



From the raw bar to the bench: Bivalves as models for human health

José A. Fernández Robledo^{a,*}, Raghavendra Yadavalli^a, Bassem Allam^b,
Emmanuelle Pales Espinosa^b, Marco Gerdol^c, Samuele Greco^c, Rebecca J. Stevick^d,
Marta Gómez-Chiarri^e, Ying Zhang^f, Cynthia A. Heil^a, Adrienne N. Tracy^{a,g}, David Bishop-Bailey^h,
Michael J. Metzgerⁱ

^a Bigelow Laboratory for Ocean Sciences, East Boothbay, ME, 04544, USA

^b Stony Brook University, School of Marine and Atmospheric Sciences, Stony Brook, NY, 11794, USA

^c University of Trieste, Department of Life Sciences, 34127, Trieste, Italy

^d University of Rhode Island, Graduate School of Oceanography, Narragansett, RI, 02882, USA

^e University of Rhode Island, Department of Fisheries, Animal and Veterinary Science, Kingston, RI, 02881, USA

^f University of Rhode Island, Department of Cell and Molecular Biology, Kingston, RI, 02881, USA

^g Colby College, Waterville, 4,000 Mayflower Hill Dr, ME, 04901, USA

^h Royal Veterinary College, London, NW1 0TU, UK

ⁱ Pacific Northwest Research Institute, Seattle, WA, 98122, USA

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ABSTRACT

Bivalves, from raw oysters to steamed clams, are popular choices among seafood lovers and once limited to the coastal areas. The rapid growth of the aquaculture industry and improvement in the preservation and transport of seafood have enabled them to be readily available anywhere in the world. Over the years, oysters, mussels, scallops, and clams have been the focus of research for improving the production, managing resources, and investigating basic biological and ecological questions. During this decade, an impressive amount of information using high-throughput genomic, transcriptomic and proteomic technologies has been produced in various classes of the Mollusca group, and it is anticipated that basic and applied research will significantly benefit from this resource. One aspect that is also taking momentum is the use of bivalves as a model system for human health. In this review, we highlight some of the aspects of the biology of bivalves that have direct implications in human health including the shell formation, stem cells and cell differentiation, the ability to fight opportunistic and specific pathogens in the absence of adaptive immunity, as source of alternative drugs, mucosal immunity and, microbiome turnover, toxicology, and cancer research. There is still a long way to go; however, the next time you order a dozen oysters at your favorite raw bar, think about a tasty model organism that will not only please your palate but also help unlock multiple aspects of molluscan biology and improve human health.

1. Introduction

Bivalves are the second largest phylum of animals after the arthropods with about 200,000 extant species (Runnegar, 1996). Bivalves, characterized by remarkable anatomical and structural dissimilarities between the species, share collective characteristics including a soft, unsegmented body consisting of a muscular foot, a visceral mass, and a mantle (Pechenik, 2000). Marine bivalves populate all latitudes, and they are particularly important in benthic-pelagic coupling filtering large volumes of water, cycling particulate matter and phytoplankton to grow the shell and the soft body fueling higher trophic levels and modifying the community composition (Newell, 2004; Smith et al.,

2018a). Bivalves also represent an important source of food and valuable goods around the world, with bivalves making up roughly 20% of aquatic animals production by weight (FAO, 2018). The phylogeny, biology, ecology, and economic importance of bivalves makes them ideal candidates for investigations targeting critical basic and applied research questions. This is facilitated by their ubiquity and amenability for maintenance in laboratory settings even in laboratories with no direct access to marine water. Bivalves are today used as experimental organisms for research in chronobiology (Perrigault and Tran, 2017), neuroendocrinology (Catapane et al., 1978) bacterial endosymbiosis (Dubilier et al., 2008), innate immunity (Cunningham and Robledo, 2015; Mydlarz et al., 2006; Song et al., 2010), biomineralization

* Corresponding author.

E-mail address: jfernandez-robledo@bigelow.org (J.A. Fernández Robledo).

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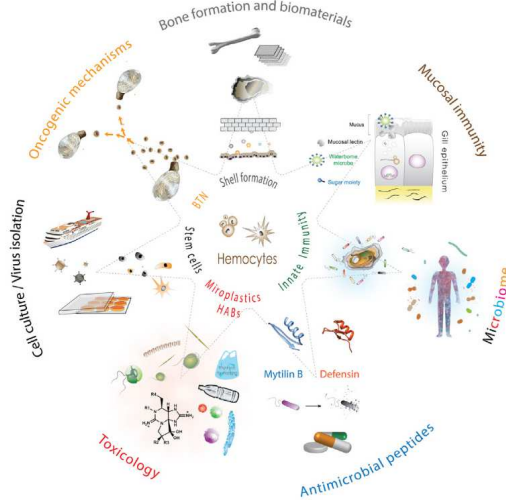


Fig. 1. Bivalves as models for human health. Hemocytes are involved in multiple aspects from the biology of bivalves with a direct implication on human health. Keystone of the immune defense at the center against a broad variety of pathogens, we can learn how organism lacking adaptive immunity (as defined in vertebrates) deal with viruses, bacteria, and protozoan. Mucosal immunity, maintaining and turnover of the microbiome in an aquatic environment rich in bacteria and as source of antimicrobial peptides in a world where antibiotic resistance is on the rise, are only a few of the aspects where hemocytes and humoral factors are involved. Bivalves can cope with detoxification during HABs and as a filter feeder can concentration of human viruses responsible for outbreaks and microplastics that are incorporated into the food chain. Shell formation is also an exciting biology aspect that has parallel mechanisms with bone formation. Contagious cancer in clams can bring some light into oncogenic mechanisms, including cancer evolution, metastasis, and the role of transposable elements in oncogenesis. Some drawings modified from IAN (<http://ian.umces.edu/>).

(Mount et al., 2004; Takeuchi, 2017), aging (Abele et al., 2009), and various biotechnological applications (Ferreira et al., 2007) as well as for the monitoring of environmental health (Vethaak et al., 2017; Zuykov et al., 2013). The concept of a model organism is applied to one organism that is particularly suited to answer a particular question in biology. These questions can be unique to the clade where the targeted organism belongs (e.g., teka formation in diatom species, apicoplast segregation in the apicomplexan group, quorum sensing) or a shared with a large number of clades (e.g., chromatin packaging, cell division). Most well-established model organisms (e.g., *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*) are the result of decades of studies and the participation of multiple laboratories; however, the world wide web and a more affordable access to the latest technologies are enabling researchers working in less-studied organisms to gain momentum for “non-model model organisms as emerging systems for tackling questions across the whole spectrum of biology (and beyond)” (Russell et al., 2017).

In this review, we focus on aspects of bivalve biology with implications in human health, (Fig. 1). We devoted section 2.1 to the shell formation. Bivalves use the shell to shield the soft body from both predators and environmental stressors, and for physiological homeostasis; in the absence of bones, the shell can be considered an exoskeleton. The biomineralization during shell formation takes place on the external surface of the mantle by specialized epithelial cells; however, there is growing evidence showing that hemocytes are also involved in biomineralization and shell formation. Section 2.2, focuses on

