From Primary Cell and Tissue Cultures to Aquatic Invertebrate Cell Lines: An Updated Overview
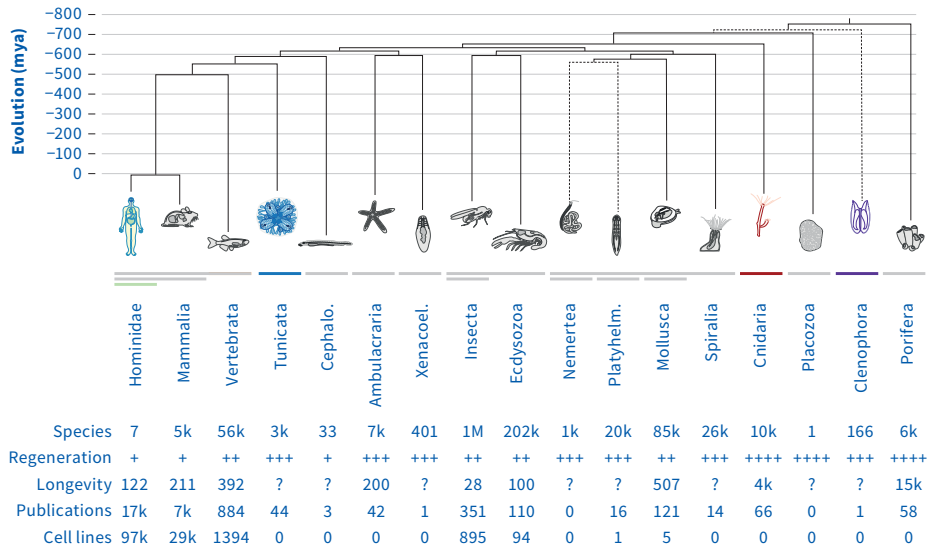
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Abstract: The stem cells discipline represents one of the most dynamic areas in biology and biomedicine. The vast majority of research on stem cells is being conducted in vertebrate models. Currently, over 98% of all cell lines are of mammalian origin, which represent only 0.4% of the extant identified metazoan evolution. In particular, aquatic invertebrates as a whole show the largest biodiversity and the widest phylogenetic radiation on Earth but have not yet significantly contributed to cell lines. Yet, with over 500 publications since the 1960s, the current lack of cell lines does not result from a lack of attempts at cultivating these cells but rather from fragmented research efforts in highly taxonomically diverse model species, a paucity in reports of negative results and persistent knowledge gaps in their in vitro metabolic requirements. To promote the establishment of aquatic invertebrate cell lines, there is thus a need for comprehensive knowledge mapping across taxa to identify adequate, possibly cell type-specific, protocols. Here, we review strategies for preparing an optimal inoculum, for optimizing culture conditions and for cell lineage authentication to monitor the quality of cell cultures. Finally, we conclude with our view on promising research perspectives towards establishing aquatic invertebrate cell lines.

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1. Introduction

Currently, the origins of in vitro cell lines are highly biased towards humans. Around 75% of the total number of established cell lines are from Hominidae origin (96,862/128,799) and over 97% are of mammalian origin (126,033/128,799) (Bairoch 2018) (Figure 1). However, mammals represent only 0.4% (1.3% when excluding the Insecta taxon) of the extant identified metazoan evolution (Zhang 2013; Wilson and Reeder 2011; Chapman 2009) (Figure 1). In addition to the scientific interest relative to their sheer diversity, non-mammalian cells have multiple potential applications, including as a source for bio-active molecules or as assays for eco-toxicological tests (e.g., Ribeiro et al. 2018; Rosner et al. 2021). Yet, with over 500 publications on aquatic invertebrate cell culture alone (Figure 1), the current limited number of invertebrate cell lines does not result from a lack of attempts at cultivating these cells but most likely from inappropriate techniques to cultivate these cells (reviewed in

Rinkevich 2005; Yoshino et al. 2013; Cai and Zhang 2014). As exemplified in insects, a breakthrough in culturing conditions (Grace 1962) initiated the emergence of a huge variety of cell lines (Bairoch 2018) (895 cell lines from 104 genera in around 50 years). There is thus a need for a sustained research effort in non-insect invertebrate cell culture to identify adequate culturing conditions and promote the establishment of cell lines. In particular, aquatic invertebrates as a whole show the largest biodiversity and the widest phylogenetic radiation on Earth but have currently contributed to only six cell lines (Figure 1).

	Hominidae	Mammalia	Vertebrata	Tunicata	Cephalo.	Ambulacraria	Xenacoel.	Insecta	Ecdysozoa	Nemertea	Platyhelm.	Mollusca	Spiralia	Cnidaria	Placozoa	Ctenophora	Porifera
Species	7	5k	56k	3k	33	7k	401	1M	202k	1k	20k	85k	26k	10k	1	166	6k
Regeneration	+	+	++	+++	+	+++	+++	++	++	+++	+++	++	+++	++++	++++	+++	++++
Longevity	122	211	392	?	?	200	?	28	100	?	?	507	?	4k	?	?	15k
Publications	17k	7k	884	44	3	42	1	351	110	0	16	121	14	66	0	1	58
Cell lines	97k	29k	1394	0	0	0	0	895	94	0	1	5	0	0	0	0	0

Figure 1. Comparison of the diversity between metazoan taxa. Depicted is the phylogenetic relation between metazoan taxa and their characteristics with respect to evolutionary radiation, regenerative capacity, lifespan and in vitro cell culture (see Appendix A Table A1 for exact values). Dashed lines represent branchings for which speciation timings have not yet been determined. Colored taxa highlight those whose publication timelines are detailed in Figure 2. Regeneration capacity of the taxa is depicted as follows: + tissue regeneration, ++ appendage regeneration, +++ whole-body regeneration, ++++ from cell aggregates. Longevity is given in years from the maximum reported characteristic of the taxon in the AnAge database (Magalhães et al. 2007). Phylogenetic tree based on Halanych (2004), species numbers on Zhang (2013), regeneration potential on Bely and Nyberg (2010), publications on manually curated online searches (Appendix B Table A2) and cell line numbers on Bairoch (2018). Source: Graphic by authors.
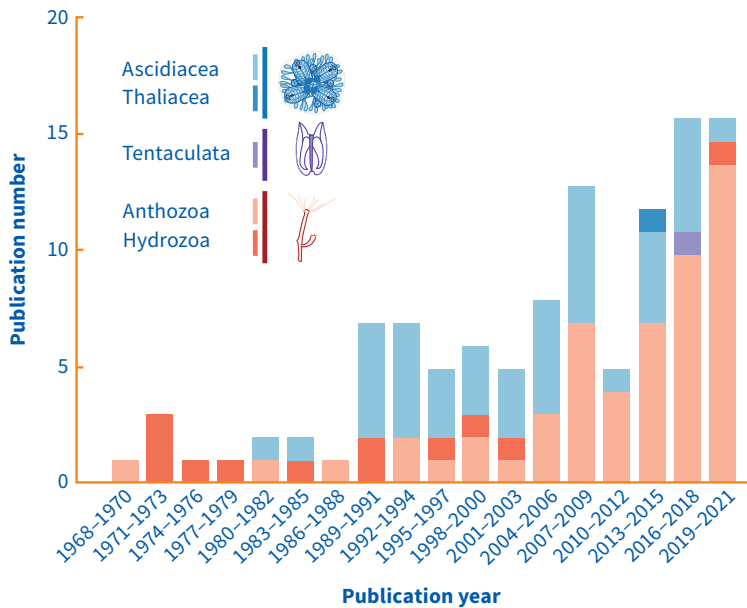
Figure 2. Five decades of research on isolation and primary culture of cells from aquatic invertebrates. The figure shows the number of publications for the phyla cnidaria (66 in total), ctenophora (1 in total), and tunicata (44 in total). Publications are grouped by classes of the species used for cell isolation, color-coded as indicated. Publications were manually curated from online searches, and detailed references are available in the Appendix B Table A2. Source: Graphic by authors.

Cell lines have been established through two main strategies (Cai and Zhang 2014; Rinkevich 2011): either by the isolation of proliferating and self-renewing cells, typically from an embryonic (Hansen 1979) or cancerous origin (Scherer 1953), or by immortalizing proliferating cells, typically through mutagenesis (Earle et al. 1943) or transfection (Russell et al. 1977). Both strategies thus require, at least transiently, a proliferating primary cell culture. The long-term culture (up to 22 months) of cells from various aquatic invertebrate phyla has been achieved by using a variety of culturing environments (Rinkevich and Rabinowitz 1993; Daugavet and Blinova 2015; Chen and Wang 1999; Kingsley et al. 1987). However, most of these in vitro primary cultures show an apparently ubiquitous cellular quiescence within three days that leads to an absence of proliferation within 1–4 weeks of primary culture (Rinkevich 2011; Cai and Zhang 2014). Yet, transient proliferation events, limited to a subset of acclimated cells, are persistently recorded across most marine invertebrate taxa ~2–4 weeks after the establishment of primary cultures at high seeding density from larval or regenerating adult tissue. For instance, DNA synthesis and mitosis have been observed both in primary cultures of explanted ectodermal tissue monolayers of

regenerating *Nematostella vectensis* (Rabinowitz et al. 2016), as well as in dissociated cell culture from regenerating tentacles of *Anemonia viridis* (Ventura et al. 2018), and dividing cells have been reported in primary culture of regenerating tissues of *Apostichopus japonicus* (Odintsova et al. 2005). The only established mollusc cell line, *Bge*, was initiated from the long-term culture of embryonic tissue of the freshwater snail *Biomphalaria glabrata* (Hansen 1979). Taken together, these results suggest that a key to setting efficient primary cultures are to use tissue with high proliferation capacity, potentially due to the presence of stem-like cells. Conveniently, aquatic invertebrates display a variety of asexual reproduction, aging and regeneration phenomena (Figure 1) that indicate high cellular plasticity, cellular proliferation and a likely involvement of stem-like cells (Bely and Nyberg 2010; Slack 2017; Bodnar 2009; Tomczyk et al. 2015; Rinkevich et al. 2022). However, established guidelines for the isolation and identification of stem-like cells are currently only available for very few species (Hayashi et al. 2006; Sun et al. 2007; Hemmrich et al. 2012; Kassmer et al. 2020). The recent improvements in next-generation sequencing techniques, and in single cell transcriptomics in particular, are enabling researchers to characterize stem-like cells in an increasing number of taxa (Hayashi et al. 2010; Siebert et al. 2019; Rinkevich et al. 2022), a first important step for their isolation and in vitro culture.

There is an ample body of work that provides numerous quantitative assessments of culturing conditions (e.g., Toullec 1999; Khalesi 2008; Dessai 2012; Maselli et al. 2018), without highlighting one ideal consensus. Given that aquatic invertebrates are phylogenetically very distant, the development of a ubiquitous culturing environment appears rather unlikely. Nevertheless, each phylum could benefit from the advances in primary cell culture made in other phyla. However, a significant fraction of the relevant research data remains unpublished in conventional peer-reviewed journals, being only accessible as chapters in master's or doctoral dissertations, conference proceedings and specialized books. Consequently, in the last five decades, the publication of research efforts has been uneven across phyla, and temporally fragmented, as illustrated for the cnidaria and tunicata phyla (Figure 2).

Here, we review three major drawbacks and limitations of this field of research and their most promising work-around (Rinkevich 2005; Cai and Zhang 2014; Rinkevich 2011; Yoshino et al. 2013): (1) seeding the cell culture with a population enriched in proliferating and potentially stem-like cells; (2) devising marine invertebrate specific in vitro culturing environment, including management of oxidative stress and cell adhesion requirements; (3) preventing culture contamination with other cell types and microbes. This review is intended to be accessible both to the non-experts and newcomers to the field of primary cell culture, while providing an updated and curated list of references on the primary cell culture of aquatic invertebrates compiled for the experienced reader.

Given the huge scope of this review (>360,000 species, >60 years of research, >510 publications), we set out to illustrate previous work on aquatic invertebrate cell culture with three summarizing tables (Tables 1–3), filled with a selection of representative publications in each taxon and focusing on stem cell cultures whenever these have been described. This review is, by nature, not exhaustive and omits, by necessity, many publications, which thus limits generalizations. We conclude this review by providing perspectives on how to solve this limitation, mainly through dramatically extending the present effort in the data mining and metacoding of published work to build an exhaustive knowledge database on aquatic invertebrate cell culture. We also highlight abiotic factors that should be further investigated. We hope that the provided perspectives will help researchers to develop robust and reproducible approaches for culturing dividing aquatic invertebrate cells, a first step towards the possible establishment of cell lines.

Table 1. Established cell isolation techniques across taxa. Indicative examples were selected among the references listed in Appendix B Table A2.

