino, Elwood & Coluccio, 2021). As an alternative to ecotoxicological testing, the development of *in silico* models in crustaceans appears to be a promising method for predicting chemical toxicity, which can offer a practical and trustworthy tool for evaluating environmental risk and ranking chemicals for testing (Cao *et al*., 2018; Varsou *et al*., 2021).

(2) Mollusca

Mollusca are the second largest phylum in the Kingdom Animalia (Fig. 1) and include seven clades: Aplacophora, Monoplacophora, Polyplacophora, Bivalvia, Gastropoda, Cephalopoda, and Scaphopoda. Molluscs are essential components of the food chain and play an important ecological role in structuring benthic communities due to their ubiquitous distribution (Fortunato, 2015). As well as being highly sensitive to pollution exposure (Mouthon & Charvet, 1999), molluscs show a variety of responses to toxic contaminants (Grabarkiewicz & Davis, 2008), have a rapid growth index, short life cycle, wide distribution, different lifestyles, bioaccumulation capability for a wide range of pollutants, various life stages with different sensitivity, and can be maintained under laboratory conditions (EPA, 2003). Bivalves and gastropods are recommended for standardised tests in water and sediment quality assessment as bioindicators (Table 1).

Most bivalves live at the water–sediment interface, exhibit burrowing behaviours, and are filter feeders (McLeod, Luoma & Luthy, 2008). These features lead them to accumulate various chemical pollutants including metals (Rzymski *et al*., 2014; Shi *et al*., 2016; Yuan *et al*., 2020), PCBs (Dodoo, Essumang & Jonathan, 2013; Milun *et al*., 2020), PAHs (Yap, Shahbazi & Zakaria, 2012; Yoshimine & Carreira, 2012), organochlorine pesticides (Tong *et al*., 2019), per- and polyfluoroalkyl substances (Cui *et al*., 2021), and pharmaceuticals released into the environment (Gomez *et al*., 2021). *Mytilus edulis* (Fig. 3A; Rosenberg & Loo, 1983), *Pecten* spp. (Fig. 3B; Metian *et al*., 2007, 2008), *Crassostrea gigas* (Fig. 3C), and *C. virginica* (Perrino & Ruez, 2019) are the recommended species for the American Society for Testing and Materials (ASTM) bioconcentration standard test (ASTM, 1994) designed to assess the ability of an aquatic species to accumulate test materials directly from the water. *C. virginica* is the standard species for two other EPA test guidelines: an acute toxicity test (OPPTS 850.1025; EPA, 1996) and a bioaccumulation test (OCSPP 850.1710; EPA, 2016*g*). The ASTM standard guide E2122-01 (ASTM, 2001) describes *in situ* bioassay

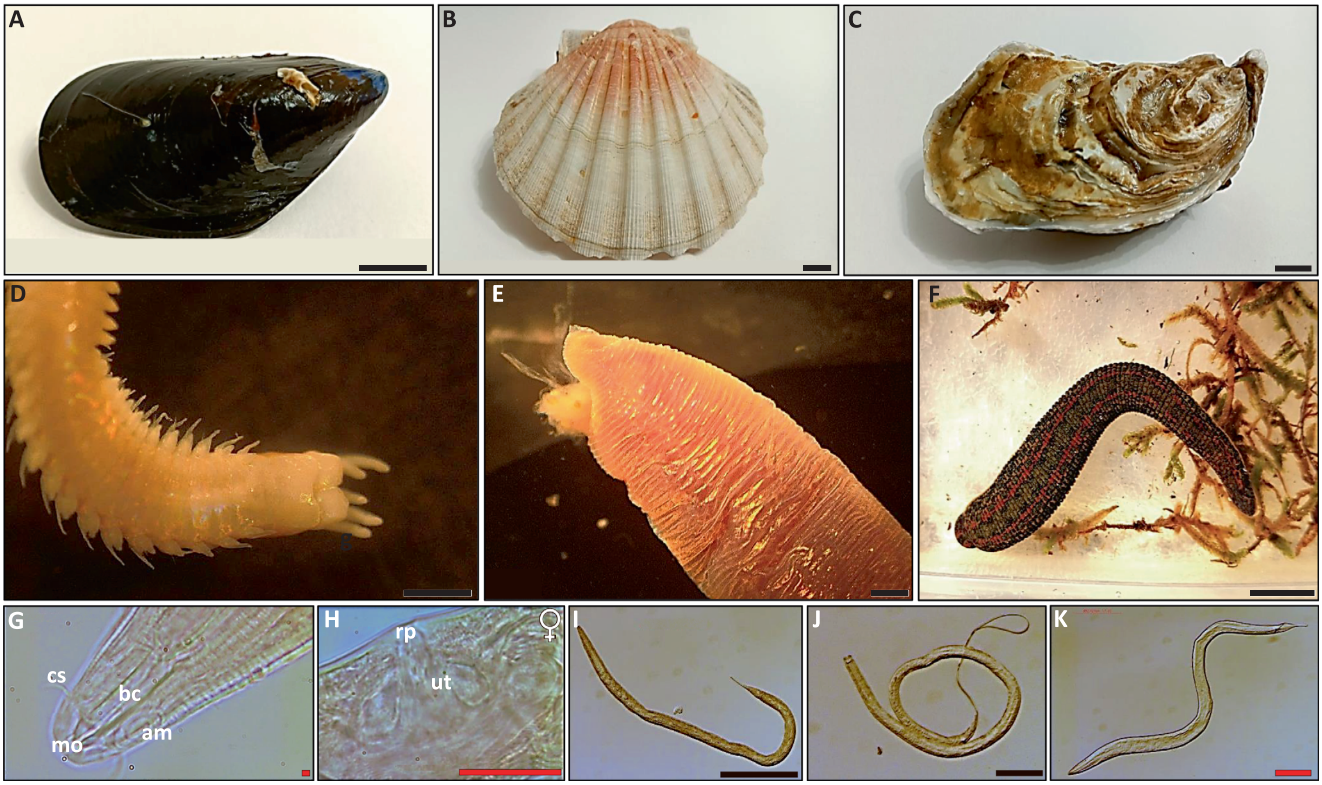


Fig. 3. (A–C) Mollusca. (A) *Mytilus edulis*. (B) *Pecten maximus*. (C) *Crassostrea gigas*. (D–F) Annelida. (D) Polychaeta, Eunicidae. (E) Polychaeta, Opheliidae. (F) Leech, *Hirudo verbana*. (G–K) Nematoda. (G) *Vasostoma* sp., head. (H) *Vasostoma* sp., tail in female. (I) *Vasostoma* sp. (J) *Thershelingia* sp. (K) *Dorylaimopsis* sp. am, amphid; bc, buccal cavity; cs, cephalic setae; mo, mouth opening; rp, reproductive pore; ut, uterus. Black scale bar = 1 cm; red scale bar = 100 μm.

using caged bivalves as allowing more precise results about the impact of pollutants in natural environments. Another asset of bivalves is the differential susceptibility of their various life stages to pollutants, and this is used by two standard guides, ASTM E724-98 (ASTM, 1998) and OCSPP 850.1055 (EPA, 2016*e*). The procedures outlined in ASTM E724-98 (ASTM, 1998) and ASTM E2455-05 (ASTM, 2005) describe how to obtain laboratory data regarding the acute effects of test materials (individual chemicals; mixtures in different environmental matrices) on embryos and larvae.

Gastropoda are the largest clade of molluscs (Frýda, 2021) and in some ecosystems, they can represent 20–60% of the total quantity and biomass of macroinvertebrates (Anderson & Smith, 2000). Due to their sensitivity to a broad variety of organic and inorganic chemicals, gastropods are widely used as effective indicators of both water and sediment quality (Tallarico *et al.*, 2014; Leomanni *et al.*, 2015, 2016) in environmental risk assessment (OECD, 2010). Freshwater gastropods have been utilised for laboratory studies on the effects of endocrine-disrupting chemicals (EDCs) on reproduction (OECD, 2010). In particular, the snails *Potamopyrgus antipodarum* and *Limnaea stagnalis* were recommended as the standard species for reproduction assays by the OECD (2016*a*,*b*), with this recommendation validated within the Conceptual Framework for Endocrine Disrupters (OECD, 2018).

Besides ecotoxicological tests based on whole-organism-related endpoints, additional molecular and cellular biomarkers have been developed in molluscs. These include: hepatopancreas metallothionein concentration for metals (Lionetto *et al.*, 2001; Lionetto, Caricato & Giordano, 2021), oxidative stress biomarkers (depletion of intracellular reduced glutathione; Lima *et al.*, 2007), alteration of the activity of antioxidant enzymes [e.g. glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT); Jena, Verlecar & Chainy, 2009], alterations of the lysosomal system (Martínez-Gómez, Bignel & Lowe, 2015), induction of lipid membrane peroxidation, cholinesterase inhibition as a biomarker of neurotoxicity (Leomanni *et al.*, 2015) and vitellogenin induction as an EDC biomarker (Tran *et al.*, 2019). In addition, genotoxicity has been assessed using the micronucleus assay applied in both laboratory and field experiments to bivalves (Bolognesi & Fenech, 2012).

In recent years, research for alternatives to *in vivo* approaches has led to the development of *in vitro* tools such as primary cell cultures from various organs (Galloway & Depledge, 2001; Nogueira *et al.*, 2013; Yoshino, Bickham & Bayne, 2013; Daugavet & Blinova, 2015) and larval stages (Van der Merwe *et al.*, 2010), allowing the use of cell cultures as models for environmental contaminant testing, the analysis of cellular responses to pollutants, and investigation of the underlying mechanisms (Rinkevich, 1999, 2005*a*). Furthermore, adult stem cells have been identified (Vogt, 2012; Jemaà *et al.*, 2014; Deryckere & Seuntjens, 2018; Cherif-Feildel *et al.*, 2019; Rodriguez *et al.*, 2020) increasing interest in developing new tools that may lead to ecotoxicological applications (Rosner *et al.*, 2021).

(3) Annelida

Annelida is a phylum of over 25,000 species of segmented worms that are found worldwide in various environments (Fig. 1; WoRMS, 2022; Glasby & Timm, 2008; Aguado *et al.*, 2014; Glasby, Erséus & Martin, 2021). They can reproduce both sexually and asexually and exhibit high regeneration potential, including the restoration of germ cells, cell repair, regrowth of body parts, and whole-body regeneration (Hyman, 1940; Berrill, 1952; Herlant-Meewis, 1964; Bely, 2006, 2014; Dannenberg & Seaver, 2018; Zattara, 2020; Kostyuchenko & Kozin, 2021).

Polychaetes (Fig. 3D, E) are highly diverse marine worms that are abundant in ocean sediments and also live in freshwater and shallow brackish waters (Verdonschot, 2015; Jørgensen & Jensen, 1978; Niederlehner *et al.*, 1984). They have soft bodies, a characteristic segmented shape, and occupy a wide range of habitats, including those in extreme conditions (Glasby, 1999; Verdonschot, 2015; Magalhães *et al.*, 2021). They play an important ecological role in benthic ecosystems through scavenging, filter-feeding, and predation (Brusca & Brusca, 1990; Hickman & Roberts, 1994). They are a crucial food source for organisms such as fish and wading birds (Verdonschot, 2015). *Platynereis dumerilii* is a well-known polychaete species with several life phases and can survive in polluted and acidic environments (see online Supporting Information, Fig. S1; Fischer & Dorresteijn, 2004; Fischer, Henrich & Arendt, 2010; Lucey *et al.*, 2015; Schenkelaars & Gazave, 2021). It is a dioecious, semelparous animal that can be bred efficiently in a laboratory (Kuehn *et al.*, 2019), producing numerous embryos for experimentation and culture populations.

Polychaetes are commonly used in marine toxicity tests to assess the impact of pollutants and toxic materials on sediments (Pocklington & Wells, 1992; Scaps, 2002; Bat, 2005; Dean, 2008). More than 48 species of polychaetes are used in standard toxicity tests (ASTM, 2000, 2007), with the six species used most frequently being *Neanthes arenaceodentata*, *Nereis diversicolor*, *Nereis virens*, *Laeonereis acuta*, *Arenicola marina*, and *Capitella teleta* (Forbes, Andreassen & Christensen, 2001; Christensen, Banta & Andersen, 2002). They are used for both acute and chronic toxicity tests (Reish & Gerlinger, 1997) and to evaluate the effects of new and emerging pollutants on reproductive, larval development, and behavioural endpoints (Lewis & Watson, 2012). Polychaetes can accumulate harmful organic compounds in their tissues and are considered good indicators of bioaccumulation (Caldwell *et al.*, 2005; Janssen *et al.*, 2010; Jørgensen *et al.*, 2005; Langston *et al.*, 2005). The possible toxic impacts of multi-walled carbon nanotubes (MWCNTs) (Baughman, Zakhidov & de Heer, 2002) and MPs on polychaetes has been studied, revealing neurotoxicity, activation of antioxidant defences, and alterations in energy-related biochemical processes (Du *et al.*, 2013; Hidalgo-Ruz *et al.*, 2012; Andrady, 2015). Exposure to MWCNTs and MPs reduces the regenerative capacity of some polychaete species (Leung & Chan, 2018). Despite their ability to accumulate harmful materials, some polychaete species are considered resistant to pollutants due to their positive responses to organic enrichment. This results in the proliferation of numerous opportunistic polychaete species and an increase in their abundance after pollution events. By contrast, amphipods are more sensitive to stress and have a higher death rate. To track significant alterations in benthic ecosystems caused by oil spills, urban sewage outfalls, and organic matter enrichment, 23 countries use P/A ratio-based indices such as benthic opportunistic polychaetes amphipods index (BOPA) and benthic opportunistic annelida amphipods index (BO2A) in estuarine and coastal environments (Dauvin, 2018).

Nearly 1100 species of freshwater oligochaetes exist (Martin *et al.*, 2008). About half of these are widely distributed worldwide (Timm & Martin, 2015) while the rest have limited distributions (Wang & Liang, 1997; Timm & Martin, 2015; Verdonschot, 2015). Oligochaetes are commonly used in ecotoxicological studies due to their abundance, cosmopolitanism, and ability to accumulate toxic substances such as metals (Helling, Reinecke & Reinecke, 2000; Corbi, Guilherme & Regiane, 2015; Gomes *et al.*, 2017). The most common species used in toxicological studies are *Pristina leidyi*, *Branchiura sowerbyi*, *Lumbriculus variegatus*, *Tubifex tubifex*, and *Allonais inaequalis* (Lobo *et al.*, 2016; Hurley, Jamie & James, 2017; Neto *et al.*, 2019, Felipe *et al.*, 2020). *L. variegatus*, *T. tubifex* and *A. inaequalis* are the most widely used species for evaluating toxic effects caused by MPs and MWCNTs (Castro *et al.*, 2020; Scopetani *et al.*, 2020; Silva *et al.*, 2021). Transmission electron microscopy showed that MWCNTs do not penetrate cell membranes, and that toxicity caused by ingesting them might result from metals solubilised from the nanotubes (Mwangi *et al.*, 2012). Ingestion of MPs did not appear to affect survival and reproductive rate, but this does not rule out the possibility of risks to biodiversity (Koelmans *et al.*, 2014; Barboza & Gimenez, 2015).