innate immunity and what mechanisms the bivalves have for dealing with pathogens, but also with food particle selection. We have broken down this section into mucosal immunity (section 2.2.1), the microbiome (section 2.2.2), and alternatives to antibiotics (section 2.2.3). The components of the mucosal immunity that Lamellibranchiate bivalves use do allow them to endure not only the myriad of waterborne microbes they are exposed to in the marine environment through their suspension-feeding mechanism but also a wide range of environmental conditions (section 2.2.1). Gills and other pallial organs are continuously encountering waterborne microbes as they enter the pallial cavity. Immune defense factors associated with the mucosal surfaces in the pallial organs combined with the open circulatory system with hemocytes on patrol makes the pallial cavity this first battleground with invading microorganisms. There is mounting evidence on the essential roles of microbiomes (bacteria, archaea, viruses, and microeukaryotes) in the biology, ecology, and evolution of eukaryotic hosts. In addition to waterborne pathogens, the convolution of pallial organs greatly increases the effective surface of these interfaces and enhance their exposure to a rich bacterial community (section 2.2.2). Bivalves can reject some of these microorganisms; some are digested as they go through the digestive tract of the bivalve, while others are retained, colonizing the gut and other organs. With clearly separated growth and reproductive seasons, it is expected that the microbiome of bivalves also changes through the seasons while maintaining a core microbiome. All these features make bivalves an attractive model to study microbiome composition and dynamics. As bivalves lack adaptive immunity, at least as we know it for vertebrates, they have evolved powerful and unique mechanisms and strategies (e.g., antimicrobial peptides) to fight and keep at-large bacterial pathogens and viruses. In section 2.3 we emphasize harmful algal blooms (HABs) and aspects of the concentration and effect on the bivalve, and detoxification mechanisms as well. As the temperature of the planet keeps rising, we are witnessing an increase in the frequency, magnitude, and distribution of harmful algal blooms. With many of these HAB species containing potent biotoxins, it becomes critical to understand better how filter-feeding bivalves concentrate these toxins, vector them to humans and/or bioaccumulate through food chains, and, ultimately, eliminated them. Microplastics (section 2.4) have recently gained attention and notoriety in society and the mass media beyond the reason why they were engineered. The plastics in the environment do not just stay in their original form; they break down, and they are passed from one organism to the next through the trophic web. Section 2.5 focuses on the tissue regeneration and stem cells, an unexplored territory with great interest still lagging in bivalves compared to other marine organisms. Bivalves can repair and regenerate at least shell and mantle, and there is considerable tissue remodeling associated with an early immune response, and it appears there is a degree of conservation between mammalian and at least oyster adult stem cells. Bivalve Transmissible Neoplasia (BTN) (Section 2.6) is a leukemia-like proliferation of cells in the hemolymph of bivalves that has been shown to be transmissible in at least four different bivalve species, and it is one of the few cases, and first in a marine organism, of leukemia-like cancers that is horizontally transmitted. Associated with overexpression of the retrotransposon *Steamer*, the BTN represents a novel model to examine the process of cancer evolution. Finally (Section 3), we review the resources and tools available, from the pioneering efforts in the 1990s for developing systems for delivering genetic material into bivalves to the numerous attempts to establish cell lines. In this section, we also highlight the need for the development of the tools that would make the bivalves an attractive model to attract new students and researchers and at the same time be competitive for funding with other well-established model organisms.

2. Unique aspects of bivalve biology

2.1. Shell formation

Bivalves are protected from predators and environmental stressors (desiccation) by shells, a sort of exoskeleton that also provides the means for physiological homeostasis regulation in the soft body. The diversity and complexity of the bivalve shells are astonishing (Aguilera et al., 2017; McDougall and Degan, 2018) and it is hypothesized to be one of the critical factors for the animal diversity explosion during the Cambrian period (Kocot et al., 2016). The shell formation involves specialized epithelial cells on the dorsal surface of the mantle that secrete the extrapallial fluid constituted of proteins, polysaccharides, glycoproteins, and lipids. This organic membrane (periostracum) is produced from a groove between the external and middle lobes of the mantle. In the eastern oyster, *Crassostrea virginica*, the formation of the shell starts early in development (14 + days from egg to spat) and an embryonic shell can be seen before the gastrulation is completed (Carriker, 2009). The general biomineralization process has been described although research is still needed to characterize it fully [reviewed by (Furuhashi et al., 2009; Kocot et al., 2016; Marin et al., 2012)]. The diversity of complexity and patterns of the shell makes bivalves an ideal model organism to study the evolution of biomineralization (Kocot et al., 2016). Comparative mantle transcriptome analysis of bivalves and gastropods indicates a lineage- or species-specific genes often including domains with rapid evolution rate and with a tendency to expand, and contract and rearrange in the genome (Aguilera et al., 2017). Genome annotation, and transcriptome and spectroscopy analysis of shell proteins indicate that biogenesis of the calcified shells is more complex than currently understood (Zhang et al., 2012) and it appears that the gene repertoire for shell formation is different in larvae and adults (Zhao et al., 2018). Osteogenic markers are induced in bone marrow-derived human skeletal stem cell growing on polycaprolactone surfaces mimicking *Pinctada maxima* nacre topographical surfaces (Waddell et al., 2018). Bivalves also provide an excellent model to study the molecular mechanisms involved in symmetry; e.g., oysters having anatomically paired mantle tissues display bilateral symmetry and recent RNA sequencing analyses of the right and left mantle indicates unique gene expression patterns, which might translate in functional differences (Wei et al., 2018a). Transcriptomic studies also reveal differentially expressed genes in the layers of the mantle (Li et al., 2017a). In the advent of the omics era, numerous studies continue to identify genes involved shell biomineralization using genomics; e.g., gene candidates for the formation of the nacreous layer, a strong, resilient, and iridescent organic-inorganic composite material made by some bivalves as an inner shell layer (Ohmori et al., 2018), formation of prismatic and nacreous layers (Funabara et al., 2014), receptor involved in regulating shell biomineralization (Li et al., 2017b), shell larvae formation (Liu et al., 2015, 2017, 2018), and shell color (Liu et al., 2015). Proteomics studies have also benefited from genomes; e.g., conservation of nacre proteins between bivalve species (Marie et al., 2017), identifying the “basic tool kit” for calcification processes (Arivalagan et al., 2017), and the proteins present in the water-soluble nacre matrix (Oliveira et al., 2012). Transcriptomics and proteomics are also used to ascertain the effect of global warming and ocean acidification on calcifiers (De Wit et al., 2018; Ivanina et al., 2013; Li et al., 2016b, 2016c; Timmins-Schiffman et al., 2014).

In bivalves, circulating hemocytes in the hemolymph represent the main component of the internal self-defense system against pathogens which involves both cell-mediated and humoral systems (Allam and Raftos, 2015; Bachère et al., 2015; Vasta et al., 2015). In addition to being the primary immune effector in bivalves against pathogens, the hemocytes are also involved in many other physiological events including nutrient transportation, detoxification, and wound repair (Mount et al., 2004). Histological, structural and fluorescent microscopy, and mass spectrometry of *Pinctada fucata* indicated the presence

of intracellular CaCO_3 crystals in circulating granulocytes thought to be released to the extrapallial fluid (Li et al., 2016d). Most recent studies provide evidence for the presence of two functional groups of hemocyte. Comparison of the hemocytes from different body fluids of *C. virginica* using stained epitopes in conjunction with flow cytometry and functional assays indicate that the cells in the extrapallial space appear to be more actively involved in biomineralization and shell formation compared to the hemolymph (Lau et al., 2017). Transcriptomic profiling of hemocytes showed a marked contrast of gene expression patterns between hemocytes collected from the extrapallial fluid and those samples from the circulatory system in Manila clam *Ruditapes philippinarum* suggesting functional specialization at the biomineralization site (Allam et al., 2014). Expression analysis of selected biomineralization-related genes in hemocytes and mantle cells from the *C. gigas* also found a different pattern of expression and distinct phenotypes based on the cell morphology, motility, and adhesion properties (Ivanina et al., 2017). A similar line of evidence in *P. fucata* is that granulocytes containing calcium-rich vesicles and crystals serve as a calcium pool in the extrapallial cavity while retaining the phagocytic activity (Huang et al., 2018). The same study showed no evidence of the shell matrix proteins in the hemocytes, suggesting that they might not be solely responsible for directing the growth of the shell. The oysters also provide a valuable model to study tissue implanting; the pearl industry produces natural pearls by inserting the mantle graft from a donor together with a nucleus into the gonad of a recipient oyster. Graft recipient is the primary driver of the pearl size while graft donor is responsible for pearl quality traits (Blay et al., 2017).

The bivalve exoskeleton formation is a fascinating biological process that has also attracted the attention of medical research as a model for bone formation and repair (Furuhashi et al., 2009; Kocot et al., 2016; Marin et al., 2012; Westbrook and Marin, 1998). The water-soluble matrix (WSM) of nacre obtained from the inner shell layer of the oyster *P. maxima* promotes an increase in alkaline phosphatase in bone marrow stromal cells (Almeida et al., 2001). The MC3T3-E1 pre-osteoblast cell line from mouse can differentiate into osteoblasts and to mineralize in the presence of beta-glycerophosphate and ascorbic acid; WSM also induces differentiation and reduces the mineralization of MC3T3-E1 pre-osteoblast (Rousseau et al., 2003). Nacre injected percutaneously into experimental cavities prepared in the lumbar vertebrae of sheep results in significant activation of bone formation and mineralization (Lamghari et al., 2001). Similar results have been found when nacre is implanted *in vivo* in bones of mice, rats, and humans (Brion et al., 2015; Lee et al., 2017; Liao et al., 2002). Proteomic studies identified three gigasin-2 isoforms, and a cystatin A2 in WSM of *C. gigas* nacre that appears to be involved in bone remodeling processes and could be responsible for the biocompatibility shown between bone and nacre grafts (Oliveira et al., 2012). Ostrea Testa, a powder made of oyster shell (*Ostrea gigas*), enhances the proliferation of primary osteoblasts, differentiation (ALP activity) and bone nodule formation of osteoblast *in vitro* and has the potential to prevent OVX-induced osteoporosis through osteoblasts activation effects (Han et al., 2007). Shell formation in bivalves is also a model system for studying biomineralization. The pearl oyster, *P. fucata* SMAD4, an orthologue of the intracellular transmitter of the BMP signaling pathway involved in biomineralization process in mammals, also appears to play a role in the regulation of the biomineralization in pearl oyster (Zhao et al., 2016). Although most of the work focuses on bone formation, oyster shell extracts also have shown lipogenesis inhibition properties (Tran et al., 2015b) and there is an increasing interest of the use of nacre as biomaterial for orthopedic applications (Gerhard et al., 2017; Rodrigues et al., 2017; Shen et al., 2014; Zhang et al., 2017).