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
Chordata (Tunicata)	Ascidiacea	*Botryllus schlosseri*	adult	circulating blood cells	mechanical, teasing apart colonial zooids	buffered washing solution with HEPES	none	none	Rinkevich and Rabinowitz (1993)
	Ascidiacea	*Botryllus schlosseri*	embryo	all cell types	mechanical, chemical	buffered washing solution with HEPES	none	none	Rinkevich and Rabinowitz (1994)
	Ascidiacea	*Botryllus schlosseri*	zooids and buds	epithelial	mechanical, enzymatic dissociations	incubation medium	collagenase	none	Rinkevich and Rabinowitz (1997)
	Ascidiacea	*Botryllus schlosseri*	zooids and buds	cup cell disease cells	mechanical, cell strainer	Fisher's medium	none	none	Moiseeva et al. (2004)
	Ascidiacea	*Botryllus schlosseri*	zooids and buds	epithelial	mechanical	filter sea water and antibiotics	none	none	Rabinowitz and Rinkevich (2004)
	Ascidiacea	*Botryllus schlosseri*	zooids and buds	epithelial	mechanical, cell strainer	buffered washing solution with HEPES	none	none	Rabinowitz et al. (2009)
	Ascidiacea	*Botryllus schlosseri*	paleal buds	epithelial	mechanical, cell strainer	buffered washing solution with HEPES	none	none	Rabinowitz and Rinkevich (2011)
	Ascidiacea	*Botrylloides leachii*	adult, hibernating colonies blood cells		mechanical	buffered washing solution with HEPES	none	none	Hyams et al. (2017)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
Chordata (Cephalochordata)	Leptocardii	*Branchiostoma belcheri Japanese*	Adult epidermis, gill, gut, spermary, ovary	all cell types	mechanical and enzymatic for ovary and spermary	FSW	100 U/mL type I collagenase	none	Cai et al. (2013)
	Leptocardii	*Branchiostoma belcheri tsingtauense*	adult, buccal cirri, tail, gill, gut and metapleural fold	all cell types	mechanical dissociation	none	none	none	Wang et al. (2009)
Echinodermata (Ambulacraria)	Echinoidea	*Anthocidaris crassispina, Pseudocentrotus depressus, Hemicentrotus pulcherrimus*	eggs	egg cortex cells	mechanical dissociation	hypotonic $MaCl_2$ (0.1 M) solution	none	sedimentation by centrifuging at 1000 RPM	Sakai (1960)
	Echinoidea	*Anthocidaris crassispina*	embryo (blastula stage)	all cell types	spontaneous dissociation	Ca^{2+} and Mg^{2+} free SW (CMF-SW) (1 h)	none	removal of suspended cells	Fujita et al. (1972)
	Echinoidea	*Strongylocentrotus purpuratus*	embryo (blastula stage)	all cell types	mechanical dissociation	Ca^{2+} and Mg^{2+} free SW (CMF-SW)	trypsin 0.1 mg/mL	two successive centrifugation	Oppenheimer and Meyer (1982)
	Asteroidea	*Asterias amurensis*	embryo (mesenchyme migration stage)	all cell types	mechanical dissociation	1.2 M glycine supplemented with 1% (v/v) nystatin-filtered sea water, 6% newborn bovine serum	none	centrifugations	Kaneko et al. (1995)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
	Echinoidea	*Strongylocentrotus intermedius*	embryo	all cell types	spontaneous dissociation	Ca^{2+} and Mg^{2+}-free SW + antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin)	0.25% collagenase	none	Odintsova et al. (2015)
	Holothuroidea	*Apostichopus japonicus*	adult	guts	Mechanical/ enzymatic	sea water	collagenase	none	Odintsova et al. (2005)
Xenacoelomorpha	Acoela	*Isodiametra pulchra*	adults	all cell types	enzymatic dissociation	nutrient-enriched f/2 ASW	CMF/1% trypsin	none	De Mulder et al. (2009)
Arthropoda (Ecdysozoa)	Malacostraca	*Pacifasticus leniusculus, Homarus americanus*	adults	hematopoietic and testicular tissue	mechanical dissociation	Medium 19	type 2 collagenase	none	Brody and Chang (1989)
	Malacostraca	*M. ensis*	adults	lymphoid organ	mechanical	sea water	none	sedimentation	Han et al. (2013)
	Malacostraca	*Penaeus vannamei*	juvenile	all cell types	Mechanical/ enzymatic	ASW	collagenase	none	Toullec et al. (1996)
Nematoda (Ecdysozoa)	Chromadorea	*C. elegans*	eggs	larval cells	Mechanical/ enzymatic	L-15 medium	pronase	none	Zhang et al. (2011)
Platyhelminthes	Turbellaria	*Girardia ystati, Schmidtea mediterranea*	adult regenerating prepharyngeal zone	neoblasts	chemical dissociation	citric acid 0.1 M with 0.5% Tween 20, 10 min RT	none	FACS-sorting (DNA-stained)	Ermakov et al. (2012)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
	Turbellaria	*Dugesia japonica*	adult (sexual and asexual strains)	all cell nuclei	mechanical dissociation	1/3 PBS hypotonic solution with 1% Triton X100	none	FACS-sorting (DNA content)	Hoshino et al. (1991)
	Turbellaria	*Dugesia japonica*	adult head	neuronal cells	chemical dissociation	5/8 Holtfreter's solution containing 30 μg/mL trypsin inhibitor	trypsin 0.25%	FACS-sorting (DNA-stained)	Asami et al. (2002)
	Turbellaria	*Dugesia japonica*	adult	neoblasts	chemical dissociation	5/8 Holtfreter's solution containing 30 μg/mL trypsin inhibitor	trypsin 0.25%	FACS-sorting (X-ray sensitive cells)	Hayashi et al. (2006)
	Turbellaria	*Schmidtea polychroa*	adult	neoblasts	mechanical dissociation	1% Digest-Eur in hypotonic solution	none	centrifugation (Percoll-density gradient)	Schürmann and Roland (2001)
	Turbellaria	*Schmidtea mediterranea*	adult tail	neoblasts and other cell types	mechanical dissociation	Ca-Mg-free buffer, with 1% BSA	none	FACS sorting (vital dye SiR-DNA; size and complexity gating)	Lei et al. (2019)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
Mollusca	Gastropoda	*A. californica*	adult pleural ganglia	neurons	mechanical	artificial seawater	none	none	Bedi et al. (1998)
	Gastropoda	*A. californica*	adult and juvenile	ganglia	enzymatic dissociation	L-15 medium	pronase	none	Schacher and Proshansky (1983)
	Gastropoda	*A. californica*	adult	ganglia	enzymatic dissociation	L-15 medium	collagenase	none	Montgomery et al. (2002)
	Gastropoda	*Helix aspersa aspersa, Helix aspersa maxima*	adult	gonadal cells	enzymatic dissociation	PBS	collagenase-dispase	none	Monnier and Bride (1995)
Annelida (Spiralia)	Clitellata	*Hirudo medicinalis*	adult, Matrigel implants	myoendothelial cells	mechanical	DMEM	none	none	Grimaldi et al. (2009)
	Clitellata	*Hirudo medicinalis*	adult, ganglia	retzius cells	enzymatic dissociation	L-15 medium	collagenase-dispase	none	Bookman and Liu (1990)
	Clitellata	*Hirudo medicinalis*	adult	microglial cells	enzymatic dissociation	L-15 medium	collagenase-dispase	none	Masuda-Nakagawa et al. (1994)
Cnidaria	Hydrozoa	*Polyorchis penicillatus*	adult nerve rings	neurons	enzymatic dissociation	Ca^{2+} and Mg^{2+} free ASW	collagenase 100 U/mL (3–5 h)	removal of unattached cells	Przysiezniak and Spencer (1989)
	Hydrozoa	*Podocoryne carnea*	adult umbrella	smooth muscle cells	mechanical dissociation	Ca^{2+} and Mg^{2+} free ASW (15 min)	collagenase 150 U/mL (6–8 h)	microdissection of striated muscle tissue	Schmid (1992)
	Anthozoa	*Leptogorgia virgulata*	adult	scleroblasts	mechanical dissociation	FSW with antibiotics	none	removal of suspended cells	Kingsley et al. (1987)

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Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
	Anthozoa	*Nematostella vectensis*	adult oral fragment	ectodermis	mucolytic agent (2% NAC for 10 s)	phosphate-buffer-saline	none	separation of epithelial layers by a reducing agent	Rabinowitz et al. (2016)
	Anthozoa	*Anemonia sulcata*	adult tentacle	epithelial and interstitial cells		Ca^{2+} and Mg^{2+} free ASW	papain 10 U/mL (1.5 h)	Percoll continuous gradient centrifugation	Apte et al. (1996)
	Anthozoa	*Anemonia viridis*	adult regenerating tentacle	adherent small round cell types	enzymatic dissociation	Ca^{2+} and Mg^{2+} free ASW then ASW	0.05% collagenase I (1 h)	fragments from 3d post-cutting tentacle	Barnay-Verdier et al. (2013)
	Anthozoa	*Anemonia viridis*	adult regenerating tentacle	gastrodermal differentiated cells	enzymatic dissociation	Ca^{2+} and Mg^{2+} free ASW with 1 mM glycine, then ASW	0.15% collagenase type I (30 min)	microdissected epithelia from 3d post-cutting tentacle	Ventura et al. (2018)
	Anthozoa	*Pocillopora damicornis*	adult	gastrodermal cell containing zooxanthellae	mechanical disruption	Ca^{2+} free ASW (3–4 h)	none	none	Gates and Muscatine (1992)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
	Anthozoa	*Stylophora pistillata, Porites lutea, Dendronephthya hemprichi, Paraerythropodium fulvum fulvum, Heteroxenia fuscescence, Clathraria rubrinoides, Plexaura* sp., *Millepora dichotoma*	adult and larva	all cell types	spontaneous or mechanical fragmentation	Ca^{2+} and Mg^{2+} free ASW	collagenase 0.05% (4 h)/pronase 0.1% (1 h)/EDTA 0.02% (2 h)	Percoll continuous gradient centrifugation	Frank et al. (1994)
	Anthozoa	*Acropora microphtalma, Pocillopora damicornis, Montipora digitata*	adult	all cell types	spontaneous dissociation	Ca^{2+} free ASW (2–4 h)	none	none	Kopecky and Ostrander (1999)
	Anthozoa	*Pocillopora damicornis*	adult	all cell types	spontaneous dissociation	Ca^{2+} free (and Mg^{2+} free) ASW (2–3 h)	none	Percoll step gradient centrifugation	Domart-Coulon et al. (2001)
	Anthozoa	*Pocillopora damicornis*	adult	all cell types	spontaneous dissociation	Ca^{2+} free ASW (3 h)	none	none	Domart-Coulon et al. (2004)
	Anthozoa	*Montipora digitata*	adult	all cell types	enzymatic dissociation	Ca^{2+} free ASW (2.5 h)	collagenase 0.15%	none	Helman et al. (2008)
	Anthozoa	*Xenia elongata*	adult	all cell types	enzymatic dissociation	Ca^{2+} free ASW (2.5 h)	collagenase 0.15%	none	Helman et al. (2008)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
	Anthozoa	*Simularia flexibilis*	adult	all cell types	mechanical/ enzymatic dissociation	ASW with 1% gentamycin–streptomycin	trypsine-EDTA 0.05%	none	Khalesi (2008)
	Anthozoa	*ystatin, Pavona divaricata*	adult	all cell types	mechanical dissociation	FSW	none	spontaneous cell aggregation into spheroids	Nesa and Hidaka (2009)
	Anthozoa	*Pocillopora damicornis*	adult	coral cell types without zooxanthellae	enzymatic dissociation	ASW	dispase 2 U/mL, lysozyme 3 U/mL, a amylase 2 U/mL, a glucosidase 0.5 U/mL, b galactosidase 0.5 U/mL, endoglycosidase H 0.25 U/mL	two successive Percoll step gradient centrifugations	Downs et al. (2010)
	Anthozoa	*Acropora millepora*	larva (planula)	"interstitial" cells	spontaneous dissociation	spontaneous dissociation	none	none	Reyes-Bermudez and Miller (2009)
	Anthozoa	*Pocillopora damicornis*	adult	all cell types	mechanical dissociation	3.3× PBS without Ca and Mg, 2% FCS, 20 mM Hepes, pH 7.4	none	FACS-sorting (size, autofluorescence, enzyme-activated fluorescence, organelle-specific fluo markers)	Rosental et al. (2017)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
	Anthozoa	*Fungia granulosa*	adult regenerating tissue (peeled off skeleton)	all cell types (coral and zooxanthellae)	mechanical dissociation	FSW with gentamicin and kanamycin (both 50 µg/mL) for 2 days only	none	spontaneous spheroid formation	Gardner et al. (2015)
	Anthozoa	*Pocillopora damicornis*	adult	all cell types	mechanical dissociation	3.3× PBS without Ca and Mg, 2% FCS, 20 mM Hepes, pH 7.4	none	FACS-sorting (size, autofluorescence, enzyme-activated fluorescence, organelle-specific fluo markers)	Rosental et al. (2017)
	Anthozoa	*Acropora tenuis*	planula	gastrodermal secretory cells, undifferentiated cells, neuronal cells, and epidermal cells	enzymatic dissociation	FSW	trypsin-EDTA + collagenase I (1–4 h)	none	Kawamura et al. (2021)
	Anthozoa	*Acropora digitifera*	adult	pluripotent cells	spontaneous dissociation (after thermal bleaching)	Ca²⁺ free ASW (2 h)	none	none	Reyes-Bermudez et al. (2021)
Ctenophora	Tentaculata	*Mnemiopsis leidyi*	adult healing lobe	all cell types	spontaneous	ctenophore mesogleal serum	0.25% trypsin/EDTA	none	Vandepas et al. (2017)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
Porifera	Demospongiae	*Axinella* sp.	adult	all cell types	mechanical dissociation	Calcium-/magnesium-free ASW + EDTA	none	none	Song et al. (2021)
	Demospongiae	*H. panicea, H. aquaeductus, H. dujardinii*	adult	all cell types	mechanical dissociation	FSW	none	none	Lavrov and Kosevich (2016)
	Demospongiae	*Spongosorites, Cinachyrella, Haliclona*	adult	all cell types	mechanical dissociation	CMFASW	none	sedimentation	Robinson (2015)
	Demospongiae	*H. oculata, H. xena, D. avara, A. polypoides*	adult	all cell types	mechanical dissociation	FSW	none	none	Schippers et al. (2011)
	Demospongiae	*Crambe crambe*	adult	all cell types	mechanical dissociation	sodium hypochlorite seawater, gentamicin, ystatin, penicillin	none	none	Garcia Camacho et al. (2006)
	Demospongiae	*S. domuncula*	adult	all cell types	mechanical dissociation	CMFSW-E/AB (penicillin, streptomycin)	none	none	Zhang et al. (2004)
	Demospongiae	*Ircinia muscarum, Dysidea avara, Suberites domuncula*	adult	all cell types	mechanical dissociation	CMFSW, ampicillin, gentamycin, kanamycin, tylosin, tetracyclin	none	none	De Rosa et al. (2001)