Leeches (Hirudinea) are a group of annelids with a variety of life-history strategies and reproductive behaviours that are primarily found in freshwater habitats, but also occur in other habitats (Apakupakul, Siddall & Burreson, 1999; Sket & Trontelj, 2008). *H. medicinalis* and *H. verbana* (Fig. 3F) are perhaps the best-known species (Trontelj, Sotler & Verovnik, 2004; Trontelj & Utevsky, 2005) and are used in medicine and research due to their ease of maintenance and well-characterised physiology and behaviour (Sawyer, 1986; Grimaldi, Tettamanti & de Eguileor, 2018; Baranzini *et al.*, 2020). Studies using the alkaline comet test on *H. verbana* haemocytes found that exposure to MWCNTs causes stress and potential risks to public health (Tice *et al.*, 2000; Mihaljević *et al.*, 2009). *In vitro* treatments of leech phagocyte cultures with MWCNTs decreased cell proliferation, increased apoptotic events, and induced the production of amyloid fibrils and reactive oxygen species (ROS), which are indicators of toxicity induced by environmental pollutants (Girardello *et al.*, 2015*a*,*b*, 2017; Torres & Fadeel, 2013). Thus, *H. medicinalis* and *H. verbana* are ideal species to conduct rapid *in vivo* and *in vitro* environmental tests with innovative methods that utilise molecular-based endpoints at the sub-cellular or cellular level (Bodó *et al.*, 2020).

(4) Nematoda

Nematodes (Fig. 3G–K) are common in aquatic habitats, mainly inhabiting sediments as free-living organisms (Hogue, 1982; Heip, Vincx & Vranken, 1985; Decho & Fleeger, 1988; Eckman & Thistle, 1988; Hodda, 1990; Hugot, Baujard & Morandi, 2001; Van Gaever *et al.*, 2006; Mokievsky, Udalov & Azovsky, 2007; Gallucci, Moens & Fonseca, 2009). Nematode distribution depends on the physico-chemical properties of the sediments, climate, oxygen and food sources (Lee *et al.*, 1977; Bell, Watzin & Coull, 1978; Montagna *et al.*, 1983; Decho & Fleeger, 1988; Blanchard, 1990; Rice & Lambshead, 1994; Brown *et al.*, 2001; Steyaert *et al.*, 2003; Fonseca & Soltwedel, 2007; Lee & Riveros, 2012; Braeckman *et al.*, 2013). In intertidal areas, some nematode species are also sensitive to the water content of the sediment (Jansson, 1968; Steyaert *et al.*, 2001). Nematodes are important in bioturbation, bioirrigation and trophic processes, and feed on a variety of resources, from dissolved organic matter to multicellular organisms, including other nematodes (Montagna, 1984; Moens, Verbeek & Vincx, 1999). Most of them have digestive and nervous systems and are dioecious. Despite being common, only 14% of estimated free-living nematode species in marine habitats are currently classified (Appeltans *et al.*, 2012). Additional information on nematode species is available online (Nemaplex, 2023).

Nematodes, as important benthic constituents and food sources for many predators, play significant roles in the trophic transfer of pollution from sediments, with some species more susceptible to environmental stressors (Hägerbäumer *et al.*, 2015). In consequence, nematodes are emerging as bioindicators for freshwater (Hägerbäumer *et al.*, 2016) and marine pollution (Ridal & Ingels, 2021), in single-species and community composition tests. Community composition tests require expertise in identifying genera and species, although this can be circumvented by using molecular tools (Avó *et al.*, 2017; Carta & Li, 2018; Knot *et al.*, 2020) and automated image recognition apparatus (e.g. FlowCAM; Kitahashi *et al.*, 2018), both of which have been shown to be credible alternatives.

Most ecological research on nematodes as bioindicators in the marine environment has focused on their responses to heavy metals, hydrocarbons, and organic enrichment (Ridal & Ingels, 2021). Studies have demonstrated species-specific susceptibility to different metals and changes in the diversity and abundance of some species in response to heavy metal pollution (Bastami *et al.*, 2017). Nematode genera have shown differential tolerance to oil spills and PAHs, with some demonstrating tolerance (e.g. *Sabateria*, *Dorylaimopsis*) and others increased mortality (e.g. Encheliidae; Allouche *et al.*, 2020*a*). Different nematode species have also shown variable susceptibility to crude oil or diesel pollution (Stark *et al.*, 2017; Monteiro *et al.*, 2018). Studies of organic and/or sewage-enriched pollution have shown inconsistent nematode abundances (Bertocci *et al.*, 2019; Sahraeian *et al.*, 2020), highlighting the need for additional pollution evaluation metrics such as an index of trophic diversity based on nematode trophic guild compositions (Kandratavicius *et al.*, 2018). Aquacultural pollution has also been found to affect nematode assemblage compositions, with a shift from long-lived, slow-growing genera to short-lived genera (Lacoste *et al.*, 2020). These experiments have demonstrated that some genera are specifically tolerant to pollutants (e.g. *Sabatieria* and *Daptonema*) while others are sensitive to pollutants (e.g. *Pomponema* and *Halalaimus*) (Ridal & Ingels, 2021). Nematode-based biomarkers have also been used to evaluate the effects of MNPs, demonstrating lethality in adults, changes in brood size and embryo number, and species-specific effects on population growth (Lei *et al.*, 2018; Mueller *et al.*, 2020). Biochemical biomarkers have also been successfully tested on nematodes, showing changes in enzymatic activities following exposure to various pollutants (Allouche *et al.*, 2020*b*; Hedfi *et al.*, 2021).

Single-organism tests complement community-level assessments of the impact of pollution. They allow a faster evaluation of the impact of pollution under controlled conditions, enabling the establishment of concentration–response relationships and the identification of pollutant mechanisms of action. Aquatic nematodes are challenging to cultivate and therefore only a few freshwater and marine species are used as models for pollution assessment (Moens & Vincx, 1998). To overcome this shortage, *Caenorhabditis elegans*, primarily a soil inhabitant (Zullini, 1988), is used as a standardised model in freshwater (Carresse *et al.*, 2021) and soil quality control (ISO 10872:2020). *C. elegans* has been tested with various pollutants, including heavy metals, PAHs, pesticides, endocrine disruptors, bacterial toxins, and nanomaterials (Hägerbäumer *et al.*, 2016). *Litoditis marina* has been proposed as a marine model due to its sensitivity to crude oil and diesel pollution, pesticide pollution, and sodium dodecyl sulfate (SDS) detergent pollution. Studies have used a wide range of parameters to measure response to pollution, including mortality assay, LC50, fecundity, egg deposition time, abundance of eggs, embryonic and post-embryonic development time, and adult sex ratio (Monteiro *et al.*, 2018; Oliveira *et al.*, 2020; Francolino *et al.*, 2021).

The low costs associated with nematode sampling should encourage their inclusion in environmental pollution assessment tests. However, the main obstacles to the large-scale use of nematodes as ecological indicators include: (*i*) the lack of useful cultivation protocols for individual species; (*ii*) the scarcity of literature on individual nematode species; (*iii*) the low number of taxonomists able to identify species; (*iv*) the paucity of metabarcoding conducted on marine and freshwater nematodes (excluding *C. elegans*); and (*v*) the absence of standardised effective metrics for each pollutant type or pollutant assembly. Some of these difficulties might be surmounted by the development of automated image recognition and improved DNA barcoding analysis techniques for the rapid identification of species.

(5) Echinodermata

Echinoderms are marine invertebrates widely distributed across all seas, including highly anthropogenically modified areas. They are important representatives of many marine ecosystems and frequently constitute the majority of the benthic macrofauna. More than 7400 living species are divided into five extant clades: Asteroidea (starfish; Fig. 4A), Holothuroidea (sea cucumbers; Fig. 4B), Echinoidea (sea urchins and sand dollars; Fig. 4C), Crinoidea (sea lilies and feather stars; Fig. 4F), and Ophiuroidea (brittle stars and basket stars; Fig. 4H). Feeding strategies depend on taxon and species and can include filter-feeding, carnivory, scavenging, grazing, and omnivory. Crinoids are generally filter-feeders; ophiuroids are carnivores, scavengers, and filter-feeders; asteroids are predators; echinoids are grazers and omnivores; and holothurians are scavengers and filter-feeders. Members of this phylum share some features including pentaradial symmetry in the adult stage, a water vascular system, a calcium carbonate dermaskeleton, mutable connective tissue and remarkable regeneration capabilities that can also be used in reproduction (Wessel, 2018). In general, echinoderms (excluding crinoids) produce feeding larvae (Fig. 4D), while some asteroids, echinoids, and ophiuroids are direct developers (McEdward & Miner, 2001). They live in close contact with substrates and sediments, and possess highly developed and permeable external epithelia, normally used for respiration and excretion. Due to these latter features, they are easily exposed to various environmental pollutants (Sugni *et al.*, 2007). As deuterostomes, they share a rather close phylogenetic relationship with vertebrates, suggesting the existence of shared mechanisms of responses to environmental contamination.

Both sea urchins (Echinoidea) and sea cucumbers (Holothuroidea) have high commercial value and are extensively exploited (Kelly, 2005; Cirino *et al.*, 2017; Hu *et al.*, 2021; Rubilar & Cardozo, 2021). Sea urchins are considered ideal models for marine (eco)toxicological tests (Goldstone *et al.*, 2006; Gharred *et al.*, 2016) as their embryos can sense adverse effects related to a wide range of environmental stressors, including metals and plastics (Pinsino *et al.*, 2017; Messinetti *et al.*, 2018; Morroni *et al.*, 2018; Bergami *et al.*, 2019; Oliviero *et al.*, 2019), polluted sediments and ocean acidification (Pagano *et al.*, 2017; Dorey *et al.*, 2018; Bonaventura *et al.*, 2021; Caetano, Pereira & Envangelista, 2021). Accordingly, assessing the embryonic and larval development of sea urchins under stress conditions is useful in monitoring and risk assessment programs (EPA, 2002; Environment Canada, 2011; Sartori *et al.*, 2017; ASTM, 2021). Chronic or sub-chronic short-term embryotoxicity tests for the analysis of environmental quality have been used since the 1960s, due to the accessibility and reliability of sea urchin embryos and the speed with which these tests can be conducted. The guidelines cover procedures for performing short-term (48–96 h) laboratory assays on sea urchin embryos using a variety of sea urchin species, such as *Arbacia punctulata* and *Strongylocentrotus droebachiensis* from the East Coast of the USA, *Strongylocentrotus purpuratus*, *S. droebachiensis*, and *Dendraster excentricus* from the West Coast of the USA, and *Paracentrotus lividus* (Fig. 4C) from the Mediterranean. The ASTM (2021) guide also includes procedures for the use of other echinoids (e.g. sand dollars). Modifications to the standard procedures can be applied. For example, the sea urchin embryotoxicity test, considered to be a valuable tool for assessing the quality of sediments in harbour areas, may result in flawed outcomes when based exclusively on normal *versus* abnormal embryos. To overcome this risk, several Integrative Toxicity Indices (ITIs) have been developed and validated (Morroni *et al.*, 2016; Bonaventura *et al.*, 2021). Among these, the ITI 4.0 index discriminates strictly between developmental delay and morphological defects from fertilised eggs to the gastrula stage (Bonaventura *et al.*, 2021). Notably, some sea urchin species are suffering from increasing anthropogenic pressures in coastal environments, including intensive exploitation. As a result, the populations of species such as the European sea urchin *P. lividus* (distributed from the northeastern Atlantic to the coasts of the Mediterranean Sea) have recently collapsed, and these species are becoming extremely rare in some regions (Yeruham *et al.*, 2015). In addition, the depletion of reproductive adults renders the procurement of an acceptable number of wild mature animals, able to release gametes of good quality, increasingly difficult (Cirino *et al.*, 2017). However, in recent years, new research opportunities have emerged, such as the use of the sea urchin for environmental immunotoxicology, and a successful culture of *P. lividus* adult immune cells has been established (Pinsino & Alijagic, 2019). This may provide the basis for the design of new approaches for monitoring the quality of the environment and estimating the hazards represented by test materials. Besides sea urchin embryos, larvae (Fig. 4D) and cultured immune cells (Fig. 4E), adult sea urchins (e.g. *P. lividus*) have also been used successfully in ecotoxicological testing and environmental monitoring studies, thus further highlighting the suitability of these organisms as diversified model species for this type of research (Sugni *et al.*, 2007; Rouane-Hacene *et al.*, 2018; Parolini *et al.*, 2020).

Other non-conventional echinoderms have been successfully used in research, particularly in assays focusing on regeneration as an alternative and complementary perspective to embryotoxicity tests (Sugni *et al.*, 2007). Regeneration is an intrinsic part of the life history of echinoderms. Alterations to regeneration can strongly affect animal performance, including survival and fitness, and thus, potentially, have an immediate impact on the entire population. Among the different model species, the crinoid *Antedon mediterranea* (Fig. 4F) is a good candidate for environmental testing and monitoring. Arm regeneration (Fig. 4G) is an extremely sensitive endpoint as it is impacted by exposure to endocrine disruptors in environmentally relevant quantities, which lead to alterations in skeletogenic

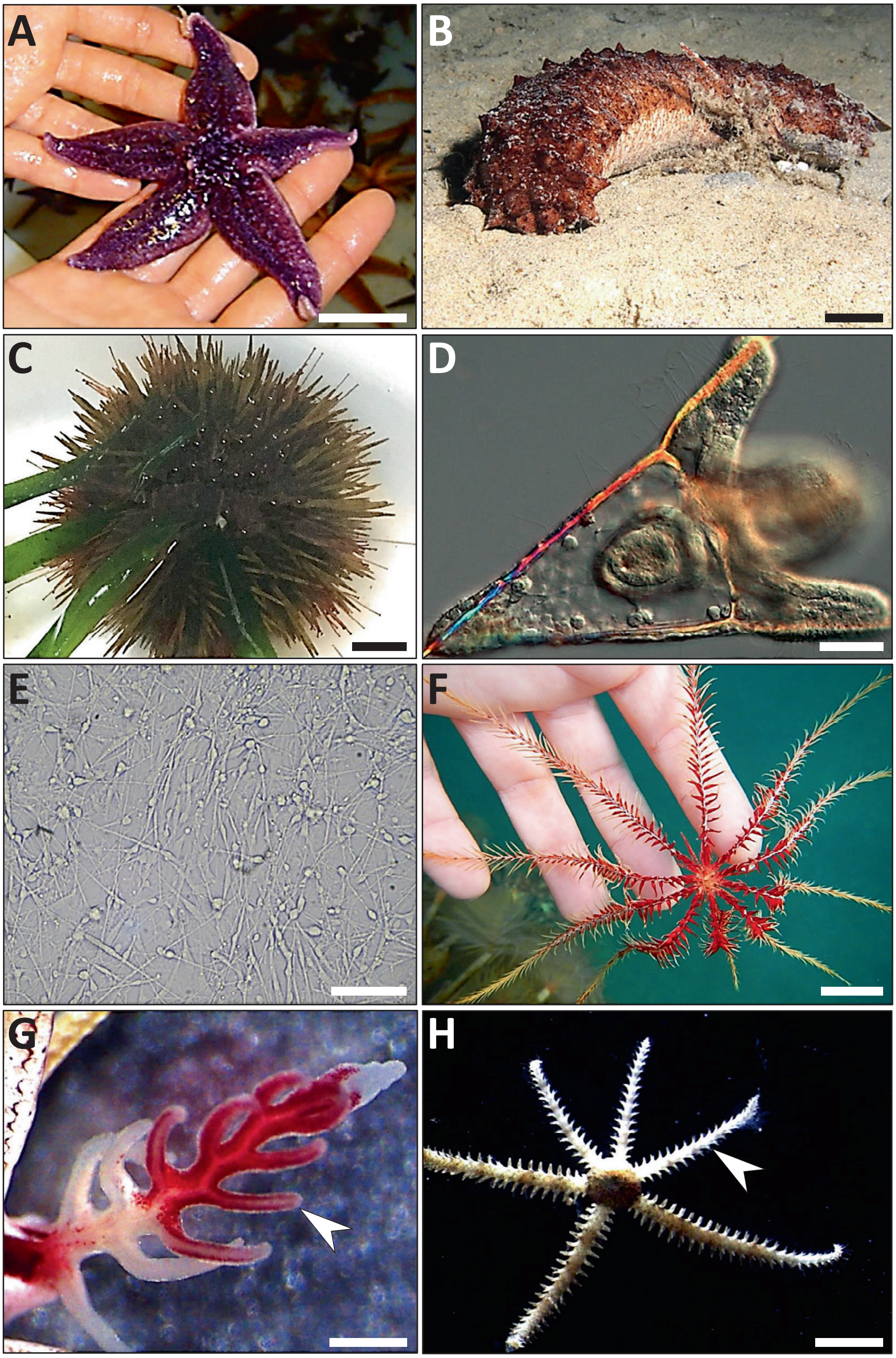


Fig. 4. Representative echinoderm experimental models used in ecotoxicological research. (A) The starfish *Asterias rubens*; scale bar = 2 cm. (B) The sea cucumber *Holothuria tubulosa* on a muddy substrate; scale bar = 2.5 cm. (C) The sea-urchin *Paracentrotus lividus* feeding on *Posidonia oceanica*; scale bar = 1 cm. (D) A pluteus larvae of *P. lividus*; scale bar = 25 μm. (E) Culture of *P. lividus* immune cells; scale bar = 30 μm. (F) The crinoid *Antedon mediterranea*; scale bar = 2 cm. (G) Arm regeneration (arrow) of *A. mediterranea* 2 weeks post-amputation; scale bar = 1 mm. (H) The brittle star *Ophiactis virens* undergoing regeneration (arrow) after asexual reproduction (fission); scale bar = 1.5 mm.