Several attempts to generate cell lines from bivalves were based on explants from the mantle edge (Awaji and Suzuki, 1998; Chen and Wang, 1999; Daugavet and Blinova, 2015). The study of the molecular mechanisms of biomineralization of the shell, pearl formation,

apoptosis, and terminal differentiation, will benefit from the availability of cell lines (section 2.5).

2.2. Innate immunology

2.2.1. Mucosal immunity

Animal mucosal tissues represent the sites of initial interactions between microbes and their hosts (Duncan and Edberg, 1995; Rayner and Wilson, 1997). For this reason, the understanding of how mucosal immune factors interact with and regulate microbes has become a central theme in biological research. Justifiably, most studies on the role of mucosal immune factors in host-microbe interactions are performed on medically-relevant models, and results highlight an intriguing central role of mucosal tissues in animal health, with an elaborate mechanism of feedback controls for the maintenance of mucosal (and host overall) homeostasis. Bivalves represent an appealing model for the examination of innate effectors involved in mucosal immunity. Their mucosal tissues are readily accessible both for *in vivo* observation (Lau et al., 2017; Mardones-Toledo et al., 2015; Pales Espinosa et al., 2016; Ward et al., 1991) and sampling (Lau et al., 2017; Pales Espinosa et al., 2016), and recent investigations show the responsiveness of these species to the knockdown of genes involved in microbial interactions in mucosal tissues (Pales Espinosa and Allam, 2018) making the validation of mucosal immune effectors within reach.

A particular feature of bivalves is their ability to process an extraordinarily large volume of water for nutrient and oxygen extraction. This is performed by the gills that pump and circulate water in the shell (pallial) cavity. In this context, the gills and other pallial organs (e.g., mantle, labial palps) represent the first tissues encountered by waterborne microbes as they enter the pallial cavity. The convolution of pallial organs greatly increases the effective surface of these interfaces and enhance their exposure to waterborne microbes. In fact, bivalve pallial cavity occupies a similar niche as that of the buccal and upper respiratory cavities of higher vertebrates (semi-confined compartment, highly regulated fluid circulation, the presence of immune defense factors associated with the mucosal surfaces) and therefore displays similar importance as a portal and barrier to invading microorganisms. It is striking that most fatal infections affecting bivalves are initiated in pallial organs or hemocytes patrolling the pallial cavity (Allam et al., 2013; Burrenson and Ford, 2004; Chagot et al., 1992; Dahl et al., 2010; Ford et al., 2002; Kleeman et al., 2002; Lau et al., 2018; Tasumi and Vasta, 2007; Villalba et al., 2004; Wang et al., 2018b) further stressing the role of pallial mucosa in host-microbe interactions. Therefore, good protection of these soft tissues is required to allow bivalves to maintain microbial homeostasis and health in a microbe-rich environment. The mucus layer, abundantly secreted by pallial organs, represents the first host matrix that interacts with waterborne microbes. Bivalve mucus tridimensional structure follows that of other metazoans (Beninger et al., 1997) with the presence of two distinct layers covering epithelial cells (Ross, 1974) (Fig. 2). The first layer is in direct contact with the cells and is often made of low viscosity mucus while the external layer is typically made of viscous secretions that entrap environmental microbes and is directed by cilia movements. Most prior work investigating bivalve pallial mucus was performed during investigations of the suspension-feeding process (reviewed in (Allam and Pales Espinosa, 2015)). But the pallial mucus layer is also the first host factor encountered by mutualistic (e.g., sulfo-oxidant bacteria that inhabit gill bacteriocytes), commensal, or parasitic microbes. We are only starting to understand the diversity and function of defense factors associated with bivalve mucosal immunity. In addition to representing an efficient physical barrier, bivalve mucus was shown to contain a wide range of molecules involved in host-microbe interactions such as galactose and mannose-binding lectins, C1q-domain containing proteins, and antimicrobial compounds (defensin, lysozyme) (Pales Espinosa et al., 2016). Some of these proteins were shown to be regulated via external stimuli as the levels of mucosal lectins was shown

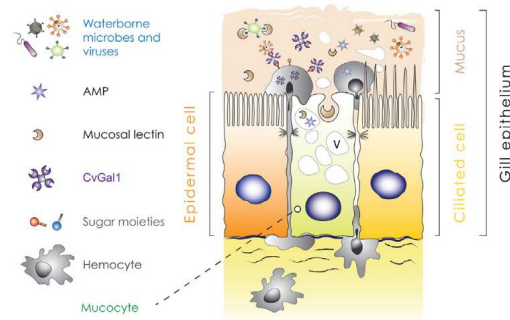


Fig. 2. Mucosal immunity model. Mucus covering pallial surfaces of bivalves plays an important role in immunity and homeostasis. Viruses, bacteria, and protozoans, but also particulate matter and microalgae present in seawater directly interact with innate immune effectors present in mucus. Mucosal hemocytes likely play a sentinel role (sensing and signaling) similar to that of dendritic cells in higher vertebrates. Based on (Allam and Pales Espinosa, 2015). Antimicrobial peptide (AMP); *Crassostrea virginica* Galectin 1 (CvGal1); vacuole (V).

to increase in oysters and mussels exposed to bacterial challenge (Pales Espinosa et al., 2010; Xing et al., 2011).

The adhesion of pathogenic microorganisms to mucosal surfaces is the first step of many infections in vertebrates and invertebrates. Due to its carbohydrate- and protein-rich composition, mucus is an excellent environment for microorganism growth (Koren and Rosenberg, 2008; Mark and Peter, 1999; Ritchie, 2006) that can, in turn, produce molecules that enter in mucus composition (Banin et al., 2001) or that alter host mucus (Brun et al., 2000). This results in the establishment of a robust, and often tightly-controlled, population of adapted microbes. Any alteration to this balance (e.g., changes in mucus physicochemical characteristics) can lead to a dramatic shift in microbial dynamics resulting in infection and disease. Previous studies on marine animals showed that mucus secretion could favor the attachment and growth of adapted microbes (Ducklow Hugh and Mitchell, 1979; Ebran et al., 1999; Nagashima et al., 2003; Ritchie, 2006; Vine et al., 2004), and to mediate symbiont recognition (Bulgheresi et al., 2006; Dufour, 2005; Nyholm and McFall-Ngai, 2003; Nyholm et al., 2000; Southward, 1986). Recent investigations showed marked tropism of the alveolate parasite *Perkinsus marinus* to specific types of mucus from its oyster (*C. virginica*) host, which induced a significant regulation of the proliferation and virulence of the parasite (Allam et al., 2013; Pales Espinosa et al., 2013, 2014). Interestingly, pallial mucus of the non-compatible host *C. gigas* was strongly inhibitory to *P. marinus* activities suggesting that host specificity may begin in the mucus (Pales Espinosa et al., 2013). The transformation of *Perkinsus olsenii* zoospores into trophozoites is also influenced by components of *R. philippinarum* gills and labial palps leading to initial infection primarily within gills and labial palps of the host clam (Wang et al., 2018a). Similarly, a recent study of the gut transcriptome of mussels *Mytilus edulis* infected with the copepod *Mytilicola intestinalis* suggested a co-evolution of this host-parasite association as indicated by the predicted mechanistic interactions between both partners at mucosal interfaces (Feis et al., 2018). In this context, bivalves provide a tractable system for the identification of effectors that regulate homeostasis at mucosal interfaces in animals (including higher vertebrates) and how specialized microbes provide benefits to their hosts.