Table 1. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Isolation Technique	Isolation Medium	Enzyme Digestion	Cell-Type Enrichment Strategy	Reference
	Demospongiae	*Suberites domuncula*	adult	all cell types	mechanical dissociation	CMFSW EDTA	none	none	Müller et al. (1999)
	Demospongiae	*Geodia cydonium, Suberites domuncula*	Adult, outer cortical layer	spherulous cells	mechanical dissociation	PBSA-SW, EDTA	none	density gradient centrifugation using Percoll solution	Koziol et al. (1998)
	Demospongiae	*S. domuncula*	adult	all cell types	mechanical dissociation	CMFSW-E	none	none	Custodio et al. (1998)

Table 2. Established media and culture conditions across taxa. Indicative examples were selected among the references listed in Appendix B Table A2. When different methods were compared, only the selected optimized protocol is listed in the table. Basal commercial formula was adjusted to seawater osmolarity by dilution of concentrated formula in seawater or by the addition of mineral salts (except for GIM and GMIM). % indicates dilution in filtered natural (FSW) or artificial seawater (ASW). DMEM: Dulbecco's Modified Eagle Medium; MEM: Modified Eagle Medium; M199: medium 199; GIM: Grace's insect medium; GMIM: Grace's modified insect medium; L-15: Leibovitz medium L-15; RPMI: xx; IPM: Isotonic Planarian Medium; TTP: Teshirogi & Tohya Planarian Medium.

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/ Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
Chordata (Tunicata)	Ascidiacea	*Botryllus schlosseri*	adult zooids	circulatory blood cells	adherent/ suspended	DME supplemented with salts	glutamine solution	none	MgSO₄, MgCl₂	penicillin, streptomycin, nystatin	plastic	dark	air	19 °C	weekly	Rinkevich and Rabinowitz (1993)
	Ascidiacea	*Botryllus schlosseri*	embryo	all cell types	adherent/ suspended	DME supplemented with salts	glutamine solution, hemolymph from Botrylloides, chick embryo extract	none	MgSO₄, MgCl₂	penicillin, streptomycin, nystatin	plastic, gelatin, poly-l-lysin, collagen	dark	air	20 °C	weekly	Rinkevich and Rabinowitz (1994)
	Ascidiacea	*Botryllus schlosseri*	colonial buds	epithelium	adherent/ suspended	RPMI-1640	fetal calf serum, HEPES	none	MgSO₄, MgCl₂	penicillin, streptomycin, nystatin	plastic	dark	air	20 °C	partly replaced every other day	Rinkevich and Rabinowitz (1997)
	Ascidiacea	*Botryllus schlosseri*	adult zooids, buds	cup cell disease	suspended	Fisher's medium	fetal calf serum		salts	gentamycin	plastic	dark	air	20 °C	partly replaced every 2w	Moiseeva et al. (2004)
	Ascidiacea	*Botryllus schlosseri*	palleal buds	epithelium	adherent/ suspended	RPMI-1640, DMEM, HAM, Fischer's, Schnider's, Grace, Iscov's, DCCM-1	fetal calf serum, DMEM, HEPES, L-glutamine, EFG		salts	penicillin, streptomycin, amphotericin, gentamycin	Plastic, collagen, Matrigel, methocel, fibronectin, poly-l-lysine	dark	air	19-23 °C	partly replaced every 2w	Rabinowitz and Rinkevich (2004)
	Ascidiacea	*Botryllus schlosseri*	palleal buds	epithelium	adherent	L-15	fetal calf serum, HEPES, L-glutamine	none	salts	penicillin, streptomycin, amphotericin	plastic	dark	air	20 °C	unknown	Rabinowitz et al. (2009)

Table 2. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/ Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
	Ascidiacea	*Botryllus schlosseri*	palleal buds	epithelium	adherent	L-15	fetal calf serum, HEPES, L-glutamine	none	salts	penicillin, streptomycin, amphotericin	plastic	dark	air	20 °C	unknown	Rabinowitz and Rinkevich (2011)
	Ascidiacea	*Botrylloides leachii*	adult, hibernating colonies	blood cells	adherent	DME supplemented with salts	glutamine solution	none	MgSO₄, MgCl₂	penicillin, streptomycin, nystatin	plastic	dark	air	19 °C	weekly	Hyams et al. (2017)
Chordata (Cephalochordata)	Leptocardii	*Branchiostoma belcheri* Japanese	Adult epidermis, gill, gut, spermary, ovary	all cell types	suspended cells	2× Leiboviz's L-15 medium	10% (FBS) + 1% BPE	none	none	streptomycin (100 U/mL), rifampicin (50 µg/mL)	unknown	unknown	unknown	25 °C	unknown	Cai et al. (2013)
	Leptocardii	*Branchiostoma belcheri tsingtauense*	adult, buccal cirri, tail, gill, gut and metapleural fold	all cell types	adherent	L-15 and F-12	20% FBS	none	none	none	plastic	unknown	air	25 °C	every 10–15 days	Wang et al. (2009)
Echinodermata (Ambulacraria)	Asteroidea	*Asterias amurensis*	embryo (mesenchyme migration stage)	all cell types	suspended cells	Millipore-filtered seawater	4% (v/v) newborn bovine albumin	none	none	penicillin G potassium (50 units/mL) /streptomycin sulfate (50 µg/mL)	plastic	ambient	air	18 °C	none	Kaneko et al. (1995)
	Echinoidea	*Strongylocentrotus intermedius*	embryo	all cell types	adherent cells	seawater	2 or 8% FCS	none	none	none	glass	unknown	unknown	17 °C	unknown	Odintsova et al. (2015)
	Holothuroidea	*Apostichopus japonicus*	adult	guts	adherent	L-15	2% FCS	none	vitamin E, insulin	penicillin, streptomycin, gentamycin	plastic	unknown	unknown	15 °C	every 3 days	Odintsova et al. (2005)
Xenacoelomorpha	Acoela	*Isodiametra pulchra*	adults	all cell types	suspended	ASW	none	none	none	none	none	unknown	unknown	unknown	cells used directly for experimentation	De Mulder et al. (2009)
Arthropoda (Ecdysozoa)	Malacostraca	*Pacifasticus leniusculus*, *Homarus americanus*	adults	hematopoietic and testicular tissue	suspended	Medium 199	10% fetal bovine serum	none	NaHCO₃	penicillin, streptomycin	plastic	unknown	air	20 °C	weekly	Brody and Chang (1989)

Table 2. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/ Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
	Malacostraca	*M. ensis*	adults	lymphoid organ	adherent	L-15	15% FBS, glucose	none	NaCl, NaHCO$_3$	Penicillin, streptomycin	plastic	dark	5% CO$_2$	26 °C	unknown	Han et al. (2013)
	Malacostraca	*Penaeus vannamei*	juvenile	all cell types	adherent	L-15 or M199	FBS 10%	none	none	penicillin, streptomycin	plastic	unknown	unknown	unknown	unknown	Toullec et al. (1996)
Nematoda (Ecdysozoa)	Chromadorea	*C. elegans*	eggs	larval cells	adherent	L-15	15% FBS	none	none	penicillin, streptomycin	glass	dark	without CO$_2$	20 °C	unknown	Zhang et al. (2011)
Platy-helminthes	Turbellaria	*Dugesia japonica*	adult head	neuronal cells	adherent cells	modified ITP medium; Teshirogi and Tohya, 1998	unknown	unknown	unknown	none	fibronectin or laminin-coated glass	dark	air	20 °C	none (4 d culture)	Asami et al. (2002)
	Turbellaria	*Schmidtea polychroa*	adult	neoblasts	adherent cells	isosmotic saline (Hepes-buffered)	FCS and BSA ("BMS"), MEM amino acids (essential and non-essential), glucose, trehalose, sodium pyruvate, glutamine	none	d-biotin, MEM vitamins	neomycin sulfate 100 mg/L	collagen/ fibronectin coated glass and plastic; planarian homologous matrix	dark	air	18 °C	every 3rd day	Schürmann and Roland (2001)
	Turbellaria	*Schmidtea mediterranea*	adult tail	neoblasts	adherent cells	IPM, KnockOut DMEM, diluted L-15, diluted Grace's medium	FBS 5%	none	unknown	none	poly-D-lysine (50 µg/mL) coated plastic	dark	5% CO$_2$–95% air (for L-15: air)	22 °C	none (6 d culture)	Lei et al. (2019)
Mollusca	Gastropoda	*A. californica*	adult pleural ganglia	neuronal cells	adherent	L-15	hemolymph supplemented	none	none	none	plastic	dark	unknown	18 °C	unknown	Bedi et al. (1998)
	Gastropoda	*A. californica*	adult and juvenile	ganglia	adherent	L-15	1% FBS	none	dextrose, glutamine	none	Plastic, poly-l-lysine	dark	air	RT	every 2 days	Schacher and Proshansky (1983)

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Table 2. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
	Gastropoda	*A. californica*	adult	ganglia	adherent	L-15	FBS	none	none	none	glass	unknown	unknown	18 °C	none	Montgomery et al. (2002)
	Gastropoda	*Helix aspersa aspersa*, *Helix aspersa maxima*	adult	gonadal cells	adherent	Medium 199	20% FCS	none	EGF, methyl cellulose	penicillin, streptomycin	plastic	unknown	unknown	unknown	unknown	Monnier and Bride (1995)
Annelida (Spiralia)	Clitellata	*Hirudo medicinalis*	adult, Matrigel implants	myoendothelial cells	adherent	DMEM	FBS 10%	none	glutamine	none	plastic	unknown	unknown	20 °C	unknown	Grimaldi et al. (2009)
	Clitellata	*Hirudo medicinalis*	adult, ganglia	retzius cells	adherent	L-15	FBS 2%	none	none	gentamycin	plastic, poly-L-lysine	unknown	unknown	20 °C	unknown	Bookman and Liu (1990)
	Clitellata	*Hirudo medicinalis*	adult	microglial cells	adherent	L-15	Glutamine, glucose	none	none	gentamycin	plastic	unknown	unknown	unknown	unknown	Masuda-Nakagawa et al. (1994)
Cnidaria	Hydrozoa	*Polyorchis penicillatus*	adult nerve rings	neurons	adherent cells and clusters	ASW	none	none	none	1% gentamycin	native mesoglea	ambient	air	10–15 °C	daily	Przysiezniak and Spencer (1989)
	Hydrozoa	*Podocoryne carnea*	adult umbrella	smooth muscle cells	adherent cells	ASW	unknown	unknown	unknown	unknown	remnants of native mesoglea	ambient	air	12 °C	none	Schmid (1992)
	Anthozoa	*Leptogorgia virgulata*	adult	scleroblasts	adherent cells	TC199	30% horse serum	none	none	penicillin + streptomycin + colimycine + bacitracine (first 2 weeks)	plastic	12L:12D	air	21 °C	daily, then every 2–3 days	Kingsley et al. (1987)
	Anthozoa	*Nematostella vectensis*	adult	ectodermis	adherent cells	20% L-15	3% FCS	none	none	penicillin, streptomycin, amphotericin b	plastic	dark	air	20 °C	weekly, half medium	Rabinowitz et al. (2016)
	Anthozoa	*Anemonia sulcata*	adult tentacle	epithelial and interstitial cells	suspended cells	ASW	none	none	none	1% streptomycine	plastic	ambient	air	16 °C	none	Apte et al. (1996)