processes, cell recruitment pathways, and proliferation (Candia Carnevali *et al.*, 2001; Sugni *et al.*, 2007, 2008, 2010). Brittle stars can also serve as optimal marine sentinels for environmental monitoring. Burrowing and deposit/filter feeder species such as *Amphiura* spp., *Microphiopholis gracillima*, or the cosmopolitan *Amphipholis squamata*, can easily accumulate contaminants (D'Andrea, Stancyk & Chandler, 1996; Gunnarsson & Sköld, 1999; Barboza, Martins & Lana, 2015) and eventually display altered physiology, for example, a reduction in natural bioluminescence (Deheyn, Jangoux & Warnau, 2000). Notably, evidence of ingestion of plastic materials by brittle stars was first reported over 40 years ago (Courtene-Jones *et al.*, 2019). Similar to *A. mediterranea*, arm regeneration in ophiuroids (Fig. 4H) is a valuable property in the assessment of environmental contamination: in the epibenthic carnivorous *Ophioderma brevispina*, the organotin compounds bis(tri-n-butyltin)oxide (TBT) and bis(triphenyltin)oxide (TPT) reduced arm regrowth at concentrations as low as 0.1 μg/L, possibly as a result of neurotoxic effects (Walsh *et al.*, 1986). Lastly, sea cucumbers (holothuroids) have emerged as bioindicators of environmental contamination (Marrugo-Negrete *et al.*, 2021). These benthic scavengers and burrowing organisms represent excellent tools for monitoring contaminants accumulated in sediments. Furthermore, their non-selective feeding strategy causes the ingestion of any available item on the substratum, thus leading to a diffuse presence of particles of anthropogenic origin in their digestive tracts (e.g. MPs; Iwalaye, Moodley & Roberston-Andersson, 2020).

(6) Standardised models – overview

Very few species among the above-mentioned taxa have been used for (eco)toxicological tests. However, many of the species used in standardised protocols possess exceptional characteristics such as high sensitivity to various chemicals, ubiquitous presence in aquatic ecosystems, ease of laboratory maintenance, and the capacity to measure numerous physiological parameters. These features drive their exploitation in the field of (eco)toxicology. In recent years, new species have been used to provide additional data about the ecotoxicity of pollutants in aquatic ecosystems. Additionally, efforts are being made to develop alternative *in vitro* and *in silico* models. The development of *in vitro* tools such as primary cell cultures and cell lines from various organs allows the use of cell cultures as models to analyse the underlying mechanisms of toxicity. From this perspective, organisms that are not currently used as standard models are of great interest for the development of new tools.

IV. NON-STANDARD MODELS

(1) Porifera

Sponges (Porifera) are cosmopolitan sessile organisms distributed in fresh and marine waters across the globe and are key invertebrates in marine benthic ecosystems (Fig. 5A, B). They are amongst the oldest of the modern Metazoa, located at the basal position of the phylogenetic tree (Wörheide *et al.*, 2012; Redmond & McLysaght, 2021). Phylum Porifera is divided into four clades: (*i*) Calcarea (calcareous sponges with calcium carbonate skeletal spicules); (*ii*) Demospongiae (demosponges, mostly with organic or silicon dioxide skeletons; a few species without skeletons), the largest group containing more than 76% of all living sponge species; (*iii*) Homoscleromorpha (the only class of sponges with a true basal lamina); and (*iv*) Hexactinellida (glass sponges, with syncytial structure and silicon dioxide spicules; not used as bioindicators due to their rarity). Porifera are diblastic organisms without organs, and many of their cells are capable of de- and trans-differentiation into other cell types. The number of cell types in sponges can exceed a dozen, and the diversity of morphogenesis during development is comparable to that of bilaterians (Simpson, 1984; Ereskovsky, 2010). The outer layer of the sponge, composed of exo-pinacocytes, is pierced by the channels of the aquiferous system. The aquiferous system consists of channels and chambers, the latter lined by choanocytes, which are flagellate cells. The beating of choanocyte flagella produces a flow of water through the aquiferous system, and the choanocytes remove food particles from the water for consumption. The space between the outer layer (exo-pinacocytes) and the wall of the aquiferous system (a layer of choanocytes and endopinacocytes) is filled with mesohyl, an extracellular matrix containing populations of resident cells, e.g. amoebocytes/archaeocytes, sclerocytes, lophocytes, and spherulous cells (Simpson, 1984). Sponges contribute greatly to ecosystem functioning as bio-filtrators: they filter more than 900 times the volume of their body per hour (Ludeman, Reidenbach & Leys, 2017), and effectively trap particles smaller than 10 μm in size (Pile, Patterson & Witman, 1996; Coma *et al.*, 2001; Yahel *et al.*, 2007). They receive dissolved organic material from the water column and make it available to higher trophic levels as detritus (de Goeij *et al.*, 2013; Rix *et al.*, 2016, 2017, 2018). Sponges are rich in symbiotic bacteria, which contribute to the overall recycling of organic material, polyphosphate production and the storage and supply of fresh photosynthate (Taylor *et al.*, 2007; Colman, 2015). Additionally, sponges are critical components of ecosystems, providing habitats for other animals and food sources for other species. As is the case for other filter-feeders, sponge cells exhibit physiological responses to pollutants present in the water.

Roveta *et al.* (2021) reviewed 50 biomonitoring studies performed on sponges. Most of the work relied on the ability of sponges to accumulate chemical elements from the water in their tissues. Heavy metal content of sponges collected from contaminated sites differed significantly from that in sponges collected from uncontaminated locations (Patel, Balani & Patel, 1985; Hansen, Weeks & Depledge, 1995; Araújo *et al.*, 2003; Perez *et al.*, 2005; Rao *et al.*, 2006; Cebrian, Uriz & Turon, 2007; Batista *et al.*, 2014). Demosponges with silica spicules are of particular interest, because during spiculogenesis,

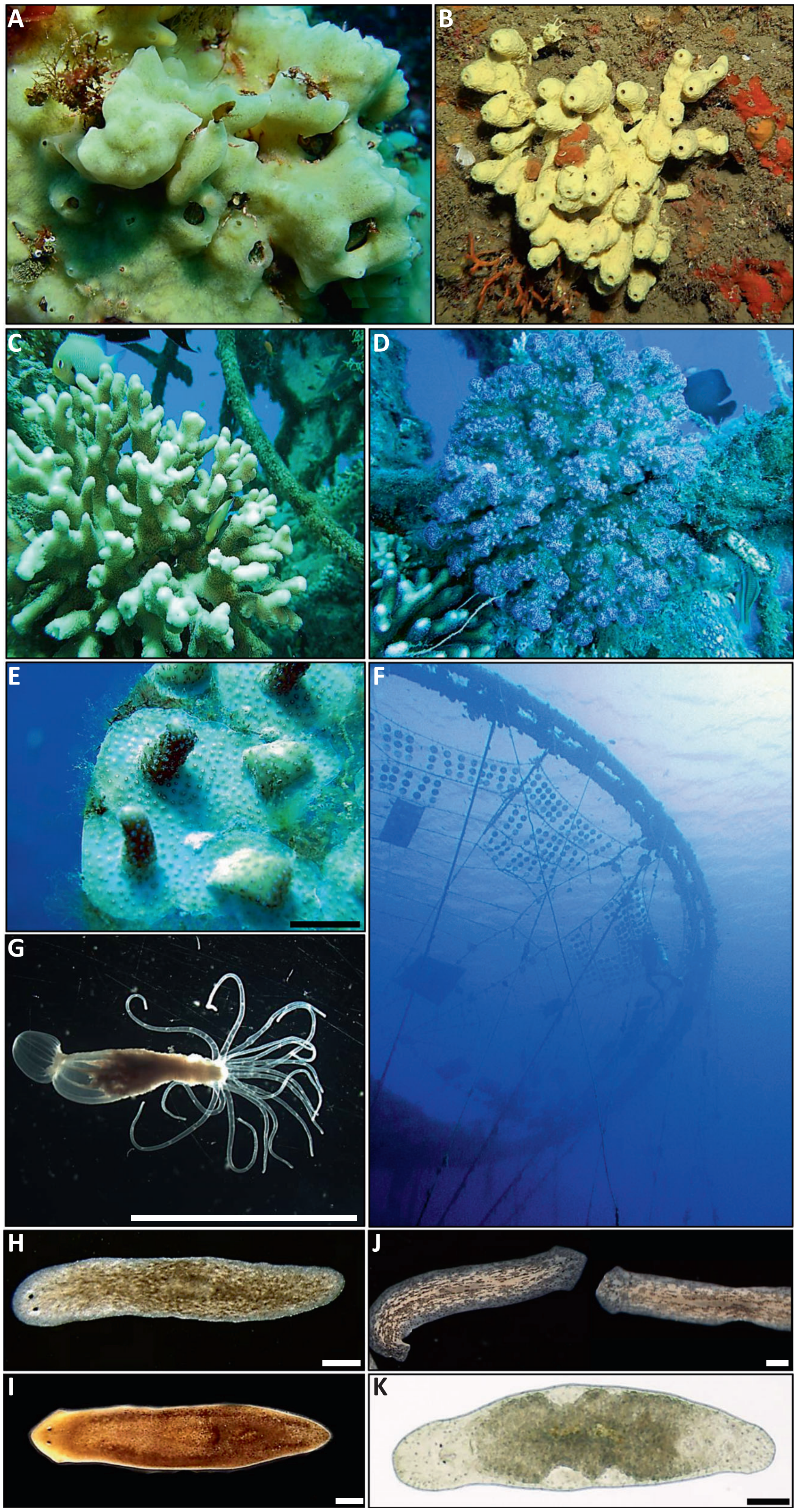


Fig. 5. Non-standard models. (A, B) Sponges. (A) *Halichondria panicea*. (B) *Aplysina cavernicola*. (C–F) Cnidarians. (C) *Stylophora pistillata*. (D) *Pocillopora damicornis*. (E) Nubbins (small fragments) of *Stylophora pistillata* portraying horizontal spread on the substrate. (F) Coral nursery. (G) *Nematostella vectensis*. (H–K) Flatworms. (H) *Schmidtea mediterranea*. (I) *Dugesia japonica*. (J) *Dugesia tigrine*. (K) *Macrostomum lignano*.

the sclerocytes accumulate not only silicon but also heavy metals in their spicules (Truzzi *et al*., 2008; Annibaldi *et al*., 2011; Illuminati *et al*., 2016). Other physiological changes that may be observed following exposure to sublethal concentrations of pollutants include changes in sponge cell behaviour (Cebrian *et al*., 2003, 2006), metabolism (Saby *et al*., 2009; Selvin *et al*., 2009), production of ROS, expression of stress genes (Müller *et al*., 1994, 1995, 1996) and increased apoptosis (Batel *et al*., 1993). For example, in the case of induction of genotoxicity by exposure to heavy metals (Akpiri, Konya & Hodges, 2017), changes in the expression of metallothionein (Berthet *et al*., 2005), post-translational modification of tubulin (Ledda *et al*., 2013), and expression of apoptosis-associated prosurvival factor (Luthringer *et al*., 2011) have been reported. Other biomarkers currently used in sponges include the altered expression or activity of heat shock proteins (HSPs) (e.g. the effect of copper on *Crambe crambe*; Agell *et al*., 2009), glutathione (GSH) and glutathione S-transferases (GST) (e.g. the effects of industrial, urban, and harbour pollution on *Sarcotragus spinosulus*; Khati *et al*., 2018). These lines of evidence support the use of sponges in retroactive assessments of water pollution (i.e. they can be viewed as providing an 'ecotoxicological memory'), particularly in long-living species. Suggested models for toxicological tests include ubiquitous species of Demospongiae like *Halichondria panicea* (Fig. 5A), which is well studied in many respects, *Aplysina cavernicola* (Fig. 5B), and *Spongia officinalis*, which is found in the Mediterranean Sea, a focal region for aquatic pollution studies.

At the cellular level, the states of cells like choanocytes, which are in direct contact with the filtered water, can be used as biomarkers. Along with archaeocytes, which are amoebocytes residing in the mesohyl (Funayama, 2013), choanocytes are part of the stem cell system of sponges and undergo constant renewal due to high rates of proliferation and the shedding of old cells into the lumen of choanocyte chambers (De Goeij *et al*., 2009). Under constant stress conditions, the pool of both stem cells in general, and choanocytes in particular, can change (e.g. in cell distribution, cell cycle phases, population size); this trait can be easily assessed by flow cytometry, as described for *Suberites domuncula* and *Haliclona oculata* (Sipkema *et al*., 2004; Schippers *et al*., 2011).