The understanding of the role of motile mucosal cells in systemic immunity of higher vertebrates remains relatively limited. Previous work in these species highlighted the role of specific neutrophils and dendritic cells in orchestrating the recognition of, and response to,

microbes at mucosal interfaces. Through transepithelial migration, these cells cross epithelial surfaces to monitor environmental microbes (Rescigno et al., 2001), although the molecular cascades involved in their transepithelial migration and signaling remain obscure (Pérez-López et al., 2016). In lower vertebrates and invertebrates, the features and functions of the mobile cellular components of mucosal immunity are, at best, extremely limited (Cima et al., 2006). Bivalves offer an attractive model for such studies. For instance, their mucosal tissues are well irrigated by the blood and hemocytes can cross the basement membrane to wander at the surface of the epithelial barrier in close association with the mucus layer (Allam and Paillard, 1998; Allam and Pales Espinosa, 2016). Through transepithelial migration, oyster mucosal hemocytes were shown to translocate within hours from pallial surfaces to underlying tissues and the circulatory system (Lau et al., 2018), suggesting these may play a sentinel role similar to that of dendritic cells in vertebrates. Interestingly, mucosal hemocytes in oysters had much higher phagocytic activity than circulating hemocytes (Lau et al., 2017). They also showed epitope signatures (surface carbohydrates and clusters of differentiation) different from those of circulating hemocytes suggesting that they represent a specialized category of hemocytes. Specifically, labelling with the cluster of differentiation 11b (CD11b; specific to integrin alpha M and characteristic of transepithelial leukocytes) and 14 (CD14, a receptor of lipopolysaccharides (LPS) and other pathogen-associated molecular patterns (PAMPs), enriched in sentinel leukocytes) were significantly higher in mucosal hemocytes as compared to circulatory cells underlining a potential sentinel role for these cells. CD14 is a common constituent of vertebrate mucus (Uehara et al., 2003) and may represent a conserved mechanism of innate mucosal immunity in metazoans. The occurrence of two-way movements of hemocytes across mucosal epithelia may facilitate infection by adapted microbes that are capable of surviving phagocytosis, as shown for some pathogens in vertebrates (Nunes-Alves, 2014). With that regard, our preliminary investigations showed the ability of the obligate oyster parasite *P. marinus* to take advantage of transepithelial migration of mucosal hemocytes to gain access to internal host tissues (Allam and Pales Espinosa, 2016; Lau et al., 2018). Microbial uptake at mucosal surfaces could be further facilitated by lectins associated with hemocytes as shown for CvGal1 (Feng et al., 2015; Tasumi and Vasta, 2007; Vasta et al., 2015). Overall, mucosal hemocytes appear to represent the missing link between the dynamic microbial landscape at mucosal surfaces and the internal immune system. More investigations are needed, however, to unravel if and how these cells monitor the microbial make-up of mucosal tissues before migrating back into tissues to inform the systemic immune system (e.g., the release of cytokines). More specifically, the transepithelial migration of hemocytes may provide a possible mechanism for the transmission of leukemia in bivalves, which was suggested to be acquired from the water column through mucosal interfaces (Metzger et al., 2015, 2016) (Section 2.6).

Finally, bivalves offer a tractable system for understanding the role of mucosal microbial communities in animal health given the interplay between mutualistic, commensal and pathogenic microbes at mucosal interfaces (Section 2.2.2). This is supported by a growing body of evidence highlighting the role mucosal microbiomes in regulating host resistance to infection either directly microbe-microbe interactions (Barr et al., 2013; Mack et al., 1999; Tuomola et al., 1999) or indirectly via immune stimulation and maturation (Russell et al., 2015). How mucosa responds to probiotics and how changes in mucus physicochemical characteristics (either caused by disease, by other beneficial/harmful microbes or by natural cycles) affect microbial homeostasis at mucosal surfaces are among the many questions that still need to be answered, and that could lead to better disease management strategies.

2.2.2. The microbiome of bivalves

The recent surge of microbiome studies in humans and other species have highlighted the essential roles of microbiomes (bacteria, archaea,

and viruses) in the biology, ecology, and evolution of eukaryotic hosts (Parfrey et al., 2018; Petersen and Osvatic, 2018; Russell et al., 2014). For example, the human-associated microbiome has been extensively studied within many contexts, especially by the Human Microbiome Project (Turnbaugh et al., 2007). Despite their economic and ecological importance, little is known about the diversity and function of bivalve-associated microbiomes. A recent extension of 'omics in bivalves includes the identification of host-associated microbial populations using 16S rRNA sequencing and functional inference. These high-throughput technologies can be used to explore and better understand a range of diverse subjects in biology and ecology, including the basic mechanisms driving selective associations between microbes and hosts (from mutualism to parasitism), and the roles of microbial-bivalve interactions in bivalve biology and health, ecosystem services, resilience to environmental changes, and human health and food safety. Bivalve microbiomes can provide a model for how human-microbe interactions may be studied, particularly within the context of host diseases and environmental change.

As described in previous sections, bivalves are exposed to a rich bacterial community while filter feeding on phytoplankton (Pierce and Ward, 2018). Many of these microorganisms are rejected during the complex process of particle selection; some are digested as they go through the organism, while others are able to colonize the gut and other organs, such as the gills. It is expected that the microbiomes of bivalves change through the seasons due to shifts in phytoplankton communities (Carrier and Reitzel, 2017). Consistent with these features of bivalve biology, the microbiomes of bivalves are proven to be highly dynamic and to respond rapidly to environmental changes. For example, adult oysters have shown differences in microbiome composition according to tissue types, seasons, environmental conditions, and geographical locations (Chauhan et al., 2014; King et al., 2012; Lokmer et al., 2016; Pierce, 2016; Roterman et al., 2015). Moreover, differences in the microbiome of mussels and oysters collected from the same location appear to reflect differences in the biology of these two species, e.g. while the functional diversity of the microbiome of oysters significantly decreased in the winter, the functional diversity in mussels in the winter remained similar to that of other seasons. These differences are consistent with the fact that mussels continue to filter feed during the winter (when temperatures are below 10 °C), while oysters do not (Pierce, 2016).

Despite the high variability in microbial communities associated with bivalves derived from variability in their environments, studies of the eastern and Pacific oyster (*C. virginica* and *C. gigas*) microbiomes have suggested the potential existence of a core microbiome in the oyster that is significantly enriched as compared to the surrounding water, often dominated by *Proteobacteria*, *Cyanobacteria*, and *Firmicutes* (King et al., 2012; Lokmer et al., 2016). Similarly, a study comparing the microbiomes in *Crassostrea hongkongensis* and the surrounding waters has shown that microbiome composition of the surrounding waters was more significantly affected by water salinity while microbiome composition of the oysters was more significantly affected by changes in water temperature (Wang et al., 2016a). Microbiome studies of *C. gigas* larvae in hatcheries and postlarvae grow-out cultivation has found that even though the microbiome in the hatchery/rearing water changes throughout the year, this background microbiome has limited effects on the larval microbiome (Asmani et al., 2016; Powell et al., 2013; Traval Fernández et al., 2014). The microbiome of gills in blue mussels collected in an estuary in the Northeast US was found to be highly dominated by an *Endozoicomonas* similar to symbionts present in another marine host (oysters and tunicates) (Schill et al., 2017). Additionally, a comparative study of two lucinid bivalves has revealed that their endosymbiotic populations are species dependent and re-establishes in the host after perturbation under starvation stress (Elisabeth et al., 2014). These studies point to the existence of selective host-microbial interactions allowing for the establishment of some species in particular tissues.

Consistent with this expectation, some molluscan species have often been used as model systems for the study of the ecology and evolution of host-microbe symbioses. The symbiotic association between the bobtail squid *Euprymna scolopes* and *Vibrio fischeri* is an example of partner fidelity in which the symbiont is acquired each generation by horizontal transmission through a selective immune response from the host (McFall-Ngai, 2014). Although microbiome sequencing approaches have been proposed as tools to discover other examples of host-symbiont coevolution, most phyllosymbiotic approaches are not sensitive enough to demonstrate partner fidelity or co-adaptation (Douglas and Werren, 2016). An initial study of 16S rDNA sequence diversity of a few isolates of *Teredinibacter turnerae*, the symbionts of shipworms (wood-feeding bivalves), did not detect evidence of host-specificity, which could only be detected after analyzing six bacterial genes (Altamia et al., 2014). It is unclear how these diverse and dynamic microbial communities in bivalves affect the fitness of the host and vice versa. A diverse but functionally resilient microbial community may support hosts and allow them to acclimate to changes in their environment (McKenney et al., 2018); e.g., bacterial isolates able to metabolize domoic acid, a potent neurotoxin associated with harmful algal blooms of *Pseudo-nitzschia* spp. have been isolated from blue mussels, *M. edulis*, and soft-shell clams *Mya arenaria* (Stewart et al., 1998). These microbial symbionts may be one of the multiple mechanisms allowing bivalves to survive the toxic effects of harmful algal blooms and pollutants and minimize toxin accumulation in their tissues (Carrier and Reitzel, 2017; Milan et al., 2018) (see also Section 2.3).