Table 2. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/ Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
	Anthozoa	*Anemonia viridis*	adult regenerating tentacle	anemone small round cell types	adherent clusters of cells	80% modified DMEM	5% FBS	none	none	1% kanamycin (100 µg/mL), 1% Amphotericin B (2.5 µg/mL) (after 3–7 d MycoKill 1× initial supplementation)	plastic	dark	air	20 °C	weekly	Barnay-Verdier et al. (2013)
	Anthozoa	*Anemonia viridis*	adult regenerating (whole) tentacle	anemone gastrodermal differentiated cells	suspended	20% GMIM	5% FBS, 1% L-glutamate	none	none	1% kanamycin (100 µg/mL), 1% Amphotericin B (2.5 µg/mL), 1% Antibiotics-Antimycotis (Sigma)	plastic	dark	air	20 °C	weekly	Ventura et al. (2018)
	Anthozoa	*Pocillopora damicornis*	adult	gastrodermal cell containing zooxanthellae	suspended cells	filtered seawater	none	none	none	none	polylysine 0.1% coated slides	NA	air	25 °C	none	Gates and Muscatine (1992)

Table 2. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/ Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
	Anthozoa	*Stylophora pistillata, Porites lutea, Dendronephthya hemprichi, Paraerythropodium fulvum fulvum, Heteroxenia fuscescence, Clathraria rubrinoides, Plexaura sp., Millepora dichotoma*	adult and larva	all cell types	adherent and suspended cells	10% L-15/DMEM/M199 + taurine 40 mg/L	10% FCS	none	none	1% gentamycin, penicillin, streptomycin, amphotericin, nystatin	plastic	NA	air	23–24 °C	weekly	Frank et al. (1994)
	Anthozoa	*Acropora microphthalma, Pocillopora damicornis, Montipora digitata*	adult	all cell types	suspended multicellular isolates	25–50% DMEM	10% FBS	none	Mg^{2+}, Fe^{2+}, Mn^{2+}	1% Antibiotic-Antimycotic Gibco	Primaria plastic	24 h light	5% CO_2–95% air	24 °C	none	Kopecky and Ostrander (1999)
	Anthozoa	*Pocillopora damicornis*	adult	all cell types	suspended cells and adherent multicellular isolates	50% DMEM + taurine 40 mg/L	10% FBS	none	Sr^{2+}	1% Antibiotic-Antimycotic Gibco	Primaria plastic	24 h light	5% CO_2–95% air	24 °C	once every 2 weeks	Domart-Coulon et al. (2001)
	Anthozoa	*Pocillopora damicornis*	adult	all cell types	adherent multicellular isolates	12.5% DMEM + taurine 40 mg/L	1.25% FCS	none	Sr^{2+}	1% Antibiotic-Antimycotic Gibco	RGD peptide-coated plastic/glass	12 hL:12 hD	air	27 °C	weekly	Domart-Coulon et al. (2004)
	Anthozoa	*Montipora digitata, Xenia elongata*	adult	all cell types	adherent cells and cell aggregates	ASW + MEM aminoacids + taurine 10 mM, glutamine 2mM, aspartic acid 20 µg/mL + glucose 0.1–3 mM	2% FBS	ascorbic acid 50 µg/mL	MEM vitamins	1% Antibiotic-Antimycotic Gibco	Primaria plastic	12 hL:12 hD	air	26 °C	weekly (every other day ascorbic acid)	Helman et al. (2008)

Table 2. *Cont.*

Phylum Class	Species	Inoculum Type	Target Cells	Adherent/Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
Anthozoa	*Sinularia flexibilis*	adult	all cell types	suspended cells and cell clusters	GIM, GMIM without salt addition	none	none	none	1% gentamycine-1% streptomycin	plastic	12 hL:12 hD	5% CO_2-95% air	24 °C	weekly	Khalesi (2008)
Anthozoa	*Fungia* sp., *Pavona divaricata*	adult	all cell types	suspended tissue balls	seawater	none	ascorbic acid 125 μM + catalase 250 U/mL; mannitol 10 mM	none	none	plastic	24 h light	air	25 °C	daily	Nesa and Hidaka (2009)
Anthozoa	*Pocillopora damicornis*	adult	coral cell types without zooxanthellae	suspended cells	RPMI + $CaCl_2$ 1 mM, NaPyruvate 1 mM, glucose 2g/L, galactose 2g/L, Na-lactic acid 0.25 g/L, Bovine Albumin 1 g/L, cysteine 0.5 mM, methionine 0.5 mM, glutamine 0.25 g/L	none	ascorbate 0.25 mM	hydroxy-cobalamin 0.01 mM, Na-folate 0.001 mM, succinate 0.05 g/L	none	plastic or teflon	light?	air	26 °C	every 3rd day	Downs et al. (2010)
Anthozoa	*Acropora millepora*	larva (planula)	"interstitial" cells	suspended cells	10% DMEM	5% FBS	none	2% marine enrichment F/2 (Sigma)	3% Antibiotics-Antimycotis Sigma	plastic	dark	air	23 °C	every 2 weeks	Reyes-Bermudez and Miller (2009)
Anthozoa	*Fungia granulosa*	adult regenerating tissue (peeled off skeleton)	all cell types	suspended spheroids	FSW without antibiotics	coralline algae whole fragment	none	none	none	glass	10 hL:14 hD 100 μmol photons m^{-2} s^{-1}	air	25 °C	every 3rd day	Gardner et al. (2015)

Table 2. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/ Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
	Anthozoa	*Acropora tenuis*	planula	gastrodermal secretory cells, undifferentiated cells, neuronal cells, and epidermal cells	cell aggregates (brown-pigmented)	10% DMEM	1.5% FBS	none	plasmin 2 µg/mL (modular protease)	penicillin 100 U/mL, streptomycin 100 µg/mL, amphotericinB (0.25 µg/mL)	plastic	dark	air	20 °C	every 3rd day	Kawamura et al. (2021)
	Anthozoa	*Acropora digitifera*	adult	pluripotent cells	suspended cell aggregates	30% DMEM	10% FBS	none	1% Glutamax	1% penicillin/ streptomycine, 0.1% fungizone	plastic	dark	air	23 °C	when 60% confluence	Reyes-Bermudez et al. (2021)
Cteno-phora	Tentaculata	*Mnemiopsis leidyi*	adult wound-healing lobe	all cell types	round ectodermal cells/ adherent giant smooth muscle cell/ suspended digestive endodermal cells	10–50% ctenophore mesogleal homogenate (in seawater)	ctenophore mesogleal serum (CMS) homogenate	none	none	1% penicillin/ streptomycin	plastic, glass	dark	air	14–16 °C	every 3rd day	Vandepas et al. (2017)
Pori-fera	Demos-pongiae	*Axinella* sp.	adult	all cell types	suspended cells and cell clusters	NSW (natural see water)	none	none	none	400 mg/L gentamicin	plastic	unknown	air	24 °C	every 1–4 days	Song et al. (2021)
	Demos-pongiae	*H. panicea, H. aquaeductus, H. dujardinii*	adult	all cell types	suspended cells	FSW	none	none	none	none	plastic	unknown	air	8–10 °C	every 48 h	Lavrov and Kosevich (2016)
	Demos-pongiae	*Spongosorites, Cinachyrella, Haliclona*	adult	all cell types	suspended cells	CMFASW	none	none	none	none	plastic	unknown	reduced air	unknown	none	Robinson (2015)

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Table 2. *Cont.*

Phylum	Class	Species	Inoculum Type	Target Cells	Adherent/ Suspended Cell Culture	Basal Formula	Supplement	Antioxidant	Trace Elements and Vitamins	Antimicrobials	Substrate	Light	Atmosphere	Temperature	Medium Change	Reference
	Demospongiae	*H. oculata, H. xena, D. avara, A. polypoides*	adult	all cell types	suspended cells	Calcium- and magnesium-free ASW EDTA	none	none	none	none	plastic	unknown	unknown	2 °C	unknown	Schippers et al. (2011)
	Demospongiae	*Crambe crambe*	adult	all cell types	suspended cells	RPMI 1640	octopus extract 5–20%	none	inorganic salts, amino acids	none	plastic	unknown	4% CO$_2$	17–19 °C	1–4 per week	Garcia Camacho et al. (2006)
	Demospongiae	*S. domuncula*	adult	all cell types	suspended cells	SW-AB	none	none	L-glutamine, pyruvate, ferric citrate, sodium silicate, RPMI 1640, Marine Broth	penicillin, streptomycin	plastic	unknown	unknown	16 °C	none	Zhang et al. (2004)
	Demospongiae	*Ircinia muscarum, Dysidea avara, Suberites domuncula*	adult	all cell types	suspended cells	CMFSW, DMEM after 3rd week	sterols in DMEM	none	none	ampicillin, gentamycin, kanamycin, tylosin, tetracyclin	plastic	Dark or light	unknown	18 or 22 °C	Every 3 days	De Rosa et al. (2001)
	Demospongiae	*Suberites domuncula*	adult	all cell types	suspended cells	CMFSW EDTA	none	none	none	penicillin, streptomycin	plastic	unknown	unknown	16 °C	daily	Müller et al. (1999)
	Demospongiae	*Geodia cydonium, Suberites domuncula*	Adult, outer cortical layer	spherulous cells	suspended cells	PBSA-SW	none	none	none	none	plastic	unknown	unknown	17 °C	none	Koziol et al. (1998)
	Demospongiae	*S. domuncula*	adult	all cell types	suspended cells	CMFSW-E	none	none	none	penicillin, streptomycin	plastic	unknown	unknown	16 °C	daily, once a week after the 2nd week	Custodio et al. (1998)

Table 3. Molecular tools to detect the most frequently recorded unicellular microeucaryote contaminants. Sequences from cloned PCR products amplified with these eukaryote primers are blasted against National Center for Marine Biotechnology (NCBI) nucleotide databases to check the potential microbial nature of long-term cultured candidate aquatic invertebrate stem cells.

Host Metazoan Taxon	Associated Microeukaryote Taxon	Opportunistic In Vitro Growth	Gene Marker	Amplicon Size (bp)	Primer Pairs	Oligonucleotide Sequences	Reference
Scleractinian coral	Chromeria velia (Chromerida: Alveolata)	mixotrophy: use of medium-provided mono/disaccharides and aminoacids (glutamate and glycine)	psbA	~2000	psbAf6	5'-GARCAC AACATHYTNA TGCAYCC-3'	Moore et al. (2003)
					psbAL1	5'-CRTGCA TWACTTC CATWCC-3'	
Scleractinian coral	Stramenopiles (Thraustochytriidae)	use of C & N from Leibovitz L-15 formula diluted in filtered seawater (and organics from mucus of coral)	18S rRNA	1500	Lab-490F	5'-TTCGG TTCCGG AGAGGG AGCCTGAGAG-3'	Siboni et al. (2010)
					Lab-2004R	5'-GCAGA ATCCGA AGAIT CACCGG-3'	
all aquatic invertebrates	unicellular holozoan Opisthokonts	predation on invertebrate cells and debris, osmotrophy	specific alpha-Amylases, alpha-glucosidases	variable	18SFU	5'-ATGC TIGTC TCAAAG RYTAA GCCATGC-3'	Tikhonenkov et al. (2020b)

Table 3. *Cont.*

Host Metazoan Taxon	Associated Microeukaryote Taxon	Opportunistic In Vitro Growth	Gene Marker	Amplicon Size (bp)	Primer Pairs	Oligonucleotide Sequences	Reference
					18SRU	5'-CWGG TTCAC CWACGG AAAC TTGTTACG-3'	
all aquatic invertebrates	Fungi (including chytrids)	saprotrophy (feed on cellular debris)	18S rDNA	2000	SR1R	5'-TACCT GGTT GATQC TGCCAGT-3'	Fliegerová et al. (2008)
					NS8.1	5'-CCGC AGGTTC ACCTACG-3'	
all aquatic invertebrates	Volvocacean algae/zooxanthellae	phototrophy (use light and inorganic C & N)	ITS rDNA	700	ITSa	5'-GGGA TCCGTTTCCG TAGGTGAAC CTGC-3'	Coleman et al. (1994)
					ITSb	5'-GGGA TCCAT ATGCT TAAG TTCA GCGGG-3'	
all aquatic invertebrates	Eukaryotes (including chytrids)	undetermined organic C & N	18S rDNA	1800	#328	5'-ACCTG GTTGAT CCTGC CAG-3'	Kupper et al. (2006)
					#329	5'-TGAT CCTTC YGCA GGTTCAC-3'	

2. Isolating Stem-like Cell Types Suitable for In Vitro Culture

Inoculum type is highly diverse, depending on targeted aquatic invertebrate taxon, tissue and life cycle stage. At the same time, selecting the proper inoculum for a given in vitro culture is certainly the most important decision towards establishing a suitable cell culture.

2.1. Selecting Suitable Sources of Cells

Based on the number of publications on primary cultures and the number of reported cell lines across aquatic invertebrate taxa (Figure 1), most research has focused on cell culture establishment from mollusks (mostly bivalves, and comparatively fewer gastropods and cephalopods), followed by porifera (mostly demosponges), cnidaria (historically hydrozoans and currently anthozoans, Figure 2), crustacea (pennaeid shrimps, crabs and crayfish), echinoderms and tunicates. Episodic attempts have also been made for one or two species representatives of ctenophores (*Mnemiopsis*), annelids (*Lumbricidae*; *Nereidae*), nematodes (*Caenorhabditis*), chelicerates (*Limulus*) and cephalochordates (*Amphioxus*). These differences in research efforts illustrate differences in the attractivity of specific taxa for cell culture, which stem from three complementary considerations, detailed below, that every researcher has to take into account when selecting the origin of the cells to be cultured in vitro.