In addition, the sexual reproduction, asexual reproduction (by budding, gemmule formation) and regeneration (the development of a new animal from a body fragment and whole-body regeneration from cell aggregates) of sponges could be of interest in the assessment of environmental stress factors. The sexual reproduction of sponges leads to the formation of free-floating larvae that, following settling, undergo metamorphosis into juvenile sponges (Ueda *et al*., 2016). Exposure of the larvae of *C. crambe* and *Scopalina lophyropoda* to heavy metals and PAHs inhibits their settling. Copper ions act synergistically with PAHs, although treatment with Cu^{2+} alone does not inhibit larval settling (Cebrian & Uriz, 2007*a*). The model of larval settlement and metamorphosis is potentially attractive and may be more sensitive than the model for adult sponges, but the molecular mechanisms that enable competence acquisition and metamorphosis are poorly understood (Conaco *et al*., 2012). Additionally, the extensive asexual reproduction and regeneration capabilities of sponges, even from adult body fragments (Baldacconi *et al*., 2010; Çelik *et al*., 2011), has enabled the production of clonal animals providing reproducible responses to contamination (Osinga, Tramper & Wijffels, 1999; Schippers *et al*., 2012) that can be used for pollution monitoring. Furthermore, primmorphs, artificial models generated from sponges belonging to the taxa Demospongiae, Calcarea, and Homoscleromorpha (Lavrov & Kosevich, 2014; Akpiri, Konya & Hodges, 2020), also have potential as *in vitro* tools for toxicological tests. Primmorphs are obtained from sponge tissues dissociated into cells by mechanical or chemical methods. These cells form aggregates and dedifferentiates, and the outer layer of the aggregated cells forms a covering layer, resulting in the formation of a radially symmetrical primmorph. The cells within the primmorph then differentiate and small fragments that attach to the substrate form new sponges by extending their structures (Lavrov & Kosevich, 2014, 2016; Ereskovsky *et al*., 2021). Differentially expressed genes, and some of the mechanisms underlying the dissociation–reaggregation phenomena in the sponge *Oscarella lobularis* have been revealed (Vernale *et al*., 2021). Heavy metals influence the behaviour of cells in the reaggregation process (Cebrian & Uriz, 2007*b*,*c*). Further studies of the effects of pollutants on the aggregation and development of primmorphs will allow the creation of effective and suitable methods for evaluating biological reactions to pollution. Additional breakthroughs in terms of *in vitro* tools include the development of cell cultures. Primary cultures of various species have been initiated (Rinkevich, Blisko & Ilan, 1998*a*; Pomponi, 2006; Urban-Gedamke *et al*., 2021) and developing cells have been cryopreserved (Munroe *et al*., 2018), but many attempts to obtain cell lines have failed (Grasela *et al*., 2012). Conkling *et al*. (2019) reported rapid cell division in primary cultures established from nine sponge species, demonstrating a cultured cell life span of 21–35 days. Such improvements may open additional avenues for the broad-scale use of sponge cell cultures in *in vitro* pollution assessment.

(2) Cnidaria

Cnidarians, especially corals (Fig. 5C, D), have been successfully used in ecotoxicological experiments (Fig. 1; Roveta *et al*., 2021) and their potential for applications in biomonitoring has been repeatedly highlighted (Rainbow, 2002). This potential is supported by the very simple tubular body morphology of cnidarians, which are diploblastic organisms consisting of two tissue layers, the outer epidermis and the inner gastrodermis, which lines the gastrovascular cavity. These layers are separated by an extracellular matrix, the mesoglea, containing some cells in Scyphozoa and Anthozoa. Both the epidermal and gastrodermal layers are in constant contact with the environment. Cnidarians can reproduce both sexually and asexually. Asexual reproduction, which characterises many cnidarian species, ensures a supply of high numbers of genotypic replicates with low variations in physiological/biochemical parameters, which is an important asset in performing reliable tests (Shafir, Van Rijn & Rinkevich, 2001, 2003, 2006). Other advantages of cnidarians for ecotoxicological testing include their wide distribution in almost all freshwater, brackish and marine ecosystems, their presence in both temperate and tropical zones (Howe, Reichelt-Brushett & Clark, 2012), and the number of cnidarian species that deposit hard skeletons. However, there is a relative paucity of ecotoxicological studies exploiting cnidarians, as shown in studies using the comet assay to examine aquatic genotoxicity (Svanfeldt *et al.*, 2014). Only seven cnidarian species were included in a recent review on the comet assay in model invertebrates and unicellular organisms (Gajski *et al.*, 2019), which covered almost 300 publications and hundreds of species: the hydrozoan *Hydra magnipapillata*, the sea anemones *Anthopleura elegantissima*, *Actinia equina* and *Bunodosoma cangicum*, and the corals *Stylophora pistillata* (Fig. 5C), *Seriatopora hystrix* and *Montastraea franksi*. Testing on cnidarians typically involves a small number of model animals for which laboratory husbandry is available, with difficulties emerging when animals collected from the wild are used, as these procedures are inherently destructive (Summer, Riechelt-Brushett & Howe, 2019).

Hydra is the most widely used hydrozoan genus in monitoring and ecotoxicological studies. *Hydra* polyps are effective for toxicity testing since they are easily adapted for mass culture in the laboratory (Loomis, 1953), forming large numbers of individuals that normally reproduce asexually by budding. *Hydra* is a reliable indicator of pollution due to its single polyp structure, its basic anatomy, and the possibility to use genetically identical or similar individuals in the same or separate tests (Beach & Pascoe, 1998): this ensures reproducible results with a low coefficient of variation (Quinn, Gagné & Blaise, 2012). The *Hydra* genome has been completely sequenced and the full range of next-generation tools is available (Chapman *et al.*, 2010). During the last three decades, several *Hydra* species [primarily *H. attenuata* (=*H. vulgaris*) and *H. magnipapillata*] have been used in toxicology studies with numerous compounds, including acute, sub-chronic, chronic, reproductive, developmental, carcinogenic, and genetic toxins (Quinn *et al.*, 2012; Patwardhan & Ghaskadbi, 2013; Zeeshan *et al.*, 2017; Murugadas *et al.*, 2019; Cera *et al.*, 2020). Many of these studies used the *Hydra* assay (Johnson & Gabel, 1992). In comparison, ecotoxicological research on jellyfish species is sparse (Faimali *et al.*, 2014; Ohdera *et al.*, 2018). While generally considered to be robust towards anthropogenic stressors (e.g. Richardson *et al.*, 2009), it has long been known that jellyfish accumulate toxic metals from the environment in high concentrations and are therefore a potential source of metal transfer in food chains (Romeo & Gnassia-Barelli, 1992) and may themselves be sensitive to environmental toxicity. The size of medusae can be a limitation in studies employing a large number of specimens, although ecotoxicological assays on ephyras have been carried out successfully, primarily on the common species *Aurelia aurita* (e.g. Faimali *et al.*, 2014; Costa *et al.*, 2015; Olguín-Jacobson *et al.*, 2020), a proposed model organism in ecotoxicology due to its high sensitivity to pollutants (Olguín-Jacobson *et al.*, 2020). In addition, some recent studies have explored the impact of chemicals on the sessile stage, the polyps (Olguín-Jacobson *et al.*, 2020; Pinteus *et al.*, 2020), a protocol that has simplified the use of scyphozoan species in ecotoxicological assays.

Members of the Anthozoa (including sea anemones and corals) are unique tools for ecotoxicological studies since they may live for prolonged periods of hundreds of years, accumulating an ecotoxicological memory, and some, like scleractinian corals, may assimilate environmental records into their skeletons (Shah, 2021). A search of *Web of Science* identified 3045 papers published in the last decade that contained the key words 'coral + (ecotoxicology or toxicology or pollution)'. One of the most diversified ecosystems on Earth is found in coral reefs that provide numerous ecosystem services, including a means of living for hundreds of millions of people (UN Environment Programme, 2023). Their monitoring requires the development of a suite of sensitive, standardised ecotoxicological tests for assessing short- and long-term anthropogenic impacts on corals (Branton, 2018). However, coral ecotoxicology at the laboratory level (Fig. 5E) is methodologically restricted, as coral husbandry is not easy. This is primarily due to the numbers of organisms required for adequate replication, as collecting corals from the field is inherently destructive (Vijayavel & Richmond, 2012). To overcome this difficulty, stocks of coral colonies (with varying numbers of coral genotypes for each species) can be developed and maintained in underwater coral nurseries (Fig. 5F; Levy *et al.*, 2010; Rinkevich, 2015, 2021; de la Cruz *et al.*, 2015), an approach with new market opportunities (Rinkevich, 2015). Numerous healthy ramets can be produced from each coral genet, opening new avenues for ecotoxicological research.

A range of laboratory-based ecotoxicological assays have been developed for corals, including histopathology, productivity levels, calcification rates, reproductive effort, and state of symbionts (i.e. bleaching events and/or photosynthetic activity), yet there is a need for the development of more defined bioassays (Branton, 2018). One such assay is the nubbins assay (Shafir *et al.*, 2001, 2003, 2006; Fig. 5E), based on the repeated use of numerous very small fragments from selected coral genotypes. This assay provides a cheap, standardised and low-variation approach, and limits the ecological impact of harvesting large numbers of corals for research. This protocol has been employed to evaluate the toxicity of organic carbon, heavy metals, household detergents, MPs, oil and oil dispersants, ultraviolet (UV) filters and more to reef corals (Ferrier-Pagès *et al.*, 2005; Kuntz *et al.*, 2005; Shafir, Van Rijn & Rinkevich, 2007; Shafir, Halperin & Rinkevich, 2014; Chen *et al.*, 2012; Vijayavel & Richmond, 2012; Svanfeldt *et al.*, 2014; Corinaldesi *et al.*, 2018; He *et al.*, 2019; Aminot *et al.*, 2020; Mitchelmore *et al.*, 2021; Xiao *et al.*, 2021). In addition, toxicity tests conducted on field-collected coral material, including planulae, gametes, and fragments, have provided important results (reviewed in

Howe *et al.*, 2012; Loya & Rinkevich, 1980) and additional methodologies, like vital staining (Shefy, Shashar & Rinkevich, 2021), have recently been added.

Sea anemones are also good candidates for routine ecotoxicological tests. The absence of a calcium carbonate skeleton is a major feature of scleractinians, and the availability of symbiotic and aposymbiotic sea anemones has been noted (Howe *et al.*, 2012). Sea anemones can be easily maintained in laboratory settings and in large cultures, and research has shown that they are susceptible to a variety of pollutants (Anjos *et al.*, 2017; Howe *et al.*, 2012, 2017; Trenfield *et al.*, 2017; Ianna *et al.*, 2020; Vitale *et al.*, 2020; Rosner *et al.*, 2023). The sea anemones *Aiptasia pulchella* (*Exaiptasia pallida*), *Anemonia viridis* and *A. equina* (Howe *et al.*, 2012; Trenfield *et al.*, 2017; Vitale *et al.*, 2020) have been suggested as the best model taxa for research. Following the genomic sequencing of *Nematostella vectensis* (Fig. 5G), ecotoxicology studies have revealed transcriptome responses to stress and pollutants (Goldstone, 2008; Elran *et al.*, 2014; Tino *et al.*, 2014; Tarrant *et al.*, 2018). The use of *in vitro* approaches (Rabinowitz, Moiseeva & Rinkevich, 2016) may further enhance ecotoxicological tests on cells derived from this sea anemone and other related species. Examples of toxicological effects on *N. vectensis* are shown in Fig. 6, including impacts on development and regeneration (Klein *et al.*, 2021).

(3) Platyhelminthes

Platyhelminthes, commonly known as flatworms, are a phylum of bilaterian acoelomate organisms. They contain distinct brain, digestive, and excretory systems, but lack circulatory, skeletal, or respiratory organs (Hyman, 1951). Platyhelminthes can be free-living, commensal, or parasitic. The phylum is subdivided into several clades, including Tricladida. This taxon includes both marine and freshwater species, some of which have been utilised in ecotoxicology over the last decade. The wide range of environments in which they live makes them ideal models to test ecosystem health. Among the Platyhelminthes, the term ‘planarian’ is used to describe the free-living flatworms belonging to Tricladida, which are the best studied models from this phylum. These animals reproduce through sexual and asexual (animal fission) strategies (Vila-Farré & Rink, 2018). The ecosystem functions of flatworms have recently been reviewed (Majdi, Kreuzinger-Janik & Traunspurger, 2016; Vila-Farré & Rink, 2018) and we refer the reader to these reviews for an extended discussion.

Many species of planarians have an immense capacity for regeneration, with small pieces of a single animal being able to generate a multitude of progeny (Ivankovic *et al.*, 2019). Their regeneration capacity depends on the presence of ASCs called neoblasts, which can represent >30% of cells in a single adult individual (Rink, 2013). Several well-studied models are available, including *Schmidtea mediterranea* (Fig. 5H). A complete genome, various transcriptomes and a culture of neoblasts are available for this species (Grohme *et al.*, 2018; Wu & Li, 2018; Lei *et al.*, 2023). Its ability to regenerate in the presence of various chemical compounds has been used in ecotoxicology. Studies have also considered behavioural endpoints, such as mobility (Deochand, Costello & Deochand, 2018; Pestana & Ofoegbu, 2021). In all experiments, a culturing methodology, following stringent standardised protocols, has been indispensable to the screening process (e.g. Wu & Li, 2018). This is rarely the case for other species. In the absence of standardisation, the effects of specific chemicals are (and must be) evaluated in different populations, as populations from different geographical areas can show differences in sensitivity (Indeherberg, Van Straalen & Schockaert, 1999). More economical and efficient technologies have been developed recently to analyse the effects of molecules on the development and behaviour of model systems. Such technologies can be applied to a variety of planarians, particularly *Dugesia japonica* (Fig. 5I), *S. mediterranea*, and *Girardia tigrina*. These methods allow cross-species comparisons, indicating the best species to be utilised for each chemical test or the optimal physical attributes to be studied (Hagstrom *et al.*, 2015; Hagstrom, Cochet-Escartin & Collins, 2016; Ireland *et al.*, 2020). Interestingly, some studies have found that, under certain conditions, planarians such as *D. japonica* possess comparable sensitivity to established models used in mammalian toxicology (Hagstrom *et al.*, 2019).

Most ecotoxicological studies use the morphology and behaviour of planarians as their endpoints due to the well-characterised morphology of many species and the availability of tests that allow the tracking of a few well-known behavioural traits. These include locomotion, thermotaxis, negative phototaxis, and the capacity to ‘scrunch’ (i.e. display a muscle-driven oscillatory escape gait) in reaction to toxic heat (Cochet-Escartin, Mickolajczk & Collins, 2015). The neuropharmacological effects of many chemicals have been assessed using planarians (Pagán, Rowlands & Urban, 2006; Yuan, Zhao & Zhang, 2012; Stevens *et al.*, 2015). Such studies focused on specific behavioural patterns following exposure to drugs that act on neural transmission (e.g. Buttarelli, Pellicano & Pontieri, 2008; Rawls *et al.*, 2008; Sacavage *et al.*, 2008; Dziedowiec *et al.*, 2018) and the effects of different drugs on the natural behaviour and regeneration of planarian species. For example, compounds such as carbamazepine, used to treat epilepsy, or fluoxetine, used to treat depression, have revealed effects on nutrition and reproduction of *S. mediterranea* (Ofoegbu *et al.*, 2019). Compounds used for the treatment of Alzheimer’s disease (e.g. donepezil, tacrine, galantamine, and rivastigmine) were studied in the related species, *Dugesia tigrina* (Fig. 5J; Bezerra da Silva *et al.*, 2016). The behaviour of planarians can now be tracked on a large scale using emerging new technologies such as automatic tracking systems (Zhang *et al.*, 2019*a*). Such platforms enable the systematic analysis of large numbers of compounds and the tracking of complex sets of parameters. Using large-scale screening platforms also allows the testing of multi-target mixtures with therapeutic potential that are derived from natural marine sources (e.g. Henry & Wlodkowic, 2019; Zhang *et al.*, 2019*a*).