The interactions between host immune responses and their associated microbiomes are being increasingly investigated within the context of disease (Lozupone, 2018). It is hypothesized that the microbiome may be one factor in the modulation of their host immune response and disease phenotype (Douglas, 2018). As an example, microbial community diversity in Sydney rock oysters was found to be affected when the host is infected with macro-parasites (Green and Barnes, 2010). The microbial diversity of the hepatopancreas of Pacific oysters was also reported to be affected by hypoxia, potentially reflecting the effect of environmental stress on the immune response of these oysters (Khan et al., 2018). Microbial dysbiosis in the marine environment and aquaculture is strongly associated with mortality events and disease (Egan and Gardiner, 2016). In response, high-throughput sequencing of microbiomes has been used as a tool for disease management of bivalve species of aquaculture interest (Bentzon-Tilia et al., 2016; Gómez-Chiarri et al., 2015a). Additional research is needed to decipher this crosstalk between the bivalve host's immune receptors and the microbiome.

Bivalves also play key roles in disease transmission to other species. As filter feeders, bivalves can act as biofilters, and therefore alter disease transmission by either decreasing concentrations of pathogens in the water through filtration and digestion (sinks) or concentrating and amplifying pathogens able to survive and, in cases, proliferate within their tissues (sources) (Burge et al., 2016). As an example, species of the *Vibrio* genus could cause a decrease in the survival of *C. virginica* larvae at a concentration of as low as 10^3 CFU per ml (Paillard et al., 2004). Oysters and their vibrio populations have been proposed as a model to study disease dynamics in wild animals within ecological and evolutionary frameworks, a model that could improve understanding on how polymicrobial assemblages and microbial-microbial interactions may affect disease initiation and progression in a variable environment (Le Roux et al., 2016). Increased understanding of microbe-host interactions would also allow developing and/or optimizing strategies to manipulate bivalve microbiomes and to prevent/treat the accumulation of human pathogens. Two studies on changes in the composition of the microbiome of mussels after depuration have revealed a decrease in bacterial diversity and evenness, but the studies differ on the outcome regarding changes in the relative abundance of Proteobacteria (Rubiolo et al., 2018; Vezzulli et al., 2018).

Better tools are needed to identify critical interactions among the

host, microbes, and the environment. While 16S rRNA sequencing has been extremely useful for estimating the population structure of the microbiome community, linking the 16S rRNA gene sequencing data with the functional potentials and activities of the microbiome is still largely unexplored and remains a challenging undertaking. In a recent study, a computational procedure was applied to infer the abundance of denitrification genes based on the distribution of 16S rRNA genes and a database of taxonomically verified function genes (Arfken et al., 2017). This approach, however, assumes the conservation of function genes among taxonomically related species and hence may overlook the genome-level variations within the same taxonomic unit. Another mostly unexplored area in the microbiome study of marine invertebrates is the application of metagenome and metatranscriptome sequencing (Martínez-Porchas and Vargas-Albores, 2015). Unlike the 16S rRNA based amplicon sequencing, the metagenome and metatranscriptome approaches target the totality of all the coding genes and gene transcripts, respectively. For example, a metatranscriptomic analysis of the gills and its microbes in a deep-sea mussel, *Bathymodiolus azoricus*, shows microbial functional acclimation to pressure changes (Barros et al., 2014). The further application of metagenome and metatranscriptome approaches to studying diverse marine invertebrates will likely bring new insights into the functional interactions both within and among the microbiome, the oyster host, and the surrounding environment.

Another area of future studies is to look for the presence, composition, and function of core microbiomes among different tissue types and host genotypes (King et al., 2012). While a few studies have suggested the presence of core microbiomes within different tissue types, the identity and relative abundance of the core microbiome requires further exploration. The temporal and spatial variation of core microbiomes could potentially be linked to variations in the host genotype, host physiology and environmental conditions. The genotype-phenotype associations of the microbiome and host could be further explored with genome-scale modeling (Steffensen et al., 2016). The models could help establish physiological understanding of the individual organisms in the microbiome and help identify auxotrophy among different microbes and the host. Genome-scale modeling has been proven useful for the prediction of changes in activity under diverse environmental conditions (Dufault-Thompson et al., 2017). It could effectively integrate the metagenomic and metatranscriptomic data into creating a holistic view of the microbiome-host-environment interactions.

Moreover, questions remain about how microbes are acquired by the host and the impact of microbiome composition and function on the biology and health of the host. In bivalves, metatranscriptomic studies will help identify potential obligate symbionts, which could not have been readily identified in bivalves due to the lack of cell cultures that would help in isolation (Carrier and Reitzel, 2017). To characterize the nature of these associations (mutualistic, commensal, pathogenic), it would be necessary to collect metatranscriptomic data at different states of the host, from health to disease, and in a variety of environments. These data may solve mysteries regarding symbiont colonization and persistence, microbiome effects on host genome evolution, roles in digestion and nutrition, defense mechanisms, and immune responses.

2.2.3. Alternatives to antibiotics

Marine life, with its incredible biodiversity, has become the focus of intensive research as a unique source of natural products which could potentially find cost-effective biotechnological applications as drugs, cosmetics, and nutraceuticals (Anjum et al., 2017). The most relevant biotechnological applications of engineered proteins from Mollusca, the largest phylum of marine metazoans, include the 2014 approval by the Food and Drug Administration of a cone snail-derived peptide (ziconotide, for sale under the name Prialt) as a commercial drug for chronic pain therapy. Other exciting products currently under development include a “medical glue” inspired by mussel adhesive byssal proteins (Barrett et al., 2013) and artificial cancer cell-binding lectins which

may find an application as diagnostics or therapeutic tools (Terada et al., 2017). The interdisciplinary paradigm that emerged from these innovations, if applied more broadly, could provide important results in the discovery of novel bioactive peptides with some potential applications. Antimicrobial peptides (AMPs), fundamental effectors of the innate immune system, have attracted considerable attention from pharmaceutical companies as alternative antibiotics for the treatment of human and farmed animal diseases (Galdiero et al., 2015; Giuliani et al., 2007; Hancock and Sahl, 2006). The focus on AMP research arises from their broad spectrum of activity and effectiveness against multi-drug-resistant bacterial pathogenic strains which cannot be targeted by conventional antibiotic treatments (Vizioli and Salzet, 2002), a growing issue that, according to some experts, may lead to a “post-antibiotic era” (Kosikowska and Lesner, 2016). These properties derive from the universal cationic nature of most AMPs that, in spite of considerable diversity regarding primary sequence and 3D structure, interact with negatively charged bacterial cell wall or membrane components, leading to the elimination of invading microbes by lytic mechanisms. Consequently, the number of changes that would be required to counteract the complex mode of action of AMPs while maintaining cell membrane function and structure are thought to make the development of AMP resistance quite unlikely (Zasloff, 2002).

As filter-feeding organisms, marine bivalves are exposed continuously to high loads of bacteria, viruses and other potentially pathogenic microorganisms (Salazar and Sunagawa, 2017; Suttle, 2007), in addition to those generally associated with their tissues as symbionts (Section 2.2.2). Over time, selective pressure is thought to have favored the development of efficient and effective mechanisms of defense towards infections, which involve the production of a plethora of bioactive defense molecules (Hughes and Fenical, 2010), far superior in abundance and diversity than other molluscan classes (Gerdol, 2017). Although bivalve AMPs have been traditionally considered as humoral factors associated with circulating immune cells (hemocytes) (Mitta et al., 2000), in recent years a growing number of studies have revealed their importance in mucosal surfaces (i.e., mantle and gills), which represent the primary interface of contact with the external environment (Allam and Pales Espinosa, 2016). At the same time, it is now clear that bivalve AMPs do not merely act as pathogen-killing effectors but are more intimately linked to the modulation of host-microbiome interactions (Destoumieux-Garzon et al., 2016) and that they may cover accessory cytokine-like functions (Balseiro et al., 2011).