The first pertinent consideration is whether to work on tissue isolated from established experimental models maintained in controlled aquarium or laboratory facilities. These animals, in contrast to wild animal sampling, provide both access to early life stages, as well as increased reproducibility for cell culturing experiments. Their use also meets the biodiversity protection regulations and traceability requirements of the Nagoya protocol. More and more clonal lineages of genotyped animals are becoming available across taxa, and establishing cell cultures from this traceable material is an additional source of reproducibility that reduces complexity and facilitates comparisons between intervention protocols. Ultimately, these biological models will help the optimization of the culturing conditions, for guidelines specific to a few model species. In this context, two attractive taxa for fundamental research are cnidaria (e.g., *Hydra vulgaris, Hydractinia echinata, Exaiptasia pallida*) and platyhelminthes (e.g., *Schmidtea mediterranea*) with well-established strains of animals. Mollusks or crustaceans of commercial importance, and with a complete life cycle obtained in captivity and traceable across generations, also represent important taxa to develop stem cell cultures.

A second important consideration when selecting a model species for cell culturing experiments is the wealth of genomic, transcriptomic and metabolomic information available for that species. In addition to allowing the identification of cell-type-specific markers, post-genomic information gained on metabolic pathways

and cellular adhesion systems can be used to formulate working hypotheses on taxon-specific in vitro cellular requirements of media components and substrates. Similarly, knowledge acquired on in vivo tissue homeostasis, dynamics of cell proliferation, and somatic stem cell niches (Martinez et al. 2022) could help in selecting a seed tissue of high proliferative potential. In this respect, the continuous decrease in sequencing costs, as well as in other omics techniques, is allowing more and more research groups working with marine aquatic invertebrates to characterize their favorite species, suggesting that omics information will fast become available for almost all taxa.

The third point to consider is the desired approach for obtaining immortalized cells. Similar to mammalian cell culture, potential sources for immortalized cell lines are artificially reprogrammed cells and spontaneously tumor-like tissue. However, immortalization methodologies are currently limited in aquatic invertebrate cells by low yields and poor stability, as observed in sponges (Pomponi et al. 2013; Revilla-I-Domingo et al. 2018), bivalve mollusks (Hetrick et al. 1981; Boulo et al. 1996), and crustaceans (Claydon and Owens 2008; Xu et al. 2018). As suggested by Odintsova et al. 2011, natural tumor-like tissue, characterized by increased (hyperplasia) or altered (neoplasia) cell proliferation patterns, is thus a promising inoculum to initiate primary cultures. However, tumor-like lesions in wild or captive aquatic invertebrate taxa have low registered frequencies (Peters 2006; Tascedda and Ottaviani 2014), and there has been repeated unexpected failures at maintaining the hyperproliferation of successfully isolated cancerous cells in vitro. For instance, in transmissible soft-shell clam (*Mya arenaria*) leukemia, cancerous hemocytes rapidly undergo in vitro apoptosis, triggered by the release of mortalin-based cytoplasmic sequestration of p53 (Walker et al. 2006). Other attempts at primary culture initiation from artificially induced tumors of carcinogen-exposed bivalves (*Crassostrea virginica*) also failed to maintain persistent in vitro cell division (Hetrick et al. 1981).

Consequently, the use of stem-like cells for seeding in vitro cultures appears key to setting dividing primary cultures. Marine invertebrates display a wide variety of intriguing cellular phenomenon, such as asexual reproduction, striking regenerative capacity, reduced aging and dormant stages, which upon arousal restore fully functional individuals (Figure 1). These mechanisms indicate high cellular plasticity, proliferation and a likely involvement of stem-like cells. Although the potency of these cells remains largely uncharacterized in most species, and the orthology between these stem-like cells remains to be assessed (Rinkevich et al. 2022), they represent a promising source of proliferating and self-renewable cell types. However, the identification, isolation and characterization of aquatic invertebrate stem cells remains a major, typically species-specific, technical challenge. With few species having established protocols for the isolation of identified stem-like cells (Hayashi et al. 2006; Sun et al. 2007; Hemmrich et al. 2012; Kassmer et al. 2020;

Reyes-Bermudez et al. 2021), their generalization and transfer by taking advantage of the vast diversity of specific approaches explored in other taxa (Table 1) appear particularly promising.

2.2. Selecting a Suitable Type of Inoculum

Aquatic invertebrates have indirect development cycles, with widespread asexual propagation strategies, including colonial budding and the generation of dormant stages, as well as high regenerative abilities, including whole-body regeneration. These developmental properties are suggestive of the presence of proliferative cells, including potential stem-like cells, which are of particular interest to establish proliferating cell cultures. Hence, they provide the following range of theoretically ideal inoculum material: embryonic/larval tissue, regenerating tissue, asexually propagating tissue and dormant stages.

Dissociated tissue from whole embryo/larva consistently yields primary cell cultures dividing over 2–3 weeks, allowing a few rounds of successive subcultures. For example, when applied to cnidarian models, whole dissociated *Acropora* planula larvae yielded subsets of dividing coral cells that could undergo several successive subcultures (see Reyes-Bermudez and Miller (2009) for *A. millepora*, and Kawamura et al. (2021) for *A. tenuis*).

Dissociated somatic adult tissue sampled from regenerating tissue has also been observed to yield dividing cell cultures that could be subcultured for several weeks. Among other examples, cultures based on regenerating tentacle tips of the sea anemone *Anemonia viridis* (Barnay-Verdier et al. 2013) could be subcultured for 2–4 weeks, and primary cultures from regenerating intestinal tissue of the holothurian *Apostichopus japonicus* displayed limited but active in vitro proliferation at ~2 weeks after evisceration (Odintsova et al. 2005).

The dissociation of asexually growing tissue similarly gave rise to cell cultures with observable proliferative activity for a few weeks. For instance, using fast-growing branch tip fragments of the *Acropora millepora* coral (Reyes-Bermudez et al. 2021), cells could be subcultured for 2–4 weeks, and delayed senescence was reported in primary cultures from extracted buds of tunicates (Rabinowitz and Rinkevich 2004).

As implied in the "live slow, grow old" adage, cold adapted hibernating freshwater sponge species from lake Baikal yielded primary cell reaggregate (termed primmorph) cultures with record (max 8 months) longevity (Chernogor et al. 2011). Sponge gemmules also represent dormant hibernation/aestivation stages rich in multipotent stem cells (Simpson 1984) that, upon hatching, regenerate a functional adult. Activated gemmules could thus constitute a promising inoculum for primary cultures. Similarly, in the colonial tunicate *Botrylloides leachii*, arousal from a cold-induced dormancy (Burighel et al. 1976) leads to the restoration of multiple adults by proliferating *piwi*+/*pl10*+ cells, two markers suggestive of stem-like

properties (Hyams et al. 2017). In both cases, investigating the mechanisms regulating arousal from dormancy may yield cues to stimulate the tissues established in vitro to switch from quiescence to active cell cycling.

In conclusion, despite their initial abundance in cells with proliferative stemness-like properties, the shared in vitro fate of all four above-cited inoculum categories is terminal cell cycle arrest and the gradual accumulation of senescent, necrotic cells in primary culture and subsequent subcultures.

2.3. Selecting Suitable Cell Isolation Techniques

Inoculum type is highly diverse, depending on targeted aquatic invertebrate taxon, tissue and life cycle stage. Although no cell culture has yet been observed to sustain its proliferative activity for long, short-term functional primary cultures are routinely established from terminally differentiated cell types of aquatic invertebrates. Differentiated cells being arrested in G_0 can survive in vitro for a limited time with intact function, and hence are best used within hours to ~3 days of isolation. Nevertheless, comparing their tissue-isolation protocols offers opportunities to survey tissue sampling and dissociation methods (Table 1), as well as the cellular interactions and defense mechanisms that may support their in vitro viability, even for short periods of time. Emblematic examples of short-term invertebrate primary culture from quiescent cells include neuron-like cells and circulating hemocytes.

Giant neuronal cells from gastropod mollusks, such as the sensory and motor neurons from the sea hare *Aplysia californica*, are used to study growth cone motility and synapse plasticity (Kaczmarek et al. 1979; Lee et al. 2008; Zhao et al. 2009; Ren et al. 2019; Suter 2011). Cultured neurons from the pond snail *Lymnea stagnalis* are also routinely used for studies on synapse formation, neuronal aging and memory (Magoski et al. 1994; Prinz and Fromherz 2000; Walcourt and Winlow 2019). The in vitro establishment of nerve cells from jellyfish bell tissue (Przysiezniak and Spencer 1989; Schmid 1992) or from the solitary tunicate *Ciona intestinalis* (Zanetti et al. 2007) have also been reported. These neuronal cell types are usually micro-dissected from their ganglion, enzyme digested with protease, immobilized on positively charged polylysine-coated coverslips and then used for short-term electrophysiology assays, providing non-conventional in vitro models in neuroscience.

Circulating cells sampled from internal fluids, are another major category of cultured aquatic invertebrate cells. When seeded at high density ($>10^6$ cells/mL), cultured adherent hemocytes can form partly complete confluent monolayers, with clusters forming in suspension above the monolayer that may then be detached and transferred to new culture dishes. Such cultures have been routinely established since the late 1960s from a wide range of species, including mollusks, crustaceans, tunicates and echinoderms, typically for in vitro cell/microbe interactions and immunopathology assays. Such cultures display short-term conserved functionality,

as shown by phagocytosis or immunomodulatory assays. Proliferation may be induced by stimulation with bacterial antigens, as shown for the bivalve *Mytilus galloprovincialis* (Cao et al. 2003), the tunicate *Styela* (Raftos and Cooper 1991) and the earthworm *Lombricus* (Bilej et al. 1994). These differentiated cell types are drawn directly from internal cavities, lacunae and sinuses using a syringe. To counter their spontaneous self-aggregation (clotting) behavior, hemocytes are collected in syringes half-filled with species-specific anti-clotting saline solution, such as artificial seawater without calcium or magnesium, artificial seawater with a calcium chelator, or Na-citrate based "Alsever" saline solutions. Indeed, hemocytes secrete their own set of taxon-specific lectins (e.g., Matsumoto et al. 2001) and extracellular-matrix components (ECM) (e.g., a fibronectin-like ECM in bivalve hemocytes (Dyachuk et al. 2015)) that support rapid adherence, within hours of sampling, to glass or poly-lysine-coated coverslips.

Aside from the two isolation techniques described above, the quantitative evaluation of various approaches for cell extraction in different species suggests that, for the rapid obtention of single-dissociated cells from soft tissues for RNAseq cell phenotyping, and thus to obtain cells as close as possible to their wild-type state, mechanical isolation is the most efficient method (Khalesi 2008; Dessai 2012; Daugavet and Blinova 2015; Maselli et al. 2018). For example, cnidarian larval tissue or demosponge and calcisponge adult tissue fragments are dissociated within minutes via shearing in calcium-free seawater and passage through a 40–70 μm nylon mesh. However, species with tough cuticles (e.g., *Lombricidae*), important extracellular matrices (e.g., *Styeliidae*) or abundant surface mucus (e.g., *Dugesiidae*) necessitate treatments with specific enzymes to liberate the cells. For instance, in the stony coral *Pocillopora damicornis,* chemical treatment with a divalent cation chelator followed by a mix of glycosidases and collagenase was reported to help dissolve the mucus and improve the yield of released cells (Downs et al. 2010). Proteolytic treatments (trypsine, dispase and other protease mixes) are routinely used to dissociate cells from solid tissues dissected from mollusks and crustaceans. Interestingly, protease treatment may induce cellular reprogramming, as shown by the collagenase-induced transdifferentiation of in vitro explanted striated muscle of jellyfish (Alder and Schmid 1987; Schmid and Alder 1984; Schmid and Reber-Müller 1995).

2.4. Selecting Suitable Cell-Type Enrichment Strategies

Cells of interest are typically mixed with other cell types after the dissociation of the inoculum. The enrichment of specific cell types, typically proliferative or multipotent ones, relies on the prior development of taxon-specific and custom-designed cell separation methods. For instance, in *Stylophora pistillata*, stem cells were not identified in the cell atlas established from both larval and adult

tissues, following either enzymatic or mechanical dissociation methods (Levy et al. 2021), which severely limits the development of stem cell-enriched primary cultures.

Sorting methods for enriching inoculum suspensions in proliferative or multipotent cell types are thus required. Initial methods were based on differential sedimentation on density gradients, including sucrose, Percoll, or mixtures of Ficoll and polyethylene glycol. To further discriminate between morphologically similar cell types, and thus target specific cell types, Fluorescence Activated Cell Sorting (FACS) methods have recently been developed and have become highly prominent. For instance, FACS has been used to separate vital-stained coral cells (Rosental et al. 2017), and to isolate cell-type subpopulations for their single-cell gene expression characterization in hydrozoan (Siebert et al. 2019), as well as in anthozoan species (Levy et al. 2021; Sebé-Pedrós et al. 2018). In these diblastic animals, which lack a circulatory system, FACS is necessary to enrich dissociated tissue suspensions in hexacorallian putative immune cells, the amoebocytes recovered from the inter-epithelial mesogleal layer typical of cnidarian, for short-term functional phagocytosis characterization (Snyder et al. 2021). In triploblastic animals, FACS has also been refined to sort tunicate cell subpopulations to study the hematopoietic system (Rosental et al. 2018). Echinoderm coelomocyte subpopulations have been further separated by FACS into distinct cell types, such as the red pigment autofluorescent spherulocytes (Hira et al. 2020).