Although the potential for automatisation is clear, most toxicological research on planarians has focused on a small number of substances and employed more traditional methodologies. These approaches have been used

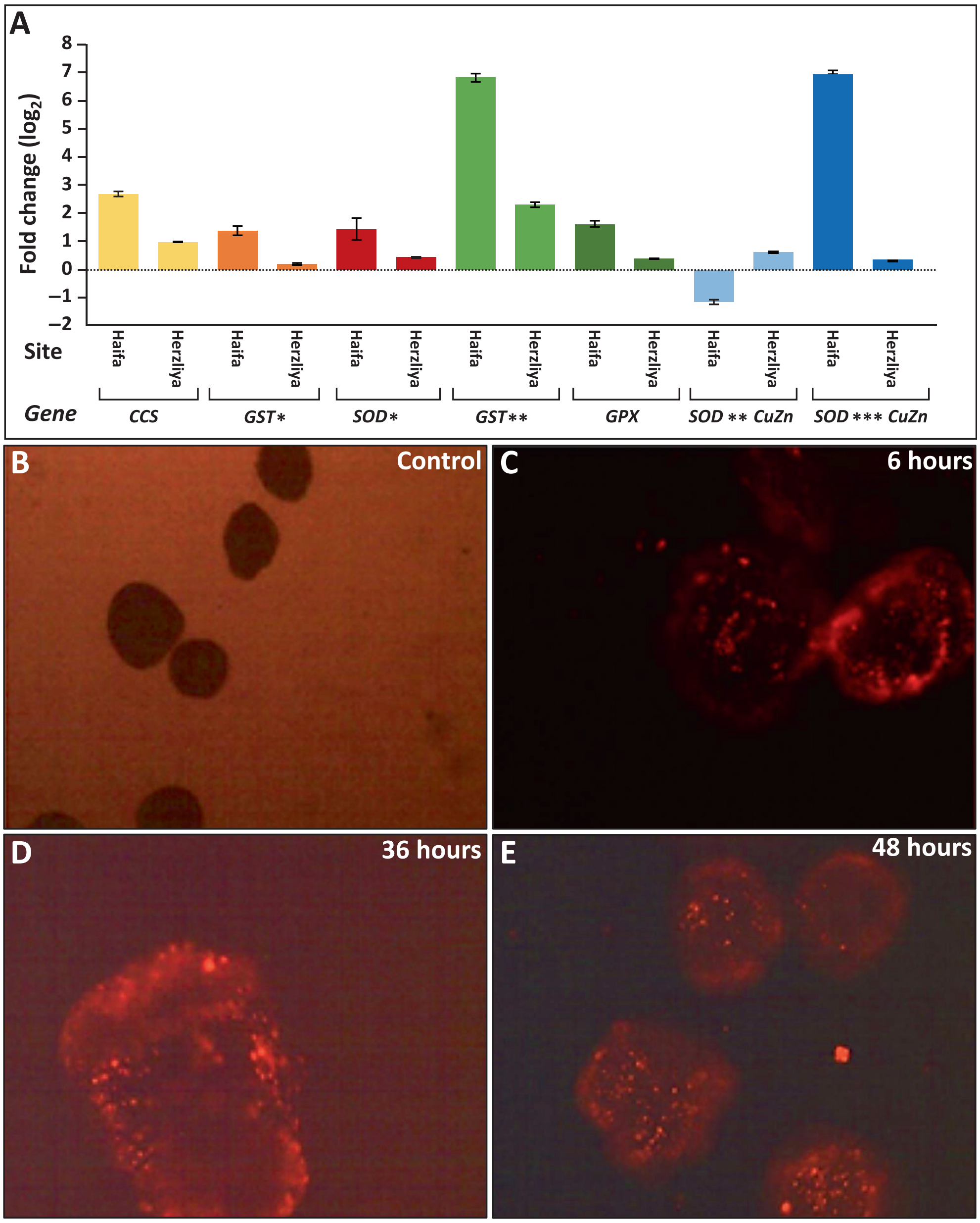


Fig. 6. Toxicological tests applied to *Nematostella vectensis* as a model organism. (A) Gene expression changes in mature *N. vectensis* after 4 days exposure to polluted sea water collected from Haifa port and Herzliya marina. Relative gene expression comparisons were made using quantitative polymerase chain reaction (qPCR) between animals exposed to polluted sea water diluted with double-distilled water (DDW; $N = 6$ for Haifa and $N = 6$ for Herzliya) in a 1:2 ratio, and animals grown in clean sea water samples from Tel Shikmona beach, also diluted with DDW in a 1:2 ratio ($N = 6$). The expression changes are presented as fold changes on the $\log_2$ scale. The tested genes represent *N. vectensis* orthologues of well-known oil pollution biomarkers retrieved from the *N. vectensis* genome database (Putnam *et al.*, 2007). Transcript id is specified in parentheses; Asterisks are used to distinguish between paralogous genes. *CCS*, copper chaperone for superoxide dismutase (227361); *GPX*, glutathione peroxidase (90698); *GST*, glutathione S-transferase (*GST** 113255; *GST*** 86756); *SOD*, superoxide dismutase (*SOD** 94316; *SOD*** 234825; *SOD**** 165732). Error bars represent standard deviations (SD). (B–E) *N. vectensis* embryos following electrophoresis with pTimer-1 Vector (Takara) under the control of the *CCS* gene promoter and exposed to the accommodated fraction of a medium containing 30 ppm crude oil. The time following electroporation is indicated. The embryos were alive at the time the photographs were taken.

to assess the impact of insecticides, pesticides, metals, and neurotoxic compounds (Simao *et al.*, 2020; Dornelas *et al.*, 2021; Silva *et al.*, 2022).

Few studies have tracked the effects of chemicals on the molecular and cellular composition of flatworms (Plusquin *et al.*, 2016). These studies rely on flatworms because they are particularly well suited for investigating how environmental stressors regulate stem cells since it is simple to observe stem cell dynamics (Stevens *et al.*, 2018). Plusquin *et al.* (2016) analysed the neoblast dynamics of the flatworm *Macrostomum lignano* (Fig. 5K) in the presence of environmentally relevant metals, using a combination of morphological, gene expression and immunochemical methodologies, allowing for detailed characterisation of the effects of metals on the proliferation of stem cells. In a similar study, the exposure of *S. mediterranea* neoblasts to the carcinogen methyl methanesulfonate (MMS), resulted in a reduction in neoblast proliferation rate, which was attributed to induction of DNA damage (Plusquin *et al.*, 2012).

In addition to existing studies on the general effects of pollutants on behaviour and regeneration, other studies have examined their specific effects on cellular components, such as DNA (e.g. chromosomal assays, nuclear DNA fragmentation) or enzymatic activities (e.g. CAT or HSPs; reviewed by Knakievicz, 2014). Environmental effects on the population level were tested by tracing reproductive fitness, such as the number of offspring or changes in sex ratios (Miyashita *et al.*, 2011) while an impact on the microbiota was demonstated by Bijnens *et al.* (2021) on *S. mediterranea*.

(4) Tunicata

Tunicates are filter-feeding marine invertebrates that are found in oceans and seas with salinities over 2.5%. They are classified as a sister group of vertebrates (Delsuc *et al.*, 2006, 2008) and are named after their 'tunic', which is the outer layer that encloses the animal body (Deck, Hay & Revel, 1967; Welsch, 1984; Van Daele *et al.*, 1992; Burighel & Cloney, 1997; Di Bella, Carbone & De Leo, 2005; Xu *et al.*, 2008; Hirose, 2009). Tunicates are subdivided into sessile ascidians, pelagic thaliaceans, and larvaceans (Stach, Braband & Podsiadlowski, 2010). Ascidians are the best-studied group of tunicates and encompass around 2300 species. They are considered reference organisms in investigations of the developmental biology, regeneration, allorecognition, and immunobiology of invertebrate chordates (Satoh, 1994, 2016; Burighel & Cloney, 1997; Stoner, Rinkevich & Weissman, 1999; Corbo, Di Gregorio & Levine, 2001; Khalturin *et al.*, 2003; Rinkevich, 2004; Jeffery, 2015; Franchi & Ballarin, 2017; Ferrario *et al.*, 2020; Gordon, Manni & Shenkar, 2019; Gordon *et al.*, 2021; Ballarin *et al.*, 2021). Most ascidians have a biphasic life history with two distinct body plans: a swimming tadpole larva and a sessile adult. The tadpole larva is considered a prototype of the chordate ancestor (Fig. 7A), with which it shares features such as a notochord, dorsal neural tube, pharynx provided with gill slits, and a muscular tail (Satoh, 1994; Burighel & Cloney, 1997). Studies have found ascidians to be reliable model organisms for ecotoxicological bioassays due to their production of eggs almost all year round, rapid development, simplicity of the larval stage (Fig. 7A), ease of management in the laboratory, and their small sequenced genome (Tosti & Gallo, 2012; Gallo & Tosti, 2015; Metri *et al.*, 2019). Ascidians include both solitary and colonial species.

(*a*) *Solitary ascidians*

Solitary ascidian species have become increasingly popular for toxicological studies. *Ciona intestinalis* is a well-studied ascidian species (Mansueto *et al.*, 1993; Bellas, Beiras & Vázquez, 2003) that has been shown to include at least two cryptic species, *Ciona robusta* (Fig. 7B) and *C. intestinalis* (Suzuki, Nishikawa & Bird, 2005; Caputi *et al.*, 2007; Nydam & Harrison, 2007; Zhan, Macisaac & Cristescu, 2010; Brunetti *et al.*, 2015). We refer to both species herein as *Ciona* spp. Additional models being investigated include *Halocynthia roretzi*, *Ciona savignyi*, *Microcosmus exasperatus*, *Phallusia fumigata*, and *Phallusia mammillata* (Pennati *et al.*, 2006; Choi *et al.*, 2014; Cahill *et al.*, 2016*a*; Gomes *et al.*, 2019; Anderson & Shenkar, 2021). The solitary ascidian phylogenetic position, a fully sequenced genome and available genomic tools make them attractive models for studying the mode of action (MOA) of toxic compounds (Dehal *et al.*, 2002; Stolfi & Christiaen, 2012).

The larvae have a simple structure consisting of only six tissue types (Fig. 7A), making it easy to assess phenotypic alterations during development and to discriminate between specific and non-specific toxicity of substances (Katz, 1983; Nicol & Meinertzhagen, 1991; Meinertzhagen, Lemaire & Okamura, 2004; Jiang *et al.*, 2005; Horie *et al.*, 2008; Hudson, 2016). The juveniles are transparent, allowing easy observation of morphological changes caused by stressors, and have been used to evaluate the impact of pollutants such as MPs, tributyltin, bisphenol A, drugs, and oil dispersants on survival and morphology (Mansueto, Cangialosi & Faqi, 2011; Mizotani *et al.*, 2015; Messinetti *et al.*, 2019; Eliso *et al.*, 2020*b*). The use of software tools such as Toxicosis (Gazo *et al.*, 2021) allows for the high-content analysis of larval phenotypes and the evaluation of embryonic malformations through scoring morphometric endpoints. Other commonly used endpoints include the percentage of normal hatched larvae and the progression of metamorphosis to adults (Fig. 7C–F). Behavioural phenotyping, such as swimming activity, also has the potential for identifying effects of toxic substances on neuro-behavioural performance (Zega, Thorndyke & Brown, 2006; Rudolf *et al.*, 2019). Comparing embryotoxicity data for chemicals in *Ciona* spp., bivalves (*Mytilus* spp.) and sea urchins (*Paracentrotus* spp.) demonstrates that *Ciona* spp. can support classical tests (Table 2).

Adult ascidians are abundant in both contaminated and pristine environments. They are important filter-feeders in many benthic ecosystems and can filter dozens of litres of water per day, retaining submicron-sized particles and accumulating low concentrations of toxicants from the water column to which they may show sensitivity (Draughon, Scarpa & Hartmann, 2010; Jacobi, Yahel & Shenkar, 2018; Tzafriri-Milo *et al.*, 2019; Vered *et al.*, 2019). Therefore, adults are useful for bioaccumulation studies

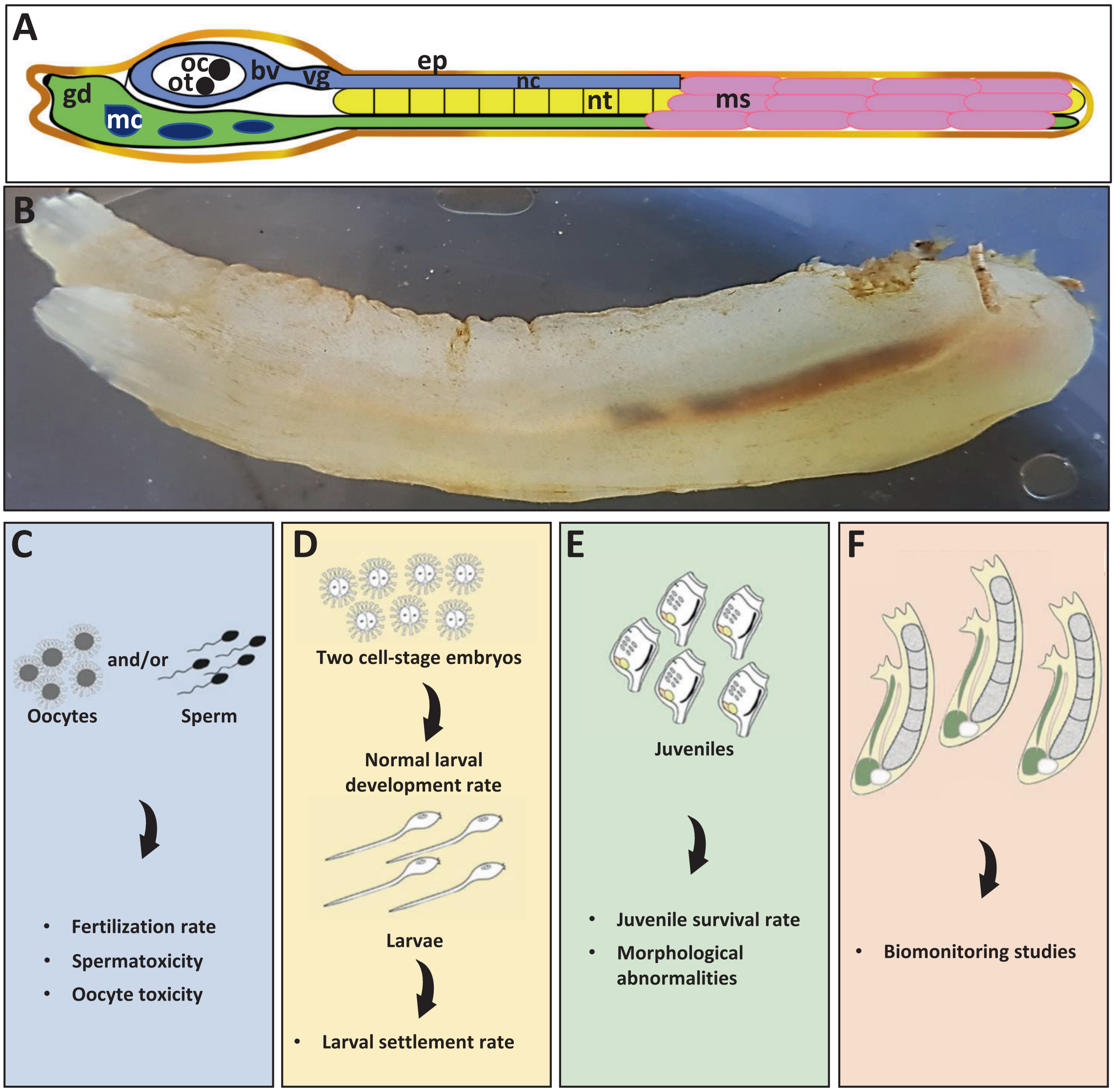


Fig. 7. Ascidians. (A) Diagram of a typical larva. Bv, brain vesicle; gd, gastrodermis; ep, epidermis; mc, mesenchyme; ms, muscle; nc, nerve chord; nt, notochord; oc, ocellus; ot, otolith; vg, visceral ganglion. (B) A typical solitary ascidian *Ciona robusta.* (C–F) Solitary ascidian life stages used in (eco)toxicological tests.

of specific tissues and organs and for evaluating how ingested toxicants may affect reproduction and development. In recent years, adult solitary ascidians, including invasive species, have been used as bioindicators to monitor anthropogenic stressors such as heavy metals, MPs, phthalate acid esters, and pharmaceutically active compounds (Vered *et al.*, 2019; Navon *et al.*, 2020). Biomonitoring methods and indices, including both chemical and physiological analyses, have been used to quantify the distribution of chemicals in tissues and organs (Tzafriri-Milo *et al.*, 2019). Increasing evidence suggests that stressful conditions stimulate the production of ROS, leading to an inability to detoxify pollutants or repair induced damage (Kaloyianni *et al.*, 2009; Tomanek, 2014; Canesi, 2015; Puppel, Kapusta & Kuczyńska, 2015; Zeeshan *et al.*, 2016). Antioxidant responses have been used as markers of stress conditions related to pollutants (Shida *et al.*, 2003; Franchi *et al.*, 2014; Drago *et al.*, 2021) in the digestive system (the first organ to contact pollutants) and circulating haemocytes (the major detoxification organ) in ascidians.