Since the isolation of the first bivalve AMPs in mid ‘90s (Hubert et al., 1996), several dozen peptides pertaining to different families have been characterized in various bivalve species, with those with commercial importance (i.e., mussels, oysters, clams, and scallops) as the main subject of research (Li et al., 2011b). Mussels (*Mytilus* spp.) in particular, represent a striking example of AMP diversity, as their arsenal of antibiotic peptides currently includes defensins, big defensins, mytilins, myticins, mytimacins, myticusins, myticalins, and mytichitins, but possibly others yet to be discovered (Gerdol and Venier, 2015) (Fig. 3). Interestingly, although multiple pathogens and disease conditions have been described in mussels (Boehs et al., 2010; Robledo et al., 1994), both natural beds and aquacultured mussels species appear to indicate a superior resilience to pathogens compared to other cultured bivalves (Gauthier-Clerc et al., 2013; Romero et al., 2014). Within this context, mussel AMPs may represent good candidates for the development of biotechnological products aimed at the improvement of aquaculture practices in bivalve species more susceptible to infection and disease. The vast majority of known bivalve AMPs are rich in cysteine residues engaged in multiple disulfide bonds, which enable a compact structure, often corresponding to a “CSαβ motif” (Dias Rde and Franco, 2015). On the other hand, AMP classes widespread in different domains of life, such as alpha-helical peptides, are seemingly absent in bivalves or, as in the case of linear peptides with compositional biases (i.e., enriched in positively charged amino acids), have only been reported on a few occasions (Gueguen et al., 2009;

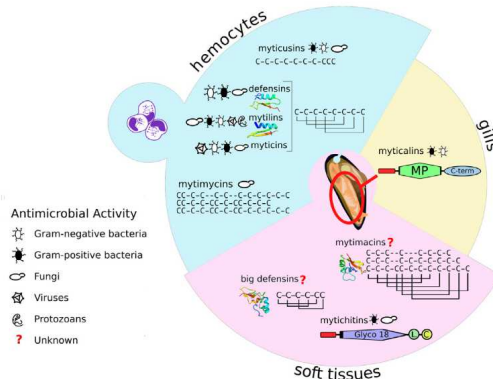


Fig. 3. Summary of the known antimicrobial peptides produced by mussels (*Mytilus* spp.). Antimicrobial activity (defined as an experimentally determined Minimum Inhibitory Concentration < 32 μM), tissue specificity and three-dimensional structure (whenever known) are indicated for each AMP family. The typical cysteine array and disulfide connectivity of cysteine-rich AMPs are also displayed. Different members of the mytimacin and mytimycin family are characterized by different cysteine arrays. The disulfide connectivity of mytimycin and myticusins has not been elucidated yet. MP: mature peptide; C-term: anionic C-terminal extension; Glyco 18: glycoside hydrolase, family 18, domain; L: linker region; C: chitinase-like domain of mytichitin.

Leoni et al., 2017).

Over the past two decades, bivalve AMP research has been mainly directed towards two interconnected areas of interests, the first dealing with the role of these effector molecules in the context of the bivalve immune system, the second focused on their possible application in the treatment of human and animal diseases. While the reader is directed to recent reviews for an overview of the biological role of bivalve AMPs in bivalve immunity (Bachère et al., 2015; Gerdol and Venier, 2015), we will here briefly outline the progress and current limitations of the use of bivalve AMPs as therapeutics for human health.

The number of functionally characterized bivalve AMPs displaying significant *in vitro* activity against biomedically relevant strains of Gram-positive and Gram-negative bacteria, fungi and, less frequently, protozoans and viruses, is steadily growing (Gueguen et al., 2009; Leoni et al., 2017; Yang et al., 2000). Interestingly, while bivalves are hosts of several metazoan parasites (e.g., nematodes, cestodes, trematodes and, in some cases, copepods), no defense molecule active against these targets has been described to date, in stark contrast with arthropods, where a few multifunctional AMPs active against parasitic nematodes (e.g., cecropins) have been described (Chalk et al., 1995). The promising results obtained with standard Minimal Inhibitory Concentration assays, evidencing biological activities at concentrations as low as 0.01 μM for oyster defensins against Gram-positive bacteria (Bachère et al., 2015), prompted the design of a few engineered AMP variants, and the evaluation of their potential for biotechnological applications.

The mussel AMP mytilin has been the target of multiple studies. In 2004, a synthetic variant was tested *in vitro* against the shrimp white spot syndrome virus, evidencing a significant reduction of mortality in shrimps challenged with viral particles pre-incubated with the AMP (Dupuy et al., 2004). Subsequent studies enabled the identification of a fragment adopting a beta-hairpin structure as the minimal active peptide (Roch et al., 2008). Two synthetic peptides derived from the sequence of *Mytilus coruscus* were subsequently produced and tested with success against some Gram-positive, Gram-negative bacteria and fungi, also displaying high stability in human serum (Liu et al., 2010). Investigations carried out on the mussel defensin MGD1 led to the development a synthetic cyclized nonapeptide (Romestand et al., 2003),

highly active against the protozoan parasites *Trypanosoma brucei* and *Leishmania major*, also capable of preventing HIV-1 viral infection in MAGIC-5B cells (Roch et al., 2004). Moreover, the rational design of a novel peptide, named Ap-S, based on an AMP isolated from the hemolymph of the Chilean scallop *A. purpuratus*, enabled the creation of a synthetic antifungal peptide showing a broader spectrum of activity compared to the native peptide (Arenas et al., 2009). However, none of these studies has led to significant developments to date, and a more recent work, which revealed the high biotechnological potential of mussel myticin C as a potent antiviral against Herpes simplex viruses (–1 and –2) infection *in vitro*, still awaits confirmation from *in vivo* testing (Novoa et al., 2016).

The current lack of marketable bivalve AMP-derived molecules is in line with the inherent difficulties in the engineering and production of peptide-based therapeutics. Globally, only a handful AMPs isolated from various animal sources are currently undergoing phase II/III clinical trials (Mahlapuu et al., 2016), which highlights the multiple issues that hamper the development of these molecules as valid alternatives to antibiotics (Hancock and Sahl, 2006). First, relevant discrepancies are often observed between *in vitro* and *in vivo* test. This is in part due to the critical influence of environmental and physiological conditions on AMP activity (e.g., pH in the case of mussel myticins) (Domeneghetti et al., 2015); in part, this is also due to the scarce knowledge on their mode of action, which, among all bivalve AMPs, has only been firmly established for oyster defensins (Schmitt et al., 2010). AMPs might also be susceptible to degradation by endogenous proteases and generally display short half-life *in vivo*, which might prevent their intravenous and oral administration, and even sensibly reduce their effectiveness as topical antibiotics (Vlieghe et al., 2010).

Although AMPs generally bear little risk due to their amino acidic nature and lack of dangerous metabolites, some potential safety concerns have been reported, in particular whenever high concentrations are required to reach a critical biological action. For example, mytilin A displayed significant antiparasitic activity and inhibition of Herpes simplex virus type I replication only at concentrations, potentially cytotoxic for human cells (Carriel-Gomes et al., 2007; Löfgren et al., 2008).

Besides the limitations mentioned above, high manufacturing costs currently represent the major obstacle towards the commercial scalability of AMP, limiting their competitiveness compared to other small non-peptidic drugs (Bray, 2003). Direct isolation of AMPs from bivalve biomass is usually impractical, and chemical synthesis, frequently applied for laboratory testing, results in costs incompatible with large-scale applications. The methodology that holds the best promises is probably the production of recombinant AMPs in heterologous microbial systems (Ingham and Moore, 2007). Although several bivalve AMPs have been produced in *Escherichia coli* (Bachère et al., 2015), *Pichia pastoris* is emerging as the preferred system as it offers many advantages over bacteria, such as the inclusion of post-translational modifications typical of eukaryotic cells (Cereghino and Cregg, 2000). Some attempts have been made at setting up large-scale production of recombinant bivalve AMPs in yeast, producing conflicting results. For example, while large quantities of mytichitin-A could be obtained with this method, the recombinant protein lost some of the biological properties of the native AMP, most notably its activity towards Gram-negative bacteria (Meng et al., 2016).

Altogether, these considerations highlight that, in spite of the enormous potential offered by marine biodiversity for AMP discovery, the pharmaceutical industry currently displays a preference for technology-intensive methods that usually lead to a fallback to traditional and more accessible resources. It is the prevailing opinion that new strategies are required to exploit the biotechnological potential of the marine environment, increasing the success of drug discovery (Desbois, 2014). The emergence of multidrug resistance in bacteria might provide the appropriate framework for a necessary investment in this field, which would see bivalves as primary targets of research.