Consequently, there is a need for stem cell markers suitable for non-invasive stemness tracing in live cells to enable their enrichment. One promising perspective comes from the few aquatic invertebrate experimental models that can be genetically manipulated for which transgenic reporters of stemness properties can be engineered (e.g., in *Hydra* (Juliano et al. 2014)). Another direction of interest is the usage of fluorescent markers conjugated with antibodies specifically labeling stem cells. However, the identification of such markers remains extremely rare for aquatic invertebrates, with the recent notable exception of the colonial tunicate *Botrylloides diegensis* for which integrin-alpha-6 was shown to specifically label pluripotent cells (Kassmer et al. 2020). Whether this specific marker can be used in other species of aquatic invertebrates to label stem cells will be important to assess.

2.5. Selecting Cleansing Techniques to Minimize Contamination

There is a wide consensus across the scientific community that the highest obstacle to continuous marine/freshwater invertebrate cell culture propagation is overgrowth by aquatic microbial contaminants (Rinkevich 2005). This problem is critical in marine invertebrate primary cell cultures for two main reasons. First, it is because the tissues sampled to initiate the primary cultures come typically from areas directly or semi-directly exposed to environmental microbes, such as the thin epithelial structures at the interface with water (e.g., in porifera and

cnidaria), tissues irrigated by a semi-open circulatory system (e.g., in mollusks, echinoderms and tunicates) or digestive and other internal tissues hosting their own microbiota (e.g., gills and hepatopancreas of mollusks). Second, commercial antibiotics/antimycotics/antiparasitic drugs have been designed against microbes isolated from terrestrial animals and mostly from humans and are thus largely ineffective against the mostly underexplored diversity of environmental aquatic microbes. To control the contamination of cell cultures by these aquatic microbes, three main strategies can be attempted.

First, microbial load can be reduced before cell isolation. The inoculum can be sampled from starved animals depurated in oxygenated sterile-filtered seawater to limit environmental microbial contaminants (e.g., for abalone mantle cell culture (Suja et al. 2014)). Microdissecting internal tissues that naturally protect from seawater by epithelial envelopes (e.g., molluscan heart tissue), and thus from aquatic microbes, would also reduce the initial microbial load of the inoculum. Collecting cells that possess natural antiseptic defenses, such as innate immune hemocytes (e.g., from mollusks, crustaceans, tunicates or echinoderms), would also have a positive impact on reducing the contamination of the culture. Alternative strategies include using short-term ubiquitous surface sterilization methods on the surface-exposed tissue, such as dipping for up to 1 hour in 10–70% ethanol (e.g., for molluscan abalone mantle, see Suja et al. (2014), and for oyster tissue, see Stephens and Hetrick (1979)) or a few seconds in $KmnO_4$ (e.g., in sea anemone tissue (Doumenc personal communication)) and treating the dissected tissue for up to days in sterile-filtered seawater enriched with a mixture of concentrated large-spectra commercial antibiotics/antimycotics/antiprotist compounds (e.g., molluscan mantle, gill or hepatopancreas tissue).

Second, if specific invertebrate cell types need to be recovered from contaminated primary cultures, the cell-type enrichment strategies established for preparing a suitable inoculum (see Section 2.4) could be reused. For instance, this approach successfully retrieved accessory nidamental gland cells pelleted from native bacteria through a 2% sucrose layer (Figure 3). In addition, the selective rinsing of adherent invertebrate cell types could help to remove cellular debris, toxins and suspended microbes.
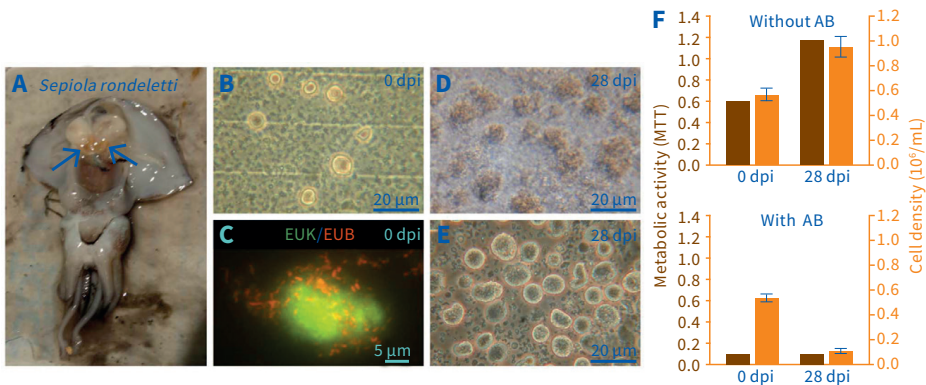
Figure 3. Primary co-culture of squid gland cells with native bacteria. (**A**) Accessory nidamental gland tissue (white arrows) from *Sepiola rondeletti* is enzymatically dissociated by trypsine (0.2% 30 min at 25 °C). (**B**) Gland cells are enriched via centrifugation through a sucrose cushion (2% in seawater), and their seeding density is controlled by Malassez hemocytometer numeration. (**C**) Glandular cell types visualized via Fluorescence In Situ Hybridization (EUK, universal eukaryote probe, fluorescein, green) are covered with surface-associated symbiotic bacteria (EUB, universal bacterial probe, Cy3, red). (**D**) Four-week-old primary culture (without antibiotics) showing high bacterial density around the cultured glandular cell types, (**E**) which can be re-enriched via sucrose cushion centrifugation. (**F**) Gland cell viability (mitochondrial enzyme activity assessed by MTT reduction assay, DO 580/630) is higher in the absence than in the presence of antibiotics (AB) and increases in primary co-culture with native bacteria, along with cell density, indicating the beneficial effect of native bacteria on the survival of cell cultures. Times are given as days post-inoculation (dpi). Source: Graphic by authors.

Third, contamination can be controlled during the primary culture itself. The main strategy for this step to reduce the unwanted mixotrophic growth of contaminants is to use a nutrient-poor basal medium formula, hence limiting the provision of carbon and nitrogen sources that typically exceeds the in vitro energy requirements of the target cells. The culture medium can also be supplemented with antibiotics/antimycotics/antiprotist drugs and changed frequently until the culture appears clean. Proliferating cultures should be closely monitored, and the primary cell cultures containing visible ciliates, bacteria, or clusters of cells with characteristic chytrid-like rhizoid morphology should be discarded.

However, these methods may rescue a subset of the targeted cell-type populations from contaminant overgrowth but carry a high cost in terms of time-consumption and cell yield reduction, for overall limited efficiency.

3. Defining Optimal Culture Conditions

While obtaining a high-quality inoculum is essential for establishing healthy cell cultures, the culturing conditions used are equally crucial. Indeed, even highly proliferative tissue will undergo terminal cell cycle arrest, typically within weeks after inoculation. Moreover, a breakthrough in the culturing conditions used for the *Bge* cell line was at the origin of a large expansion in cell lines.

3.1. Selecting Suitable Culture Media Composition

Media formulation should strive to provide adequate levels of carbon and nitrogen sources to meet the nutritional needs of each isolated aquatic invertebrate cell-type population. However, the metabolism of stem cells and their nutrient requirements are poorly documented across aquatic invertebrate taxa. Consequently, a large variety of culture media have been tested for their in vitro culture (Table 2). Based on the hypothesis of the conservation of major metabolic pathways across animal phyla, a widespread approach is to use commercial basal formulas originally designed for vertebrate cells, typically MEM, DMEM or Leibovitz L-15, supplemented with salts to adjust to the targeted osmolarity of the specimen's original environment and generally diluted to 10–50% (Maramorosch and Mitsuhashi 1997; Mothersill et al. 2000). An even simpler option is to provide a minimal medium composed of seawater with pyruvate as a carbon source, and glutamic acid as a nitrogen source. This approach has been used with sponge primmorph spontaneously aggregated from dissociated cells. These media have, however, persistently failed to sustain the in vitro division of cells of aquatic invertebrates. Another much more complex option is to entirely custom design the media's formula based on an extensive biochemical characterization of internal tissue or fluid composition from the targeted animal species (e.g., molluscan hemolymph). However, these taxon-specific media have not yet demonstrated sufficient benefits to justify their development cost.

A more integrated and personalized approach is to adapt the media formulations to meet the needs of the targeted invertebrate cell subpopulations. To check nutrient consumption in vitro, individual uptake experiments of targeted organic carbon (glucose, lipids, etc.) or nitrogen (amino-acid) substrates (see Apte et al. (1996) for amino-acid transport into sea anemone cells, and Heude-Berthelin et al. (2003) for glucose uptake and glycogen metabolism in oyster cells) may now be updated to metabolomics-based global approaches. Indeed, the search for changes in the metabolite profiles of media sampled at various timepoints in cultured mammalian CHO-CK1 cell lines has helped identify factors that sustain growth and affect in vitro behavior (Mohmad-Saberi et al. 2013). A recent breakthrough was reached using this approach to develop an amino-acid-enriched sponge cell culture medium that sustains cell division in primary cultures (Conkling et al. 2019). The team used a genetic algorithm to identify suitable amino acid components to supplement a

commercial basal formula (M199) for improving the in vitro metabolic activity of *Dysidea avara* sponge cells (Munroe et al. 2019).

A striking feature of successful insect culture media that support proliferating primary cultures and cell lines is the addition of lipid-rich supplements, with a trophic role and potential protection against oxidative stress. Lipid addition has been shown to transiently increase metabolic activity (mitochondrial MTT reduction) in cultured oyster heart cells (Domart-Coulon et al. 1994). The lipid-rich "Grace" commercial formula was shown in cnidarian primary cultures to increase octocoral cell numbers (Khalesi 2008), and is used to obtain a subset of dividing cells and a few rounds of subcultures from cultured sea anemone tentacle (Barnay-Verdier et al. 2013). However, a more global picture of the impact of lipids on culture media for aquatic invertebrate cells is currently lacking.

Medium renewal strategy should be aimed at striking a balance between a conditioned medium supply of undefined trophic factors and cytokines and the removal of senescent cells, debris and toxins from the aging primary cultures. Manipulating inoculum cell densities is an efficient way to facilitate confluence and thus maintain cell-to-cell contacts necessary for the secretion of cytokines that, although currently undefined, are certainly necessary for sustaining cell survival. Old-time tissue explantation methods that rely on the slow outward migration of mixed cell types from a dissected tissue fragment adherent to a culture dish yield the successive outgrowth of distinct morphotypes characterized at minima by their in vitro shape and behavior. These cells can broadly be classified by the following three categories: fibroblast-like, epithelial-like and amoeboid-like cell types (Vago 2012), and can be selected for their ability to survive in vitro on residual native extra-cellular-matrix components. Insect cell lines have emerged from such long-term maintained explant cultures of lepidopteran imaginal discs (Echalier 1997). More recently, the explantation of ectodermal monolayers of regenerating starlet sea anemone yielded mitotically active, mixed cell types, primary cultures (Rabinowitz et al. 2016).

In addition, culture medium can be complemented with a number of factors to promote cell proliferation: C-type lectins have been shown to have cytostatic effects on the hemocytes of the tunicate *Polyandrocarpa misakiensis* (Matsumoto et al. 2001); lectins from another tunicate, *Didemnum ternatanum*, promote the adhesion of a range of marine invertebrate cells (Odintsova et al. 1999); insulin and insulin growth factor, as well as other vertebrate growth factors, were shown to have a positive impact on the transient proliferation of molluscan bivalve cells (Domart-Coulon et al. 1994; Giard et al. 1998); and retinoic acid-related molecules are known to be involved in the dedifferentiation process of multipotent cells as reported for tunicate hemocyte cultures (*Polyandrocarpa misakiensis*) (Kawamura and Fujiwara 1995).

3.2. The Oxidative Stress Problem

Very few and exclusively freshwater taxa among the large diversity of aquatic invertebrates have given rise to cell lines, including the snail *Biomphalaria* (*Gastropoda*). Salinity is thus a major difference between the primary culture systems that have given rise to cell lines and the unsuccessful attempts based on aquatic invertebrate species. One possible cause for this difference is that higher salinity correlates with lower dissolved oxygen. Consequently, dissolved oxygen levels in the cell cultures might be an important yet overlooked physico-chemical parameter of culture conditions. To date, primary cultures of aquatic invertebrate cells are indeed mostly conducted under standard atmospheric conditions (i.e., ~20% O_2), with the cells covered by a thin layer of culture medium where dissolved oxygen is equilibrated by diffusion with the surrounding air. Except for a few cases of full-strength Modified Eagle Medium (or derivatives, osmotically adjusted by salt addition), which requires a bicarbonate/5% CO_2 buffer system, the gaseous atmosphere of most cell cultures is thus composed of air (Table 2). The widely used, amino-acid rich, Leibovitz L-15-based media do not require a 5% CO_2 atmosphere. Seawater/freshwater diluted commercial or custom-made media rely on the addition of Hepes (~20 mM) for pH buffering at 7.4–7.6, depending on species (Tris-HCl is used for sponge cells grown at pH ~8.0). Hence, under typical laboratory conditions (air and 15–25 °C), in vitro aquatic invertebrate cells are exposed to ~20% O_2, which is largely more than in their natural aquatic environment, and could likely expose them to in vitro oxidative stress.