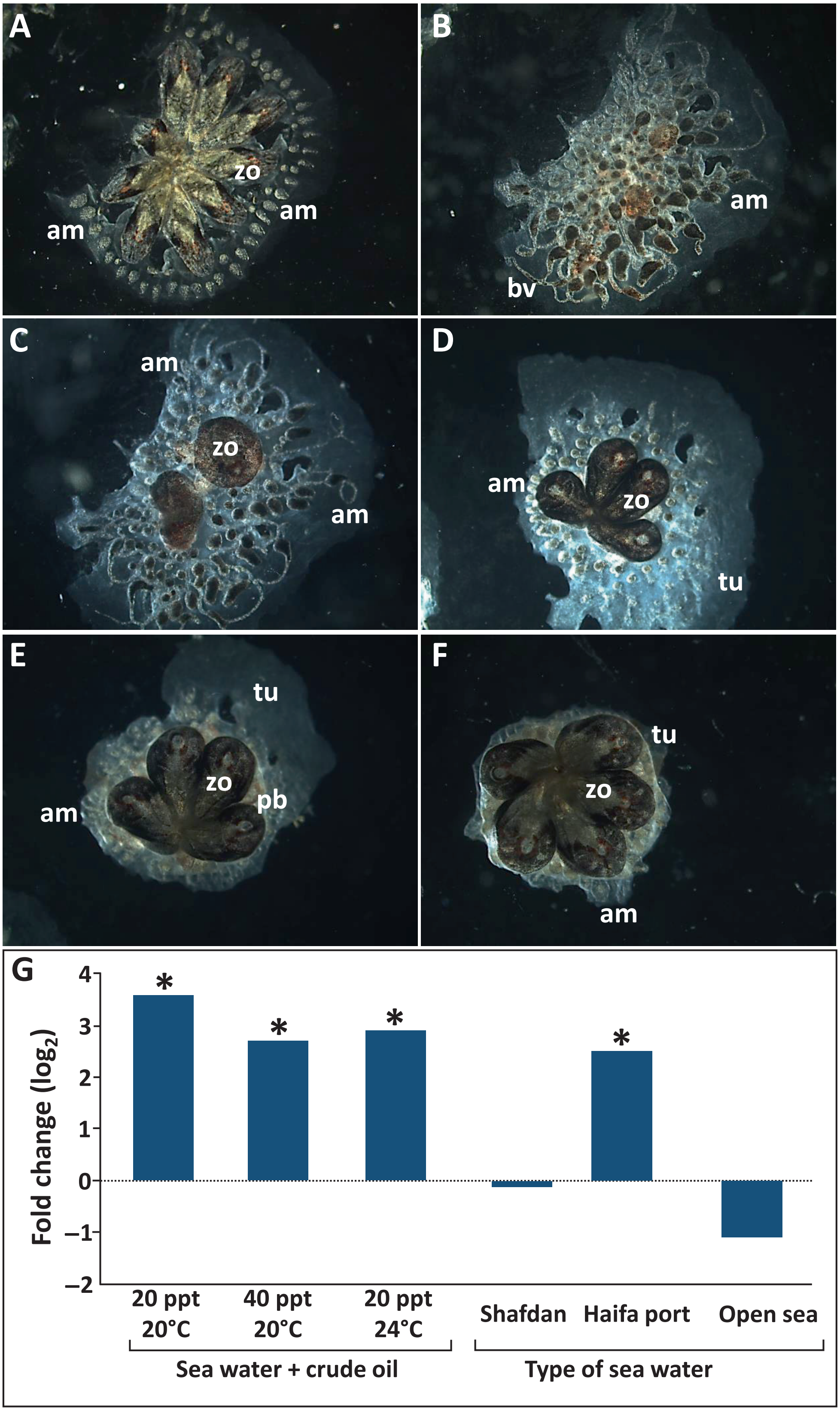
(*b*) *Colonial ascidians*

Colonial ascidians are the only chordates capable of asexual reproduction (Manni *et al.*, 2007) and have been widely used in studies investigating asexual reproduction (Manni & Burighel, 2006; Manni *et al.*, 2014, 2019; Rosner *et al.*, 2014; Gasparini *et al.*, 2015; Kowarsky *et al.*, 2021), regeneration (Fig. 8A–F; Rinkevich, Shlemberg & Fishelson, 1995*b*; Rinkevich *et al.*, 2007*a*; Voskoboynik *et al.*, 2007;

Table 2. Comparison of embryonic sensitivity towards inorganic and organic compounds among three marine invertebrates: *Ciona* spp., *Paracentrotus* spp. and *Mytilus* spp.

Compounds	EC50* *Ciona* spp. (20 h)	EC50* *Paracentrotus* spp. (48 h)	EC50* *Mytilus* spp. (48 h)	References
Copper	0.72 μM	1.8 μM	0.05 μM	His *et al.* (1999); Bellas *et al.* (2001); Fernández & Beiras (2001); Gharred *et al.* (2016)
Mercury	0.27 μM	0.04 μM	0.61 μM	
Cadmium	7.46 μM	3.1 μM	19.6 μM	
Tributyltin	7.1 μg/L (0.022 μM)	0.309 μg/L (0.009 μM)	0.377 μg/L (0.0012 μM)	Bellas *et al.* (2005)
Lindane	4412 μg/L (15.2 μM)	>91,000 μg/L (>313.5 μM)	1992 μg/L (6.8 μM)	Bellas *et al.* (2005); Beiras & Bellas (2008)
Chlorpyrifos	5666 μg/L (15.7 μM)	300 μg/L (0.83 μM)	154 μg/L (0.44 μM)	
Sodium dodecyl sulphate	5145 μg/L (17.8 μM)	4100 μg/L (14.18 μM)	2353 μg/L (8.2 μM)	
Naphthalene	1.9 μg/L (15 μM)	4.72 μg/L (37.3 μM)	6.55 μg/L (51.7 μM)	Bellas *et al.* (2008)
Phenanthrene	>2400 nM	>2400 nM	809 nM	
Pyrene	>640 nM	>640 nM	>640 nM	
Fluoranthene	>1250 nM	>1250 nM	>1250 nM	
Chlorothalonil	123 nM	25 nM	33 nM	Bellas (2006)
Sea-Nine 211	372 nM	43 nM	38 nM	
Dichlofluanid	846 nM	1881 nM	244 nM	
Tolylfluanid	625 nM	1165 nM	213 nM	
Irgarol 1051	8346 nM	15871 nM	6076 nM	
Bisphenol A	168 μg/L (0.74 μM)	710 μg/L (5.7 μM)	3.68 μg/L (0.016 μM)	Özlem & Hatice (2008); Matsushima *et al.* (2013); Fabbri *et al.* (2014)
Paclitaxel	3 μM (MTC**)	10 μM	–	Semenova *et al.* (2006); Mizotani *et al.* (2015)
PS-NH_2***	7.52 μg/mL	2.61 μg/mL	0.142 μg/mL	Della Torre *et al.* (2014); Balbi *et al.* (2017); Eliso *et al.* (2020*a*, 2023)

The data demonstrate that *Ciona* spp. can support classical embryonic tests previously performed with species already incorporated in standardised protocols.
EC50*, half maximal effective concentration; MTC**, minimum toxic concentration; PS-NH2***, polystyrene nanoparticles amino-modified.



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Brown *et al.*, 2009; Rosner, Kravchenko & Rinkevich, 2019), stem cells (Rinkevich *et al.*, 2010; Voskoboynik *et al.*, 2008; Rosner *et al.*, 2013) and allorecognition (Sabbadin, 1962; Karakashian & Milkman, 1967; Oka, 1970; Mukai & Watanabe, 1974; Koyama & Watanabe, 1982; Saito & Watanabe, 1982; Taneda & Watanabe, 1982*a*,*b*; Rinkevich & Weissman, 1992; Ballarin, Cima & Sabbadin, 1995; Ballarin *et al.*, 2002; Rinkevich, Porat & Goren, 1995*a*; Rinkevich, Tartakover & Gershon, 1998; Cima, Sabbadin & Ballarin, 2004; Rinkevich, 2005*b*; Voskoboynik *et al.*, 2013; Taketa & De Tomaso, 2015; Franchi & Ballarin, 2017). Very few colonial ascidians have been used as sentinel organisms in toxicological assays and most data refer to the species *Botryllus schlosseri* (Fig. 8G). Toxicological tests on *B. schlosseri* larvae have primarily focused on antifouling paints. Results suggest that antifoulants interfere with larval adhesion and metamorphosis, cause developmental delays and increase mortality (Cima & Ballarin, 2004; Cima, Burighel & Ballarin, 2006).

The effects of exposure to xenobiotics on adult colonial ascidians have mainly been studied on haemocytes. At high concentrations, xenobiotics can change many parameters, from phagocytosis capability and membrane permeability to increasing cell mortality by inducing apoptosis. At lower concentrations, they can alter cell shape by changing cytosolic levels of Ca^{2+}, hence affecting the cytoskeleton. Such endpoints have been used to screen various antifouling compounds and compare their toxicity (Cima *et al.*, 1995, 1997, 1998*a*, 2002; Cima, Spinazi & Ballarin, 1998*b*; Cima, Bragadin & Ballarin, 2008; Cima & Ballarin, 1999, 2000, 2004, 2012, 2015; Menin *et al.*, 2008; Matozzo & Ballarin, 2011; Matozzo, Franchi & Ballarin, 2014; Cima & Varello, 2020) or to evaluate the impact of cadmium (Franchi & Ballarin, 2013).

Responses of whole colonies to pollutants were also tested. The response of *B. schlosseri* depends on pollutant concentration and exposure duration, ranging from changes in transcription of genes involved in glutathione synthesis and antioxidant responses, detectable only in haemocytes (Franchi, Ballin & Ballarin, 2017), to phenotypic modifications such as disconnected zooid oral siphons, reduced circulation, and darkened ampullae, or even colony death (Gregorin *et al.*, 2021). *Pseudistoma crucigaster* colonies transplanted for 6 months to polluted areas of the Catalan coast with high concentrations of dissolved copper exhibited negative effects on growth (Agell *et al.*, 2004). Other experiments have shown an increase in the activity of enzymes like SOD and CAT in *Botryllus* colonies transplanted to sites exposed to high anthropogenic impact (Tasselli *et al.*, 2017). Alterations in GPX gene expression due to exposure to crude oil and environmental pollutants were also detected (Fig. 8G).

To date, the use of colonial ascidians in ecotoxicology has been limited to a few species, as reported above. Important features of colonial species that have been neglected, such as their asexual reproduction and their huge regenerative ability could be exploited as reliable biomarkers in environmental monitoring.

(*c*) *Appendicularia*

Appendicularia, free swimming tunicates, are another group of tunicates proposed as bioindicators for monitoring the quality of estuarine areas. The density of *Oikopleura longicauda*, *Oikopleura dioica*, *Oikopleura fusiformis*, and *Fritillaria haplostoma* populations has been used to evaluate water quality in Rio de Janeiro state (de Carvalho, Bonecker & Nassar, 2016).

(5) Non-standard models – overview

Many hitherto underused species (poriferans, cnidarians, planarians, and colonial ascidians) have exceptional characteristics such as enormous regenerative ability, and the possession of populations of pluripotent ASCs, capable of differentiation into both soma and germ lineages (Rinkevich *et al.*, 2022; Rosner *et al.*, 2021; Ballarin *et al.*, 2022). These features can be exploited in the field of (eco)toxicology in many ways: (*i*) production of many ramets from a specific genet, all with identical genetic information and epigenomes,

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Fig. 8. (A–F) Various time points during regeneration of a *Botryllus schlosseri* colony after removal of all buds from the colony (budectomy). (A) A colony immediately after budectomy. (B) Morphological changes occurring in the budectomised colony upon entering stage D (takeover stage). These changes include the destruction of zooids, dilation of blood vessels, and increased pigmentation of ampullae. (C) Formation of new zooids. (D, E) Healing of the colony through normal blastogenic cycles. (F) The recovered colony. am, ampulla; bv, blood vessel; pb, primary bud; tu, tunic; zo, zooid. (G) Changes in *B. schlosseri* glutathione peroxidase (GPX) messenger RNA (mRNA) expression following the submersion of colonies ($N = 3$ for each test) in crude oil-polluted sea water samples and environmental seawater samples. Each colony was subcloned into several ramets. A ramet was submersed in tested seawater samples while the matched control was submersed in clean sea water originating from Tel-Shikmona (near Haifa). GPX expression was measured using relative quantitative polymerase chain reaction (qPCR) analysis and is expressed as fold changes at the $\log_2$ scale. Some of the tested samples consisted of clean sea water (from Tel-Shikmona) spiked with 20 or 40 parts per thousand (ppt) crude oil while others contained only sea water without any additional additives. Shafdan is near Tel Aviv, where regional treated sewage was discharged into the Mediterranean Sea until the end of 1996. The Haifa port sample was collected from a highly polluted region inside Haifa port and the 'open sea' sample was taken from open sea outside Haifa port. Each experiment was performed with three different genets. Statistical analysis was performed by the paired *t*-test method; statistically significant upregulation of GPX mRNA following treatment ($P \leq 0.005$) is marked by an asterisk (A. Rosner, unpublished data).

for testing various endpoints under controlled laboratory and field experiments; (*ii*) the possibility of assessing the impact of various pollutants on regeneration (Best *et al*., 1981; Best & Morita, 1982) using new models like artificial embryos (Johnson *et al*., 1982; Johnson & Gabel, 1983), the nubbins assay (Shafir *et al*., 2003) and the use of primmorphs (Akpiri *et al*., 2020) that include endpoints such as the inhibition of regeneration, regeneration time and formation of teratomas; (*iii*) evaluation of the direct effects of pollutants on stem cell populations, and the use of these systems to design effective interpretations of *in vitro* outcomes in order to understand whole-body impact better; and (*iv*) investigation of the epigenetically mediated inheritance of the impacts of pollution by unexposed descendants (Ellis, Kissane & Lynch, 2021*b*; Rosner *et al*., 2021).