AMPs are not the only secretory components of the bivalve immune system that hold biotechnological potential for applications aimed at the improvement of human health. Indeed, the production of antimicrobial effectors and the regulation of immune response at the cellular and systemic levels depend on the activity of cytokines, small glycoproteins that guarantee a fast and effective immune response even at a very low concentration in body fluids. Due to their role, human cytokines and their receptors have been the target of intensive biomedical research and several analogs, derivatives and competitive inhibitors have been developed for the treatment of multiple pathologies, including cancer, AIDS, asthma and autoimmune disorders (Fry and Mackall, 2002; Gordon et al., 1996; Leonardi et al., 2008; Noble and Goa, 1997; Vatrella et al., 2014).

Cytokines have long been thought to be a prerogative of organism endowed with an adaptive immune system until the first evidence supporting the existence of invertebrate cytokine-like factors started to surface in the early 2000's (Beschlin et al., 2001). Little progress has been made in bivalves since, mostly due to the elusive nature of such molecules and the lack of sequence similarity with well-characterized vertebrate interleukins. Functional studies have so far targeted a few evolutionarily conserved cytokines (i.e. interleukin-17, the macrophage migration inhibitory factor MIF and the allograft inflammatory factor AIF-1), whereas research is still at its very early stages for others which have been discovered very recently (for a review on the subject, see (Gerdol et al., 2018)).

Large uncertainties remain about the ability of bivalve cytokines to interact with the highly divergent cytokine receptors expressed by human cells. To the best of our knowledge, just two studies have been able to confirm *in vitro* the immunomodulatory effects of exogenous bivalve cytokines on vertebrate cell lines. In detail, it has been revealed that recombinant MIF protein from the scallop *Azumapecten farreri* could induce sheep fibroblast migration into scraped spaces, suggesting potential usefulness for wound healing (Li et al., 2011a). A second study was able to demonstrate the interaction of the oyster Tumor Necrosis Factor with TNF receptors in the human cell line HEK293T through the activation of a reporter gene (Qu et al., 2017). Our knowledge of bivalve cytokines is still minimal compared to AMPs, and this lack of knowledge most certainly currently hampers the development of biotechnological products at this stage. Yet, the multifaceted immunomodulatory properties of cytokines may lead to surprising innovations once these obstacles are overcome in the years to come.

2.3. Harmful algal blooms (HABs): toxin impacts, metabolism, and detoxification

A worldwide increase in the frequency, magnitude, and distribution of harmful algal blooms was first recognized in the late 20th-century (Anderson, 1989; Smayda, 1989). While this increase has been attributed to any one of many causes depending on location and the algal species involved, the trend is predicted to continue as climate change increasingly impacts these blooms (Hallegraeff, 2010). Many of these HAB species contain potent biotoxins which may be concentrated by filter-feeding molluscan shellfish and subsequently vectored to humans and/or bioaccumulated through food chains (Landsberg, 2002). Although the specific HAB toxin differs depending on the algal species involved, which in turn can vary with location, bloom timing and the shellfish species involved, these blooms constitute a significant current and future threat to both cultured and natural harvests of filter-feeding molluscan shellfish (Shumway, 1990).

Bivalves can serve as vectors for biotoxin transfer to humans via both natural harvest and aquaculture activities, especially oysters and mussels (Farabegoli et al., 2018). Although filter-feeding bivalves have a low sensitivity to phycotoxins, they can accumulate high concentrations of these toxins (Haberborn et al., 2010a, 2010b) and thus are often routinely monitored for these toxins (Anderson, 2009). Originally thought to be exempt from accumulating toxins (see (Shumway, 1990)

for a historical review), oysters and mussels can accumulate a variety of algal toxins, including saxitoxin(s), brevetoxin(s), domoic acid, and okadaic acid. These toxins can be subsequently vectored to humans via consumption of contaminated oysters and result in syndromes which include paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP), and diarrhetic shellfish poisoning (DSP). These algal toxins represent a variety of chemical structures ranging from tetrahydropurines to ladder polyethers, and the amino acid domoic acid, and hence exposure results in a variety of *in vitro* physiological effects in humans due to differences in the mode of action of individual toxins (see (Baden and Trainer., 1993) for review). A commonality, however, is the ability of bivalves to bioaccumulate and tolerate these algal toxins while only experiencing sublethal impacts themselves. Bivalves also can metabolize and biotransform toxins in toxin analogs via complex chemical and enzymatic conversion mechanisms (Manfrin et al., 2012).

The vast majority of algal toxicological studies have focused on human cell lines and model vertebrate systems (Manfrin et al., 2012). The variety of genomic, genetic, behavioral, and pathological responses documented in bivalves, as well as the sublethal nature of the majority of these impacts in response to algal biotoxin exposure, suggests that bivalves are a potentially suitable model organism for the study of toxin metabolism, including modification and detoxification. Additionally, the complete genome of the several bivalve species has been assembled (Gómez-Chiari et al., 2015b; Powell et al., 2018; Wang et al., 2017a; Zhang et al., 2012). This makes bivalve species excellent choices to potentially serve as a model system/organism to both study the impacts of these toxins and to serve as potential tools for human health monitoring by developing sensitive, cost-effective bioindicators or cell markers (e.g., cell damage and detoxification responses) of toxin presence, especially at low algal cell concentrations. The following summary is a brief overview of the variety of sublethal impacts of these algal toxins on oyster species, with the goal of fostering and advancing the potential use of oysters as model systems for the study of the impacts and monitoring of these toxins.

Impacts of algal toxins on bivalves can be grouped into either behavioral, pathological, genetic, or genomic effects (Fig. 4). As bivalves have no adaptive immunity to algal toxin exposure, their responses are limited to their innate immune responses to stressors (e.g., pathogens, parasite and physical injury (Janeway, 1994)). Bivalves take up and accumulate algal cells as food, and often exhibit short-term behavioral responses to toxin exposure. If toxic cells are not immediately rejected

as pseudofeces (Hégaret et al., 2007b; Mafra et al., 2010), toxins accumulate in the bivalve digestive gland (Bardouil et al., 1993; García et al., 2015; Guéguen et al., 2008; Haberkorn et al., 2011), and immediate behavioral effects include changes in valve activity (Basti et al., 2009; Borcier et al., 2017; Haberkorn et al., 2011; Mat et al., 2013; Nagai et al., 2006; Tran et al., 2010), respiration (Shumway and Terry, 1987), and reduced clearance, and filtration rates (Hégaret et al., 2007b; Lassus et al., 1999; Lesser and Shumway, 1993; Wildish et al., 1998). These negative impacts extend to embryonic and larval stages and include less active spermatozoa (Haberkorn et al., 2010b), decreased sperm viability (Rolton et al., 2015) and impairment of larval survival and reproductive development (Basti et al., 2016; Gainey Jr and Shumway, 1988), and clearance rates (Leverone et al., 2007). Extreme behavior effects such as paralysis of the adductor muscle in *C. virginica* have been reported on exposure to the dinoflagellate *Alexandrium fundyense* (Hégaret et al., 2007a). Some bivalve adaptive mechanisms are possible when feeding on toxic algae however. *M. edulis* was able to alter absorption kinetics of biochemical compounds and digestive gland enzymes on exposure to *Alexandrium catenella*, allowing exploitation of the toxic algae as a food source with minimal impacts (Fernández-Reiriz et al., 2008).

Although toxins accumulate primarily in the digestive gland, toxins do directly affect the bivalve immune systems, resulting in inflammation and impaired immune function (Hégaret and Wikfors, 2005a, 2005b). Immune response in bivalves is mediated by hemocytes and exposure to algal toxins directly affect bivalve hemocytes. Hégaret et al. (Hégaret and Wikfors, 2005a, 2005b) demonstrated changes in the number and proportion of hemocytes in *C. virginica* on exposure to the dinoflagellate *Prorocentrum minimum*. *M. edulis*, conversely, experiences diapedesis of hemocytes into the intestine when exposed to *P. minimum*, aggregating the *P. minimum* cells and potentially isolating them to minimize *M. edulis* tissue damage (Galimany et al., 2008). Changes in oyster hemocyte counts and viability are commonly reported upon exposure to algal toxins (Basti, 2011; Haberkorn et al., 2010b; Hégaret et al., 2011; Medhioub et al., 2013; Mello et al., 2013), however there are some exceptions. Hégaret et al. (Hégaret et al., 2007a) reported that the toxic dinoflagellates *A. fundyense* and *catenella*A. had no impacts on *C. virginica* hemocyte morphology or function. However, toxin production is highly variable in some toxic algal genera (Burkholder and Glibert, 2006), which may explain reported difference in hemocyte impacts. Additional immune-related responses to toxin exposure include hemocyte phagocytosis (Ford et al., 2008; Mello et al., 2013), higher mucus production in gills (Haberkorn et al., 2010b), and histopathological lesions (Pearce et al., 2005) including myopathies in the adductor muscle (Haberkorn et al., 2010b). *In vivo* exposure of *Mytilus galloprovincialis* to *Prorocentrum lima* causes oxidative DNA damage resulting in early genotoxicity in hemocytes (Prego-Faraldo et al., 2016). In addition to the toxins, there is new evidence that bioactive extracellular compounds produced by the microalgae also have an effect on valve-activity behavior and hemocyte mobilization (Castrec et al., 2018).