To circumvent this potential problem, the first step will be to monitor invertebrate intracellular oxidative stress, for instance, via a fluorescent general oxidative stress indicator, such as CM-H_2DCFDA, which has been used on the spheroid tissue of *Fungia* coral exposed to short-term acute thermal stress (Gardner et al. 2017). Upon the confirmation of oxidative stress, the second step could be medium supplementation with exogenous antioxidants (for example, ascorbic acid (Helman et al. 2008), catalase enzyme (Domart-Coulon et al. 1994)) or native pigments with high antioxidant properties (e.g., sea urchin spinochrome (Ageenko et al. 2014) and shrimp astaxanthin (Lee et al. 2021)). Both approaches have reproducibly led to the increased maintenance of the primary cell cultures. An alternative when establishing cultures of tissues containing photosynthetic endosymbionts (e.g., Cyanobacteria-containing sponges, and Symbiodiniaceae-containing sea anemones, corals and octocorals) is to maintain the cultures in the dark to inhibit the photosynthetic processes that generate oxygen and thus increase oxidative stress (Table 2).

3.3. Understanding Adhesion and Cell-to-Cell Contact Requirements of Aquatic Invertebrate Stem Cells

To optimize the proliferation of culture cells, transferring genomic knowledge obtained in each aquatic invertebrate taxon on cell-to-cell and cell-to-ECM adhesion systems will be particularly useful for selecting suitable ECM-coatings of culture dishes.

Shifting from classical 2D monolayer culture to 3D "spheroid" culture systems offers opportunities to facilitate the maintenance of cell-to-cell interactions and of native secretions within the cell cluster. Cells from the earliest branching aquatic metazoans, such as poriferans and cnidarians, display spontaneous aggregation properties after tissue dissociation into single cells, sometimes leading to whole-body regeneration (see Simpson (1984) for sponge, Gierer et al. (1972) for hydra and Vizel et al. (2011) for coral). This re-aggregation property is being harnessed for spheroid formation (coral "tissue balls", sponge primmorphs) and their establishment for primary culture (Figure 4A–C). Hemocytes drawn from mollusks, crustaceans, tunicates or echinoderms also self-aggregate into clusters, through sequential migrations of adherent cells on the culture substrate followed by the putative secretion of self-recognition lectins (Figure 4D–F). A recent breakthrough using 3D cultures of sponge cells in ultra-low-gel agarose hydrogel microdroplets has been reported to support cell–ECM interactions and to facilitate the survival of differentiated *Geodia neptuni* demosponge cells (Urban-Gedamke et al. 2021).

Similarly, improving the in vitro microenvironment of isolated stem cells could potentially sustain their proliferation. Adapting the cellular microenvironment to mimic stem cell niches of a target organism should be pursued in each model taxon, as such information becomes available. In addition, primary cultures that gave rise to cell lines (e.g., insect imaginal disc cells) can provide mechanistic insights into the cellular microenvironment needed to maintain stem cell self-renewal. As shown in mammalian systems, multidirectional signaling by co-culturing stromal "feeder" cells with the target cells (e.g., neurons from the gastropod *Aplysia californica* (Montgomery et al. 2002); stem-like cells on a monolayer of confluent cephalopod hemocytes (Figure 4E)) might help to generate a microenvironment suitable for stem cell maintenance, proliferation and differentiation, typically by providing cell adhesion molecules, growth factors, hormones and other secreted proteins (see Girard et al. (2021) for hematopoietic stem cell niche, and Ootani et al. (2009) for intestinal stem cell niche). Furthermore, supplementing the culture media with specific growth factors (e.g., Wnt fusion proteins for ISC (Ootani et al. 2009)) can lead to the expansion of stem cells with sustained proliferation and multilineage differentiation. As in vivo information on the regulation of aquatic invertebrate stemness becomes available, transferring such information will be particularly important to design optimized cell culture media.
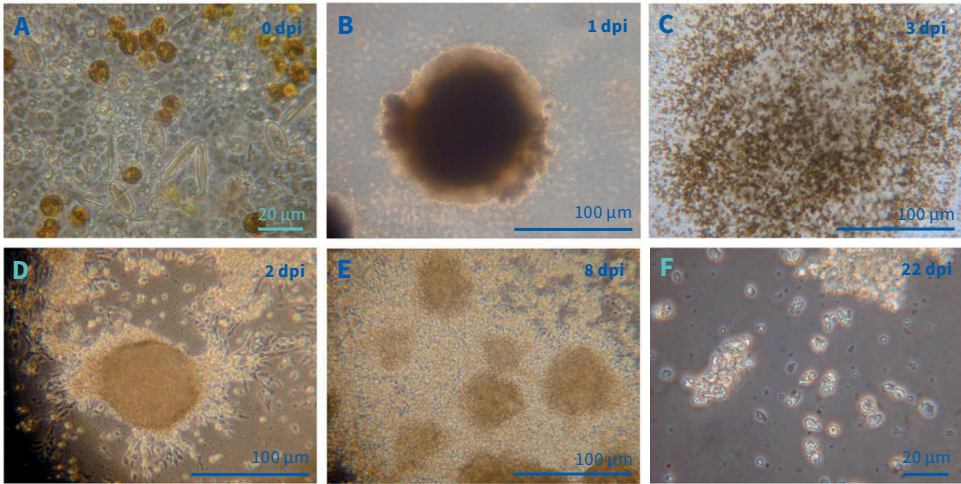
Figure 4. Aggregate vs. dissociated primary tissue and cell culture, on plastic dish-culture substrates. The figure shows micrographs of individual cells or multicellular aggregates at the indicated days post-inoculation (dpi). (**A**) Scleractinian coral cell types and their *Symbiodiniaceae endosymbionts* (within coral gastrodermal host cell, or free-living in the culture medium). (**B**) Suspended coral multicellular aggregates spontaneously formed in explant culture of colonies of *Pocillopora damicornis*. (**C**) Spontaneous dissociation into multilayered, mixed-cell-type culture, containing translucent coral cells and brown-pigmented microalgal symbionts (*Symbiodiniaceae*). (**D**) Cephalopod hemocytes from *Nautilus pompilius* aggregate in cell culture when seeded at high seeding density (>10^6 cells/mL, 2 dpi). (**E**) Confluent primary culture 8 dpi, showing networks of adherent hemocytes and proliferating cell clusters (300–500 μm in diameter), which can be detached and transferred (passaged) to new culture dishes. (**F**) Subcultured hemocytes (14 days post transfer from cells detached from clusters in 8 days post-inoculation-primary culture) remain quiescent and do not grow to confluence. Source: Graphic by authors.

To further mimic the in vivo microenvironment of the isolated cells, and their interactions with their environment in particular, new "physiomimetic" approaches should be developed using, for instance, versatile hydrogels to concentrate cells in a 3D microenvironment (see Otero et al. (2021) for a review on such experimental approaches for vertebrate cell systems). The ongoing development of commercial hydrogels (synthetic or derived from jellyfish, i.e., "Jellagel") provides new 3D substrates to test on aquatic invertebrate cells. To determine whether these cells behave in vitro similarly to in vivo, live-cell or live-tissue observations based on the micropropagation of tissue in microfluidic devices should be further established (Januszyk et al. 2015).

4. Controlling the Purity and Quality of Cultured Cells

Upon isolation from their initial tissue microenvironment for establishment in culture dishes, aquatic invertebrate cells change morphology and are notoriously difficult to identify by their in vitro shape and behavior (Rinkevich 2011; 2005; Cai and Zhang 2014). Moreover, cultured cells are morphologically highly plastic, changing shape, granularity and sometimes pigmentation with culture age and substratum composition (i.e., with or without surface coating with positive charges or ECM compounds). For cell lineage authentication, checking phenotype and genetic identity is imperative, not only upon culture initiation but also throughout the primary culture and derived subcultures, at least at the time of use for functional assays and before/after cryopreservation.

4.1. Proliferation

The monitoring of in vitro cell proliferation is traditionally based on monitoring cell densities (via subsampling a fraction of the culture followed by cell numeration on Malassez- or Neubauer-type hemocytometers, or time-lapse image analysis of microscopy fields of view) and attentive changes in the total protein content or DNA content extracted from cell pellets or monolayers. These methods overestimate live cell densities as they integrate dying cells to the viable cells. Another widely used method relies on the miniaturized high-throughput colorimetric quantification of mitochondrial oxidative phosphorylation (MTT or XTT reduction assays) by the cultured cells. First adapted for screening medium nutritional factors and physico-chemical parameters for molluscan cells (bivalve oyster *Crassostrea gigas* (Domart-Coulon et al. 1994)), it has also been adapted to sponge cell mitochondrial activity evaluation in primary culture (Zhang et al. 2004) and to the monitoring of coral larval cell density in primary cultures (Kawamura et al. 2021). However, this type of MTT test detects not only oxidative phosphorylations of the animal cells but also that of bacterial associates in primary culture (e.g., of cephalopod holobiont) tissue (Pichon et al. 2007). The fluorescence monitoring of cellular esterase activity is also a common method to quantify viable cells in cultures. However, their use for aquatic invertebrate taxa can be limited by the widespread co-occurrence of autofluorescent cell types with fluorescence spectra overlapping those of the enzyme substrates.

By quantifying the proportions of cells in each phase of the cell cycle, flow cytometry allows us to check the proliferative status of the collected tissue sample before culture establishment, and to monitor cell cycling in the derived primary cultures and potential sub-cultures. Applied, for example, in the early 2010s to primary cell cultures from five demosponge species, this flow-cytometry-based approach revealed rapid changes in the cell cycle distribution of a mixed-cell-type suspension over time in primary culture (over a short-term 2–10-day timescale)

(Schippers et al. 2011). The rapid accumulation of cells with low DNA content together with a drop in the proportion of quiescent (G_1/G_0) and cycling cells (S & G_2/M) could be visualized, supported by the parallel detection of activated (caspase3) apoptosis pathways. This evidence supported the hypothesis of the rapid senescence of cultured sponge cells, with the accumulation of cellular debris (demonstrated by widely scattered cell size distribution), despite stable or slowly declining cell counts, by only minus ~20% over the 10-day culture period. This observation calls to cautious interpretations of stable or slightly growing cell densities, counted from image analyses of microscopic fields or enumerated on Malassez-type slides, as round empty cell bodies cannot be unambiguously discriminated from living cells based on morphology only, even when using vital stains assays (e.g., neutral red or trypan-blue). Another point of caution when using this approach is the ploidy of the studied samples, and in particular, the presence of mixoploid cell populations that could bias their cell-cycle profile (Ermakov et al. 2012).

4.2. Phenotyping

Autofluorescent markers (e.g., Green Fluorescent Protein-rich intracellular granules of cnidarian cells,) or chromophore/pigments of specific cell types (e.g., red "echinochrome" pigments of echinoderm coelomocytes) can be used to sort cell types among a mixed cell suspension. However, care should be taken to minimize irradiance energy during fluorescence microscopy examination as it may damage the living cells by DNA photodamage or lipid peroxidation, and thus limit their subsequent in vitro survival. Enzyme activity assays (e.g., phenoloxidase of mollusk and crustacean immune cells), biochemical phenotyping and phagocytosis assays have also been used to characterize the in vitro functionality of hemocytes from molluscan hemolymph, tunicate hemolymph and echinoderm coelomic fluid.

Immunophenotyping requires the prior development and validation of polyclonal or monoclonal antibodies against epitopes of cell-type-specific proteins or membrane preparations. Although labor-intensive and time consuming, this strategy provides the advantage of the unambiguous localization of immuno-positive phenotypes in initial tissue and in primary tissue or mixed cell culture. For instance, low abundant small round coral skeletogenic (calicoblast) cell types were labeled with a polyclonal antibody raised against the biomineral organic matrix (Puverel et al. 2005) and antibodies were raised against the *Botrylloides piwi* sequence to label a specific population of hemocytes (Rinkevich et al. 2010). This antibody-based approach has been successfully used to trace self-sorting processes during cell-to-cell aggregation from mixed-cell-type dissociated tissue suspensions (Schmid et al. 1999) and for cell fusion experiments (Pomponi et al. 2013). This has an interesting yet still overlooked potential for cell-type enrichment via the antibody panning of immuno-positive cell types (Auzoux-Bordenave and Domart-Coulon 2010).

Novel phenotyping methods have recently been developed from cutting-edge single-cell RNA sequencing methods, which are applied to cultured cells. However, these techniques currently have the three following drawbacks: (1) the prior definition of cell-type-specific markers is needed, through the data-mining of single-cell RNAseq libraries obtained from dissociated tissue suspensions, which is still available for a limited number of species of established model organisms (e.g., for the starlet sea anemone (Sebé-Pedrós et al. 2018), for planarians (Hayashi et al. 2010) or for the scleractinian coral (Levy et al. 2021)); (2) molecular markers should be specific to metabolic pathways restricted to the targeted invertebrate taxon and exclude pathways that are also active in potential contaminating protists/microeucaryotes; (3) assessing the polyclonality (mixture of cell types) versus clonality (single cell line) of the culture requires the quantification of the percentage of reads obtained for each claimed phenotypic marker, relative to the total number of reads.