V. NEW OPTIONS FOR BIO-MONITORING USING NON-STANDARD MODELS FOLLOWING THE DEVELOPMENT OF INNOVATIVE METHODS

Advances in omics and bioinformatics methodologies over recent decades have expanded the use of non-model organisms (Pai *et al*., 2018; Amil-Ruiz *et al*., 2021), and enabled fast and effective studies on the impact of environmental pollution at different organisational levels, from the cell to the population. Omics approaches cover genomics for studies at the DNA landscape level, transcriptomics for studies on RNA repertoires, metabolomics for studies on metabolites and epigenomics to study epigenetic modification.

Transcriptomic analyses performed on aquatic invertebrates exposed to various pollutants (Srivastava *et al*., 2010; Elran *et al*., 2014; Riesgo *et al*., 2014; Ereskovsky *et al*., 2017; Kenny *et al*., 2018, 2020; Zhang *et al*., 2019*b*; Luter *et al*., 2020; DeLeo *et al*., 2021; Rubin *et al*., 2021) have shed light on species-specific differences in response to environmental pollution. Tests based on the mode of regulation and expression of genes of the aryl hydrocarbon receptor signalling pathway (Hahn, Karchner & Merson, 2017) and xenobiotic-metabolising enzymes have become prominent examples of these approaches, serving as reliable pollution biomarkers in various organisms, including non-model organisms (Zhou *et al*., 2020). Proteome profiles have also been used as pollutant-specific biomarkers (Diz & Calvete, 2016; Gouveia *et al*., 2018). For example, a differential expression of proteins has been observed in solitary ascidians (Kuplik, Novak & Shenkar, 2019) and corals (Tisthammer *et al*., 2021) exposed to pollution. Metabolomic analysis revealed that microbiome-derived bioactive metabolites contribute significantly to the overall organism metabolites, with changes observed in the metabolome of the amphipod crustacean *Hyalella azteca* exposed to the anti-inflammatory drug Diclofenac (Fu *et al*., 2021). In the sponge *S. officinalis*, exposure to anthropogenic pollutants containing the synthetic surfactant coconut diethanolamide (C11 DEA) resulted in the discovery of new metabolites, while increased levels of metabolites were found in the coral *S. pistillata* following exposure to polyethylene MPs (Bauvais *et al*., 2017; Lanctôt *et al*., 2020). Site-associated differences in metabolome were also observed in the ascidian *Ciona intestinalis* (Utermann *et al*., 2020).

Pollution can also modify the epigenetic signature of organisms, and this can persist for several generations, even after pollutant removal. Such alterations can change the sensitivity of organisms to other chemicals, resulting in hormesis and resistance (Vaiserman, 2011; Roberts & Gavery, 2012; Oziolor, De Schamphelaere & Matson, 2016; Calabrese & Mattson, 2017; Ellis *et al*., 2021*b*). However, some of these techniques rely on sequenced genomes, therefore, to bypass such obstacles, new techniques have been developed [such as reduced-representation bisulfite sequencing (RRBS-Seq), epigenotyping by restriction-site associated DNA sequencing (EpiRADseq), bisulfite-based restriction-site associated DNA sequencing (BsRADseq), and epi-genotyping by sequencing (epiGBS(; Schield *et al*., 2016; Trucchi *et al*., 2016; Van Gurp *et al*., 2016]. Although invertebrate epigenetic research is in its infancy, epigenetic changes can be a valuable source of information for the development of innovative tools for monitoring the effects of pollution (Brander, Biales & Connon, 2017). In a recent comprehensive review on the impact of pollution on invertebrate epigenomes, Šrut (2021) highlighted various exposure scenarios, epigenetic endpoints and methods for detecting epigenetic impact. However, much work still needs to be done before such tools can be used in models to predict the impact of specific pollutants. Alterations in the epigenetic profile may also be linked to changes in the microbiome and changes to organism resistance to environmental factors (Barno *et al*., 2021). The microbiome can also be directly affected by pollution, ultimately leading to adverse effects in the host (Lederberg & Mccray, 2001; Haiser & Turnbaugh, 2013; Claus, Guillon & Ellero-Simatos, 2016; Adamovsky *et al*., 2018). Studies of the impact of pollution on the microbiomes of non-model aquatic hosts include tests on the Manila clam *Ruditapes philippinarum* (Bernardini *et al*., 2021), some sponge species (Yang *et al*., 2011; Pita *et al*., 2013; Turon *et al*., 2019), reef-building corals of the genus *Acropora* (Littman *et al*., 2009; Ziegler *et al*., 2018, 2019), the planarian *S. mediterranea* (Bijnens *et al*., 2021) and ascidians (Cahill *et al*., 2016*b*; Evans *et al*., 2018; Goddard-Dwyer, López-Legentil & Erwin, 2021). These results suggest high potential for microbiomes as tools to test the impact of pollution; however, the standardisation of protocols is required to avoid methodological bias (Evariste *et al*., 2019).

Environmental pollution can result in changes to population composition, including the loss of native species and the emergence of invasive species. Pollution can also impact phenotypic plasticity, which may adversely affect the accuracy of traditional morphology-based taxonomic classifications of organisms (Abdelhady *et al*., 2018). DNA barcoding is a genetic-based method for species classification that utilises highly conserved regions flanking species-specific sequences, which are amplified by polymerase chain reaction (PCR) and sequenced (Hebert & Gregory, 2005). One commonly used gene for this purpose in many animals is the mitochondrial cytochrome oxidase subunit 1 (CO1) gene. The Barcode of Life Data System (BOLD), a global database established in 2005, stores species-specific information obtained using this approach from all over the world, along with Linnaean classification, photographs and collection sites (Ratnasingham & Hebert, 2007). BOLD facilitates the identification of cryptic and invasive species (Douek *et al.*, 2020, 2021; Galil *et al.*, 2021) and was also used to identify phenotypic plasticity induced by pollution (Weigand *et al.*, 2011, 2019). DNA metabarcoding (Pawlowski *et al.*, 2018), which applies the same principles as DNA barcoding, is a cost-effective tool for observing biodiversity alterations across environments. This approach involves using PCR amplicons from a variety of species in environmental samples and then subjecting them to high-throughput sequencing and bioinformatic analyses. Alternatively, Droplet Digital PCR (ddPCR) can be employed for water samples (Wood *et al.*, 2018; Hernandez *et al.*, 2020). Standardised methods for collecting environmental samples, such as from river streams or sediments, are crucial for the reliability of such methods (Emilson *et al.*, 2017; Holman *et al.*, 2019).

In addition to the use of genetic material extracted from organisms, environmental DNA (eDNA) and environmental RNA (eRNA) analyses of water or sediment samples are promising approaches for detecting genetic material from various sources, including microscopic organisms, physical damage to animals and reproductive products (Wood *et al.*, 2020). eDNA- and eRNA-based methods offer benefits over conventional methods, such as non-invasive sampling, lower costs and improved detection sensitivity (Bohmann *et al.*, 2014; Rees *et al.*, 2014; Valentini *et al.*, 2016). eDNA is already used to record aquatic organism distribution (Lamy *et al.*, 2021), while eRNA experiments are at a proof-of-concept stage, enabling the detection of alternative gene spliced transcripts or non-coding RNA species *via* an RNA sequencing (RNA-seq) technique.

Quantitative trait loci (QTLs) are used to evaluate the impact of pollution on species diversity by linking pollution resistance with specific genomic regions/loci (gene–environment) or gene–gene epistatic interactions. These approaches involve crosses between resistant phenotypes and the comparison of genetic polymorphisms of exposed/unexposed individuals. Comprehensive methods like pooling-based sequencing (pool-seq) sequencing and population resequencing are replacing traditional methods like amplified fragment length polymorphism (AFLP), microsatellites, single nucleotide polymorphisms, and restriction site-associated DNA sequencing (RADseq) to detect the responses of multiple regions to multiple selective stressors and have successfully been applied to fish populations. The coral species *A. millepora* and *Pocillopora damicornis* have also successfully been used to correlate environmental stressors with specific QTLs for thermal stress, water clarity, eutrophication and high-temperature stress conditions (Lundgren *et al.*, 2013; Jin *et al.*, 2020).

These are just a few examples of the new methods that are continually emerging and can be applied to non-model aquatic invertebrates. Consequently, they eliminate boundaries in ecotoxicological studies, allowing broader ecotoxicological research that considers the diversity of the animals, the representation of their natural habitats and their role in the food chain.

VI. INTEGRATION OF DATA FOR REUSE IN (ECO)TOXICOLOGY AND ENVIRONMENTAL RISK ASSESSMENT

Environmental risk assessment (ERA) is the process of predicting the risk of adverse effects on the environment caused by pollutants through successive steps of hazard identification, hazard characterisation, exposure characterisation and risk assessment. The main outputs of ERA are risk management and communication plans (Blasco & DelValls, 2008). Recent reports (including from the World Health Organisation) have demonstrated that the integration of health, safety and environmental (HSE) risk assessment approaches is needed to protect human health and the environment, a so-called One Health approach (Aguirre *et al.*, 2016). Integration improves the efficiency and quality of decisions related to human health and ecological risk assessments (Suter *et al.*, 2005). In addition, the integration of HSE risk assessment and risk-mitigation measures requires the integration of scientific data, information and knowledge.

Integrated approaches in toxicity testing and assessment (IATA) are already included in OECD testing recommendations. IATA is a practical, scientific approach to the analysis of chemical danger relying on an integrated study of the available data along with the development of new data (experimentally and/or computationally; OECD, 2021). The IATA approach of integration of knowledge, information, and data may enable more comprehensive safety profiling (Rivetti *et al.*, 2020). The first step in ERA is hazard assessment, but it is unrealistic to identify the hazards presented by all pollutants released into the environment. Therefore, the reuse and integration of existing data, information and knowledge represents a way forward to a more holistic approach to ERA (Bennekou, 2019). Data, information and knowledge integration in (eco)toxicology is facing many challenges, which are not only related to informatics and technical issues, but to an even greater degree to the assessment of the weight of test outcomes and their overall relevance (Neagu & Richarz, 2019). For example, available studies vary in study design, methodology, and in the level of detail reported. Regulators must therefore evaluate each individual study for reliability in accordance with good research practice (Schwab *et al.*, 2022). Adherence to metadata content standards, i.e. an agreed list of common metadata items and the standardisation of terminology and definitions for these items is a prerequisite for quality data sharing, reuse and integration. In addition to metadata standards, data reporting templates can also play an important role in ensuring quality data sharing, reuse and integration. Data reporting templates provide a standardised format for reporting data, including metadata, and can help ensure that important information is not omitted, and that data are reported consistently across different data sets. At present, over 50 harmonised data entry templates for various types of studies have been elaborated in the nanomaterial safety community, including the physicochemical characterisation of materials, hazard assessments (cell viability, genotoxicity, environmental organism dose–response tests, omics) and the reporting of data from exposure and release studies. The templates can be used in other research domains and have already been extended and adapted for MPs and advanced materials research. The harmonised templates aim to make data presentation, interlaboratory comparisons and meta-analyses more reliable and to streamline the evaluation and regulation process. Data entry templates are created collaboratively with the active involvement of data providers. Once the layout and content are agreed, the templates are integrated into the Template Wizard, which is an online tool allowing the sharing and downloading of dynamically customisable templates. The tool is designed to be user-friendly and attractive to data providers, as well as to improve awareness and reuse of existing templates in new projects and when adding new (or extended) endpoints. The online template validator allows self-evaluation of the template and transformation by the open-source parser into a machine-readable format (e.g. json or rdf) compliant with the FAIR (findable, accessible, interoperable, reusable) principles (Jeliazkova *et al.*, 2021).

The integration of data and knowledge is outlined by the FAIR data principles (Wilkinson *et al.*, 2016). This set of principles is focused on ensuring that research objects are reusable, and will be reused, and thus become as valuable as possible. The FAIR principles put specific emphasis on enhancing the ability of machines automatically to find and use the data, in addition to supporting its reuse by individuals. The idea of being machine-actionable applies in two contexts – first, when referring to the contextual metadata surrounding a digital object (‘what is it?’), and second, when referring to the content of the digital object itself (‘how do I process it/integrate it?’). This means that domain experts should also have a basic understanding of how to organise, document, store and share data, to ensure they are properly managed and can be understood and (re)used in the future.

VII. CERTAINTIES AND UNCERTAINTIES IN ASSESSING AQUATIC ECOTOXICOLOGY

Species sensitivity distribution (SSD) models have been established as a key tool for the ERA of chemicals (Posthuma, Suter & Traas, 2002). SSDs consist of a statistical approach to predict the potential biological impact of a chemical in nature. SSDs simulate the range of sensitivity of various species to a variety of chemical concentrations. These tools are used to estimate the potentially affected fraction (PAF) of species that will be harmed by exposure, and to establish threshold concentrations. When using SSDs, it is assumed that the species toxicity data represent a random sample from a statistical distribution that is typical for a community or ecosystem. The precision of SSDs in predicting environmental toxicity impact will rise as more data become accessible for a variety of species. SSDs have been useful tools for risk assessment purposes for decades, despite their numerous limitations (Belanger & Carr, 2019).

De facto testing of the effects of chemicals is only possible on a restricted number of species. Ecotoxicologists, therefore, face the major problem of translating the measurements acquired from the tested species into predictions of effects on the wider range of species in aquatic ecosystems. A broad comparison of the toxic concentrations of various classes of compounds for some aquatic invertebrates has highlighted that species belonging to highly diverse phyla, such as arthropods and molluscs, exhibit a wide range of sensitivities (Rosner *et al.*, 2021). For example, a study on arthropods comparing *Deleatidium* spp. and *D. magna* exposed to heavy metals showed similar sensitivities to Cr^{6+} and Cu^{2+}, whereas a significantly higher sensitivity was observed in *Daphnia* exposed to Cd^{2+} and Zn^{2+} (Hickey & Vickers, 1992). Differences in sensitivity can be observed even among cryptic species. For example, comparisons of two cryptic species of the polychaete annelid *Capitella* found that *Capitella* sp. I possessed a greater ability to biotransform fluoranthene than *Capitella* sp. S (Selck, Palmqvist & Forbes, 2003; Li, Bisgaard & Forbes, 2004). The observation of such differences in invertebrates, as opposed to vertebrates, could be explained by the huge molecular diversity (divergence in protein sequences, for example) arising during their evolutionary trajectories. This molecular diversity could be directly linked to disparities in inherent sensitivities to chemical compounds dependent on specific monoamine oxidase (e.g. inhibition of an enzyme or receptor by xenobiotics *via* their attachment to specific sites of action; Chaumot *et al.*, 2014) and is further illustrated by the high sensitivity of arthropods to acetylcholinesterase (AChE)-inhibiting insecticides compared to rotifers, molluscs, and annelids (Van Wijngaarden *et al.*, 2005; Bally *et al.*, 2016). This wide molecular diversity also results in unexpected xenobiotic toxicity in specific groups of invertebrates; for example, the biocide TBT is responsible for the abnormal development of the genital tract, with masculinisation of females, in about 100 species of marine gastropods (Migula, 2005).

To overcome the limitations of SSDs, trait-based approaches have been proposed as complementary tools for ERA. These approaches allow the deciphering of the mechanisms behind the effects of exposure and prediction of the responses of species to chemicals with the same MOA. For example, the SSD for various MOAs highlighted groups of species with different sensitivity levels. Arthropods were classified as more sensitive than average, followed by nematodes, molluscs and annelids. However, bryozoans, cnidarians and flatworms were never of above-average sensitivity (Van den Berg *et al.*, 2019). It is interesting to note that species used routinely for ecotoxicological tests, e.g. *Daphnia*, are not the most sensitive species (Van den Berg *et al.*, 2019).

Another way to improve the accuracy of ecotoxicological tests on aquatic organisms consists of designing adverse outcome pathways (AOPs; OECD, 2017). AOPs serve as the basis for mechanistically driven toxicological approaches using a variety of toxicological data including details of chemical interactions with a specific biomolecule, commonly called the molecular initiating event (Ankley *et al.*, 2010). Toxicokinetic (TK) pathways together with toxicodynamics (TD) are also key elements in the design of AOPs, as TK pathways determine the concentration of chemicals in an organism by considering adsorption–distribution–metabolism–excretion (ADME) traits (McCarty & Mackay, 1993; Nyman, Schirmer & Ashauer, 2012), while TD describes the interaction between the chemicals and the target(s) (McCarty & Mackay, 1993; Nyman *et al.*, 2012). The OECD actively supports AOP development to standardise testing methods to assess substance toxicity. However, to date, AOPs in aquatic organisms have not been fully developed. They range from putative to partially characterised forms, even in cases where toxicant effects are highly conserved across species, such as those of organophosphates due to the presence of conserved AChE receptors (Brockmeier *et al.*, 2017). Indeed, for other xenobiotics, such as heavy metals, interspecies variation has been observed due to microevolutionary processes leading to the acquisition of resistance mechanisms (see review by Posthuma & Van Straalen, 1993; Chandrangsu, Rensing & Helmann, 2017). Modification in the expression of metallothioneins has been shown to cause a change in the resistance capacity of adapted species. For instance, genotypes with a specific metallothionein promoter are detected most frequently in cadmium-tolerant populations of the springtail *Orchesella cincta* in comparison to other *O. cincta* populations (Costa *et al.*, 2012). This adaptation is also observed in the springtail *Folsomia candida*, which exhibits constitutive metallothionein expression. Additional examples of adaptation have been observed in natural populations of oligochaetes, molluscs, crustaceans (Isopoda), myriapods, arachnids, apterygotes and insects (Migula, 2005). Nevertheless, it is important to remember that these adaptations are species dependent. For instance, the isopod *Porcellio scaber*, considered an accumulator of metals, has a smaller body size and starts to reproduce earlier in polluted sites than in unpolluted sites, and shows different responses to *O. cincta*, which is a very rapid eliminator of metals (Drobne, 1997; Migula, 2005). In addition to these species' adaptations to environmental stresses, increasing evidence is accumulating on the potential contribution of the host-associated microbiome to adaptation to environmental stress. A prominent example is the change in the mammalian gut microbiome in laboratory animals that results in altered concentrations of inorganic arsenic metabolites in urine and in increased sensitivity to arsenic toxicity (Lu *et al.*, 2013; Chi *et al.*, 2019). Speciation of organoarsenic species in aquatic organisms also highlights the importance of the microbiome (Langdon *et al.*, 2002; Rahman, Hasegawa & Lim, 2012; Liebeke *et al.*, 2013). The degradation of organic chemicals, such as pesticides, by the host-associated microbiome has also been demonstrated (Daisley *et al.*, 2018; Fernandez *et al.*, 2019; Wang *et al.*, 2020). In *Hydra*, an increased tolerance to copper has been associated with the presence of microalgal symbionts (Karntanut & Pascoe, 2005), while in metal-rich hydrothermal vent environments, symbiotic bacteria on mussels (*Bathymodiolus*; Hardivillier *et al.*, 2004) protect their host by transforming absorbed metal ions into metal particles. Additionally, microbial symbionts of aquatic invertebrates frequently provide their hosts with crucial micronutrients (Stock *et al.*, 2021). As an example, the settling of poriferan, cnidarian and mollusc larvae requires bacteria. Chromatiales supply the amino acid L-arginine to *Amphimedon queenslandica* to allow larvae to settle successfully and metamorphose. Overall, even if the role of the underlying mechanisms of microbiomes in pollution adaptation are not always evident, case studies suggest that associated microbiota can play a relevant role (White & Torres, 2009).

VIII. DISCUSSION

This review summarises many (eco)toxicological studies on chemicals affecting the aquatic environment, with a particular focus on several major aquatic invertebrate phyla, including those represented by non-model organisms. It is clear that interspecies and even intraspecies differences in sensitivity and responses to toxicants do occur (Hendriks *et al.*, 2013). The key issue remains the heterogeneity of the species and the number of endpoints required to enable the design of reliable predictive toxicity platforms, including tools for the estimation of ecological risks and decision-making processes. Currently, standardisation by the main international organisations is available for only a few species representative of a limited number of invertebrate phyla: Arthropoda, Mollusca, and Annelida (Table 1). Sea urchins and sand dollars are the only echinoderms for which there are standardised protocols, while *C. elegans*, the nematode model species recommended for freshwater toxicity studies, is not even typically an inhabitant of fresh water. In some cases, the selected model organisms include invasive species such as *Corbicula fluminea* [ENV/JM/MONO(2019)11]. By contrast, many ecosystem engineers and keystone species like sponges and corals are not represented in the standardised protocols, and some pollution-related properties like hormesis, microbiome changes and population diversity properties are studied *de facto* using varied protocols. This is the current status of the field, although a growing body of information has been accumulated on corals, sponges and ascidians. Moreover, for predictive models like SSD, recommendations regarding the minimum number of species to be tested exceed this: eight for the US EPA and 5–8 for the European Union (for SSD; TenBrook *et al*., 2009). It is therefore logical to extend testing to additional taxa. However, as demonstrated in Fig. 1, most of the aquatic (eco)toxicological research performed in the last decade still used vertebrates (54.2%), and the most studied invertebrates were arthropods (12.5%) and molluscs (19%). Performing an additional search of *Web of Science* (see Q3 and Q4 in Table S1 for search terms) for the genera listed in Table 1 demonstrated that 58% and 32% of all tests performed with arthropods and molluscs, respectively, used only this small subset of model species. Clearly, only a limited and biased subset of taxa still make up a substantial part of toxicity testing.

The available results from non-model animals from Porifera, Cnidaria, Platyhelminthes and Tunicata encourage the use of species in these phyla as reliable model organisms for acute and chronic tests (Fig. 9), with similar endpoints to those used for testing the impact of chemicals on standard models (Table 2) or with additional new tests. Some tests based on non-standard species are relatively well established and generally accepted (although non-validated), such as *H. attenuata* reproduction, survival, and development tests used as indicators of teratogenic effects [ENV/MC/CHEM(98) 19 (Johnson *et al*., 1982; Johnson & Gabel, 1983)]. A drawback of the lack of standardisation is the creation of multiple laboratory-specific protocols, which affects the outcomes and limits or complicates the inclusion of the resulting data into predictive models. In other cases, certain types of tests may be performed with unsuitable models, because standardisation does not exist for the best animal model. Additionally, major regulatory frameworks, including the Canadian Environmental Protection Act, the US Toxic Substances Control Act, and the ECHA REACH initiative, all encourage increased reliance on *in silico* approaches, with the latter being based on the acute toxicity of pollutants to a few species only, such as *Daphnia* and fish (Zhou *et al*., 2021). The Danish QSAR Database (Danish QD; DTU, 2018), the virtual models for property evaluation of chemicals within a global architecture (VEGA; Benfenati, Manganaro & Gini, 2013), the Kashinhou tool for ecotoxicity (KATE; Furuhama *et al*., 2010), the toxicity estimation software tool (TEST; EPA, 2016*h*), the QSAR toolbox developed by the OECD (OECD, 2014), and the ecological structure activity relationships (ECOSAR; Mayo-Bean *et al*., 2012), are several of the *in silico* technologies created for ERA and utilised to support chemical regulation.

The preservation of aquatic biodiversity is now widely recognised as an important conservation goal, which requires the use of effective toxicology and ecotoxicology tools as well as modern safety assessment technologies. Hazard identification and prompt responses are necessary for pollutants such as waste, micro- and nanopollution (including nanoplastics), endocrine disruptors, and persistent chemicals, as they can have harmful effects. In parallel, newly emerging supporting technologies are becoming increasingly affordable and accessible. These include various omics-based applications, microbiomics, metabarcoding (Pawlowski *et al*., 2018),

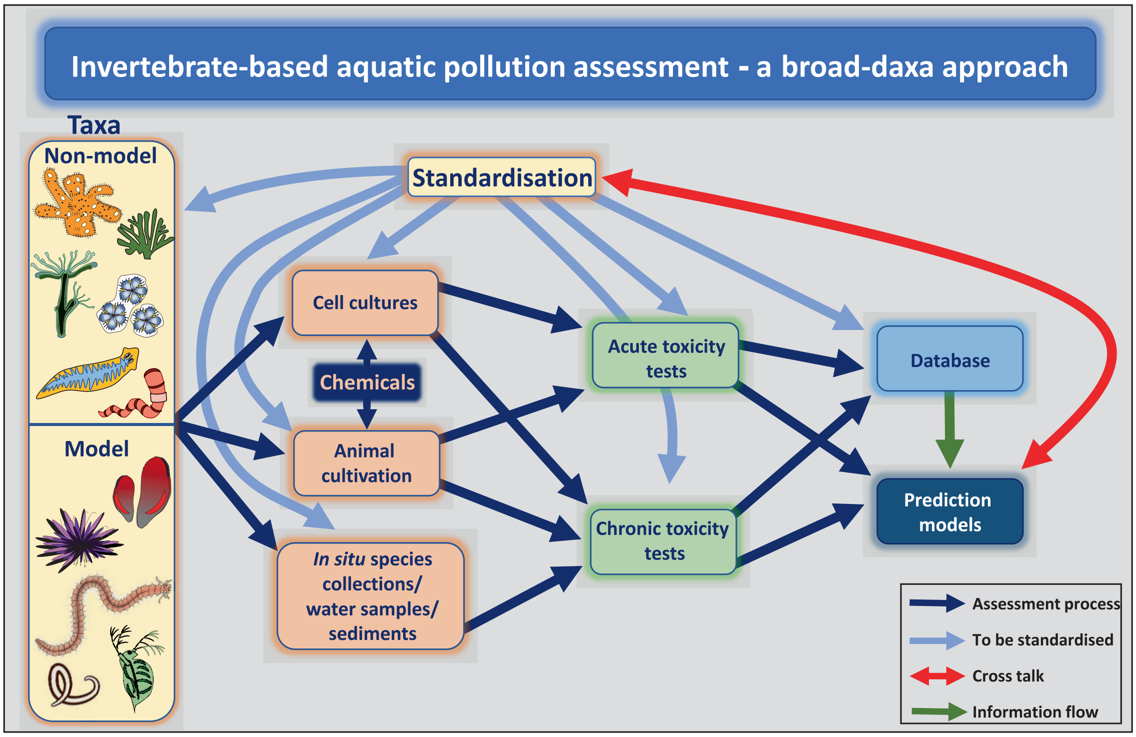

Fig. 9. Summary of the broad-taxa approach proposed herein for environmental assessment and ecotoxicological studies.

automated species identification technologies (Gorsky *et al.*, 2010; Le Bourg *et al.*, 2015; Kalafi, Town & Dillon, 2018; Wäldchen & Mäder, 2018, First *et al.*, 2021), *in vitro* technology (Rosner *et al.*, 2021), bioinformatics, big data processing techniques and increased computing power. Omics technologies will facilitate studies on non-model organisms; *in vitro* technology will enable the simultaneous analysis of samples from many species using a large number of tests; while technologies like DNA barcoding (Weigand *et al.*, 2019; Paz & Rinkevich, 2021) and automated species identification will reduce reliance on professional taxonomists, facilitating the processing of large amounts of data originating from (eco) toxicological tests performed on a variety of aquatic species. By increasing the number and diversity of species used in toxicity assessment studies and incorporating these advances, predictive models can adopt a more precise 'broad-taxa approach' (Fig. 9). Transformation in research approaches is evidenced by the recent increase in tests using non-model species performed using newly emerging techniques (Fig. 1), especially with coral and sponges. It is important for the scientific community to take a more proactive role in integrating large volumes of experimental data into formats that are user-friendly and readily accessible, further influencing the ways (eco)toxicity studies are performed under international regulations, such as the EU Chemicals Strategy for Sustainability (https://www.chemistryworld.com/news/eu-commits-to-overhaul-of-chemicals-legislation/4012615.article) and the EU Green Deal environmental programme (https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal_fr). Integrated approaches to test and assess are already recommended by the OECD *via* IATA, but our goal should be holistic. The term 'holism' describes the necessity for knowledge governance between various knowledge sources and decision-making levels, represented by scientists, experts, citizens and laypeople, as well as administrative and political decision-makers (Giebels *et al.*, 2020). This necessitates problem-focused collaboration between multiple scientific fields and non-scientific actor groups in order to produce transdisciplinary knowledge.

Rigorous reporting of research results is crucial for the scientific community to advance. A minimum annotation checklist for reporting research results needs to be extended beyond omics research and should be applied to other fields, including environmental sciences. The application of artificial intelligence has opened up new and exciting possibilities in a holistic and integrative way, to study the complexity of biological systems (Johnson *et al.*, 2021).

IX. CONCLUSIONS

(1) The preservation of the aquatic environment and its biodiversity necessitates the application of state-of-the-art toxicological and ecotoxicological tools and the development of accurate prediction tools.

(2) The field of (eco)toxicology is experiencing the emergence of new methods and technologies that can be applied to all living organisms, including *in vivo*, *in vitro*, and *in silico* methods, which may eventually lead to a reduction in animal experimentation.

(3) Accurate evaluation of the impact of toxicants, and the prediction of their mode of action requires the use of a wide range of animal models that encompass both interspecies and intraspecies variations, as well as the normal geographic distribution and habitat of each model.

(4) A broad range of endpoints, spanning multiple levels of organisation (from subcellular to population level) and including aquatic non-model invertebrates, is necessary to gain a comprehensive understanding of pollution impacts.

(5) Standardisation and predictive models are needed to compare data obtained by different groups and on different animal models, further leading to a comprehensive global approach to aquatic toxicology.

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XII. SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The lunar-controlled life cycle of *Platynereis dumerilii*. After fertilisation, the zygote starts segmentation, giving rise to a nectochaete larva (72 h) (stomodeum and prototroch highlighted in green). After the first metamorphosis, the juvenile worm forms and starts to feed. After 8–10 days of benthic feeding, the small worm undergoes cephalic metamorphosis to become an atoke worm inside its tube, which then grows continuously until sexual maturation. After sexual maturation (female shown in yellow; male in red), the epitoke worm leaves its tube and swims into the water column, eventually to take part in mass spawning coordinated by the lunar cycle. Drawing modified after Schenkelaars & Gazave (2021) and Fischer & Dorresteijn (2004) by K. M. Karahan and A. Karahan.

Table S1. List of search terms used in *Web of Science*. Q1 was used to identify all publications on marine and freshwater species in the domains of ecotoxicology, toxicology, and pollution in the last 10 years (see green numbers in Fig. 1). Q2 was used to identify all publications on marine and freshwater species based on omics technology in the domains of ecotoxicology, toxicology, and pollution in the last 10 years (see red numbers in Fig. 1). Q3 and Q4 are the search terms used to assess the proportion of studies that focus only on the subset of taxa listed in Table 1 as common model taxa used in standardised regulatory tests.

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