4.3. Genotyping

Despite the proper isolation and cleansing of cells, cultures can easily be overgrown by undesired cells. An undetermined fraction of these aquatic microbes survives the tissue aseptization treatments prior to dissociation or explantation and co-occurs along with metazoan cells in the mixed-cell-type suspensions obtained from soft tissue dissociation or hemolymph syringe-drawings. This large diversity of aquatic microbes is hard to monitor as it requires taxa-specific specialist microbiology knowledge and molecular tools for accurate identification. It is especially difficult to recognize their morphological traits in a mixed-cell-type primary culture that combines the morphological and behavioral plasticities of both the microbe and microbial life stage, and the targeted invertebrate cell types.

To address this problem, genetic markers specific to a species (e.g., *Axinella corrugata* demosponge, (Lopez et al. 2002)) or to a genus (e.g., *Acropora* scleractinian coral (Shinzato et al. 2014)) have been developed and validated for identifying cells from the targeted taxon in the initial tissue and over time in primary cultures and subcultures. Marker development is based on molecular genetics methods, such as DNA fingerprinting, amplified fragment length polymorphism (AFLP), single-locus DNA sequence analyses and microsatellites markers designed by next-generation sequencing population genetics methods.

As microbes tend to proliferate more actively than the cells of primary interest of the in vitro culture, it is crucial to check the potential microbial nature of long-term cultured candidate aquatic invertebrate stem cells.

4.4. Microbial Contaminants Authentication

Detecting genetic markers specific to the invertebrate taxa of interest does not exclude the potential co-occurrence of microbial contaminants. In fact, because

molecular detections are highly sensitive, the detected invertebrate cells could even represent a very small fraction of the cell culture. Thus, it is highly recommended to also systematically use molecular probes for microbial taxa to detect potential culture contaminants (Table 3).

Culture contamination is a major obstacle to the development of aquatic invertebrate in vitro models. Indeed, it is widely acknowledged that microbes persistently take over the cultured aquatic invertebrate cell types (Rinkevich 2005), putatively as a result of antagonistic interactions (predation and competition for nutrients) or metabolic plasticity and better adaptation to the in vitro growth conditions. Culture media are commercially designed for vertebrates (e.g., DMEM, Leibovitz L-15) or insects (e.g., Grace Insect Medium) and partly diluted in seawater (or freshwater) or formula custom-prepared to mimic the microenvironment of the sampled tissue, and they are nutrient-rich. Although they may not adequately meet the largely unknown growth requirements of the cultured invertebrate cell types, they provide abundant organic carbon and nitrogen sources that facilitate the overgrowth of opportunistic resident microbial associates. Indeed, epibiotic or endobiotic microbiota (especially unicellular microeukaryotes that are hard to discriminate from animal cells) have repeatedly been shown to take advantage of the medium-derived nutritional resources to fuel their fast heterotrophic growth; see, for example, the consumption of mono and disaccharides, glycerol, glutamate and glycine by the opportunistic unicellular Alveolate *Chromera velia* (Foster et al. 2014). Predatory opisthokonts, ubiquitous in aquatic environments, have a highly plastic morphology, with in vitro growth alternating between a unicellular 'spindle-shape' stage and aggregative or clonal (partly fused) multicellular stages, and they are known to feed on metazoan tissue or derived cells (Tikhonenkov et al. 2020a).

Such eukaryotic microbes, collectively defined as protists, may feed on cellular debris from senescent or dead host/aquatic invertebrate cells, taking over the initial host cell population in long-term primary cultures or their successive subcultures.

5. Perspectives

While marine invertebrates as a whole show the largest biodiversity and the widest phylogenetic radiation on Earth, they have contributed very little to the in vitro cell lines discipline. The culture of marine invertebrate stem cells and/or their progenitors could thus create new perspectives for fundamental research as well as for biomedical applications. To reach this objective, we thus recommend two main actions.

First, a systematic map of knowledge, built in the form of a database of publications with metacoded information on taxon population, intervention strategies (e.g., cell isolation methods, culture media and physico-chemical conditions) and outcomes (e.g., cell viability, proliferation and differentiation) would be an important

tool for increasing the visibility of protocols and know-how in the fragmented scientific community. Furthermore, it would help to incorporate typically unpublished results, including negative results, a scientific status that is rarely highlighted in the refereed literature (Grasela et al. 2012). Such database would help to build a comprehensive knowledge map to identify optimized culturing conditions for each aquatic invertebrate taxon and cell type, adapted to the expected timescale of utilization. The multiple usage of primary cell and tissue cultures from aquatic invertebrates ranges from short-term use (within hours to <7 days for physiology and cytotoxicity testing) to the long-term (bi-weekly to monthly) selection of subpopulations of dividing cells for serial sub-culturing attempts. Each type of inoculum implies distinct culture media and condition strategies to balance cellular yield, functional stability and proliferation potential. The curated list of 511 relevant publications compiled in Appendix B Table A2 provides a start to this database, allowing us to assess by taxon the extent of research efforts to initiate or develop cell cultures. It should be maintained and completed by the scientific community, for more exhaustive listing and optimized visibility.

Second, best practices would be to develop and adopt robust cell-type authentication protocols applicable to insect or vertebrate cell lines and primary cultures (Lynn 2001; Dominici et al. 2006), and to systematically deposit live or cryopreserved vouchers of "cell lines" in cell repositories. This could lead to the identification of more general stem cell markers for aquatic invertebrates, which would be crucial for obtaining a robust inoculum for in vitro cell cultures. Most recurrent past claims of successfully established aquatic invertebrate cell lines have turned out *in fine* to be cultures overgrown by microeukaryote contaminants, with examples in each taxon (porifera, colonial cnidarians, crustaceans and others).

Overall, these two actions taken together could help to standardize aquatic invertebrate cell culture to facilitates comparisons between intervention protocols and thus help to optimize the standardized protocols. Given that aquatic invertebrates are phylogenetically very distant, the development of a ubiquitous culturing environment appears rather unlikely. Nevertheless, each phylum could benefit from the scientific and technological advances in primary cell culture made in other phyla.

In particular, the assessment of whether the list of three identification criteria, defined for vertebrate stem cells, are conserved in aquatic invertebrate stem cells, would be of particular interest. The first criterion is whether the stem cells adhere to plastic, and more generally if a specific culture method, such as 3D Matrigel, could lead to decisive improvements (Urban-Gedamke et al. 2021). The second criterion explores the expression of specific surface markers that would allow the robust isolation and enrichment of stem cells/progenitors, as has been attempted by using a single marker in a colonial tunicate (Kassmer et al. 2020). The third criterion aims to define protocols for assessing stem cells' potency differentiation potential, typically

by using predefined induction cocktails combined with markers for differentiated cell types, both of which require a precise characterization of gene expression profiles specific to each cell-type for every species of interest (Sebé-Pedrós et al. 2018).

An alternative to identifying suitable stem cells is to immortalize cells of interest in a reproducible manner. One suggested approach is to manipulate adult stem cells of aquatic invertebrates similarly to the approach implemented in mammalian induced pluripotent stem (iPS) cells (Rinkevich 2011). The second route, probably the most promising and reliable approach, is to control the process of tumorigenesis in aquatic invertebrates, as already suggested (Odintsova et al. 2011). Research on this topic is currently very scarce (Gardner 1993; Robert 2010) primarily due to the facts that tumorigenesis in aquatic invertebrates is not as commonly observed as in vertebrates (Vogt 2008; Tascedda and Ottaviani 2014), that tumor-like lesions in aquatic invertebrates possess a low mitotic index (Odintsova et al. 2011) and that the definitions of tumors and tumor cells in aquatic invertebrates are less familiar to pathologists (Tascedda and Ottaviani 2014). Yet, the tool of tumorigenesis may constitute a very important route for future research, and a potential approach is to use the trait of the vertebrates' cancer cells (Vincent 2012) as a guiding list for tumors in aquatic invertebrates. A third concept proposes the use of regeneration processes as the source of tumor development (Oviedo and Beane 2009), which is particularly interesting given the broad involvement of aquatic invertebrates' stem cells in regeneration processes, including whole-body regeneration (Rinkevich et al. 2022). For each one of these three approaches, the development of suitable tools for the controlled editing of genetic material of cells, typically through viral transfection, could enable the knockdown of suppressor genes, similar to standard approaches in mammalian cells (Yang et al. 2007). One such advance is the successful induced stem cell neoplasia in the marine hydrozoan *Hydractinia echinata* by the ectopic expression of a POU domain transcription factor (Millane et al. 2011). However, even immortalized tumor-like cells will need appropriate culturing conditions to proliferate properly. Lessons may be drawn from the failure to sustain in vitro the neoplastic hemocyte proliferation observed in vivo in spontaneously occurring clam leukemia, with research pointing to a role for the stress protein mortalin in the induction of apoptosis in cancerous hemocytes (Walker et al. 2013). The RNA-seq approach may be applied to compare gene expression patterns in cultured cells and initial tissue, with a focus on essential cell proliferation and cell cycle arrest regulator genes, in order to develop future strategies for immortalization, as recently explored for developing shrimp cell lines (Thammasorn et al. 2020).

As a supplementary approach to support the development of cell lines from aquatic invertebrate stem cells, studies on metabolomes of cultured cells, and their secretomes in particular, could be considered. Such an approach may provide important insights into the requirements in media composition that support

proliferative activities. Ample information has been gained on this issue in mammalian cell cultures (Čuperlović-Culf et al. 2010; Mohmad-Saberi et al. 2013). Yet, the study of the secretome of aquatic invertebrates has seldomly been undertaken (Kocot et al. 2016), but data on the metabolome of whole organisms in the context of marine natural product discoveries are becoming quite common (Reverter et al. 2020). Furthermore, high-precision tool development specific to seawater are now available (Sogin et al. 2019).

Finally, future research should also address the still largely overlooked abiotic factors, such as testing hypoxia and pressure stimuli, on primary cultures.

Regarding hypoxia, parallel research in cultured mammalian cell models has highlighted the better survival and proliferation of stem cells in low oxygen environments (Zhu et al. 2005; Hung et al. 2012; Ramirez et al. 2011). A shift from oxidative phosphorylation to aerobic glycolysis, known as the Warburg effect, has been documented in the context of proliferating cancer cells: the glucose consumed in high amounts to fuel the growing biomass of cancer cells is fermented to lactate rather than oxidized, even when there is sufficient oxygen to convert glucose to CO_2, although the process is less efficient in terms of ATP synthesis (reviewed by DeBerardinis and Chandel 2020). Hyperactive glycolysis involving lactate supports the tumor energy metabolism of cancer stem cells in mostly hypoxic environments, and similar pathways might support the metabolism of aquatic invertebrate stem cells. A similar Warburg effect has indeed been documented in *Crassostrea gigas* oyster tissue. First discovered during the response to viral infection with ostreid herpesvirus-1 (Corporeau et al. 2014), it is thought to be a mechanism to adapt the oyster metabolism to extreme (salinity and oxygen) changes in the intertidal environment (Corporeau et al. 2019). In agreement with this finding, preliminary data obtained on oyster heart primary cell cultures showed transient increased proliferation between 2 and 4 weeks post-inoculation in a 2% O_2 atmosphere (obtained by incubation in a 95% N_2/5% air incubator), compared with 20% O_2 atmosphere (air) (Domart-Coulon, unpublished), when medium was supplemented with growth factors, lipids and antioxidants (Domart-Coulon et al. 1994).

Regarding pressure, research on the primary cultures of vertebrate (Wharton Jelly's) mesenchymal stem cells has shown the combined positive effects of pressure and hypoxia (Park et al. 2020). In response to pressure stimuli, cell proliferation was increased, and stemness was maintained. Cellular adhesion and confluency were higher in 5% O_2 hypoxia with 2.0 PSI pressure conditions relative to standard 5% CO_2–95% air conditions, and hypoxia alone yielded a mild increase in stem cell adhesion and confluency. Thus, we propose the inclusion of these abiotic parameters in future invertebrate stem cell culture optimization efforts.

Appendix A

Table A1. All the detailed values used for building Figure 1.

Taxon	Species	Regeneration	Longevity	Publications	Cell Lines
Hominidae	7	organ	122	16,851	96,862
Mammalia	5480	organ	211	7238	29,171
Vertebrata	56,508	appendage	392	884	1394
Tunicata	2760	WBR	-	44	0
Cephalochordata	33	organ	-	3	0
Ambulacraria	7111	WBR	200	42	0
Xenacoelomorpha	401	WBR	-	1	0
Insecta	1,015,897	appendage	28	351	895
Ecdysozoa	202,423	appendage	100	110	94
Nemertea	1200	WBR	-	0	0
Platyhelminthes	20,000	WBR	-	16	1
Mollusca	85,000	appendage	507	121	5
Spiralia	26,099	WBR	-	14	0
Cnidaria	9795	aggregates	4265	66	0
Placozoa	1	aggregates	-	0	0
Ctenophora	166	WBR	-	1	0
Porifera	6000	aggregates	15,000	58	0

Appendix B

Table A2. The full curated list of 511 references, sorted per taxa. (This table was not included in the print version of this book, to view the table please visit https://www.mdpi.com/books/pdfview/edition/5071).

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