Recent research on genetic and genomic responses of bivalves to algal toxins suggests that these responses may provide a promising approach to identify biomarkers of toxin contamination. Mat et al. (2018) report that toxin load is a primary driver of transcriptomic variation; the whole transcriptome of the digestive gland of *C. gigas* exposed to *Alexandrium minutum* exhibited differences in transcript abundance between oysters with different toxin loads (1098 transcripts) rather than between exposed and non-exposed oysters (16 transcripts); only 70 of 29,000 oyster genes described transcripts modeled the toxin load. *A. minutum* also disrupts circadian rhythmicity in the cryptochrome gene (CgCry) in exposed *C. gigas*, and gene transcription remains at a constant low level throughout a daily cycle with exposure (Tran et al., 2015a) and also provokes activation of oyster antioxidant system at the transcription level (Fabioux et al., 2015; García-Lagunas et al., 2013). Other oyster genes have also been shown

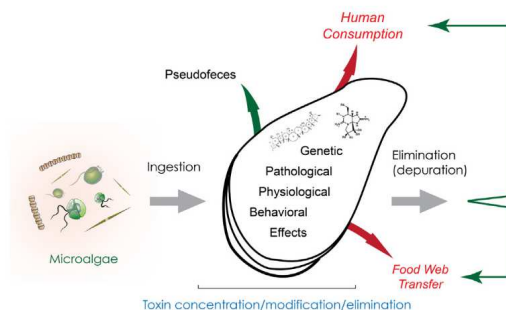


Fig. 4. Transfer routes and effects of toxins in bivalves. HAB species containing biotoxins are ingested through the filter-feeding process. Some species are rejected in the pseudofeces. However, the ingestion of the HAB species may result in the concentration and modification of the toxins, resulting in genetic, pathological, physiological, and behavioral effects. Accumulated toxins are vectored to humans and/or bioaccumulated through the food chain. Interestingly, the bivalves also can eliminate (depurate) the toxins. Some drawings modified from IAN (<http://ian.umces.edu/>).

to be upregulated by toxin exposure, including pro-apoptotic genes (Bax and Bax-like) implicated in the mitochondrial pathway, two caspase executor genes (caspase-3 and caspase-7) and five (Bcl2, Bcl-1, IAP1, IAP7B and Hsp70) inhibitors of apoptosis-related genes (Medhioub et al., 2013) as well as genes involved in antioxidant defense (copper/zinc superoxide dismutase), cell detoxification (glutathione S-transferase, and cytochrome P450), intermediate immune response activation (lipopolysaccharide and beta-glucan binding protein), and stress responses (glutamine synthetase) (García-Lagunas et al., 2013). Overexpression of these genes indicates the activation of a protective mechanism, whose answer depends on both toxic cell concentration and exposure time (García-Lagunas et al., 2013). Exposition of *M. galloprovincialis* to *P. lima* downregulates the expression of genes involved in antioxidant stress (Prego-Faraldo et al., 2017). Similarly, transcriptomic profiles in response to the experimental exposition of mussels to okadaic acid results in an increase in proteasomal activity, molecular transport, cell cycle regulation, energy production, and immune activity in the digestive gland and gill tissues (Suárez-Ulloa et al., 2015).

All of these impacts will vary depending on biological and environmental variables at the time of exposure, including the algal species, the toxicity of the clones and the specific toxin(s) involved, the oyster species, its life cycle stage, nutritional status and size, as well as local environment conditions which may favor bloom development and exposure periods. Interactions between algal toxins and other stressors also have impacts on oysters. Lassudrie et al. (2015) demonstrated increased dermo susceptibility in *C. virginica* infested with the trematode *Bucephalus* sp. when exposed to *A. fundyense*. Conversely, exposure to the dinoflagellate *Alexandrium catenatum* reduced the prevalence of herpes infection in *C. gigas*, and the oysters accumulated fewer toxins (Lassudrie et al., 2015). Whether the host-pathogen interaction was modified by behavioral, physiological or direct interaction effects is unknown. While extended exposure to toxic algae may ultimately result in reduced bivalve survival and growth (Shumway, 1990), the variety, extent and commonality of the sublethal impacts of toxin exposure, which include genomic, genetic, behavioral and pathological effects (Fig. 4), suggest that bivalves have the potential to serve as a model system for the study of direct and indirect impacts of algal toxin on biological functions on a variety of timescales.

2.4. Microplastics in the ocean

The ocean and other aquatic environments are considered sinks for anthropogenic contamination. Microplastics have recently gained attention and notoriety in society and the mass media beyond the reason why they were engineered. The release of plastics into the environment during manufacture, transport, and as waste is increasing by approximately 25 million tons a year (Mato et al., 2001; Waite et al., 2018). Since plastics are engineered to have a slow rate of degradation, they build up in the environment making them a hazard for future generations (Galloway and Lewis, 2016; Law et al., 2010). These plastics do not just stay in their original form, the ocean breaks them down into millions of microplastics (MP) through mechanical degradation such as wave action or chemical processes like photodegradation, thermal degradation, and biodegradation (Law and Thompson, 2014; Smith et al., 2018b; Waite et al., 2018). Technically, MPs are defined as any plastic less than 5 mm, but plastics less than 1 µm have also been reported in the environment (Smith et al., 2018b). Their origin comes from clothing fibers and microbeads in cosmetic and hygiene products being deposited into the ocean by rivers, tides, runoff, cargo dumps and even lost fishing gear (Law and Thompson, 2014). The most prevalent plastic chemicals found in ocean debris are polypropylene, polyethylene, and polyvinylchloride (PVC) (Wright et al., 2013). In addition to the chemical toxicity, MCs are a dynamic mixture of chemical and microbial contaminants that bind to them changing toxicity and bioavailability (Galloway et al., 2017; Law and Thompson, 2014). Studies have found

high concentrations of harmful chemicals (polychlorinated biphenyl, dichlorodiphenyldichloroethylene, and non-phenols) in polypropylene resin pellets in both ambient seawater and ocean sediments (Mato et al., 2001). These pellets are highly absorbent and could absorb other harmful chemicals beyond those tested (Mato et al., 2001). The MPs are causing issues for trophic systems, as they are small enough to be eaten by zooplankton (Law et al., 2010; Law and Thompson, 2014). Since many zooplankton species are not highly selective in their food choice, subsequently MPs can be passed from one organism to the next through the trophic web (Law et al., 2010; Law and Thompson, 2014; Wright et al., 2013).

The complexity of current systems in the ocean makes very challenging to determine the exact concentration of MPs (Law and Thompson, 2014); however, there are report of MPs in bivalves is increasing worldwide (Kanhai et al., 2018; Li et al., 2018a, 2018b; Naji et al., 2018; Santana et al., 2016). Marine snow contributes to mobilize MPs away from the surface into deep environments (Porter et al., 2018). High-density plastics, such as PVC, float down to sediments (Wright et al., 2013) allowing them to be consumed by benthic organisms, like bivalves. Aggregates of microbeads form in upper layers of the ocean via currents and sink to the sediment regions. On the seafloor, bivalves are more likely to ingest these aggregates rather than loose plastics, which incorporates MPs into the food web (Wright et al., 2013). It is estimated that the sediment in coastal waters contains a range of 2–30 MPs per 250 mL (Law and Thompson, 2014). Plastics in the range of the 16.5 MP pieces per individual have been reported in oysters (*C. virginica*) (Waite et al., 2018) and 1.1–6.4 MPs per individual in blue mussels (*M. edulis*) (Li et al., 2018b). As bivalves accumulate MPs, higher trophic levels are at risk for exposure to the chemicals found in MPs. Globally, 6.7% of human protein consumed is seafood (Smith et al., 2018b). These MPs have the potential to be both toxic and cause physical issues; however, the full extent of this risk must be studied further.

Some studies have found that MPs can cause physical blockages, chemical changes, and biological change in European flat oysters (Green, 2016). While bivalves do not have the highest amount of selectivity for trapping food particles, they are capable of selecting their food particles using various criteria, including size (Pales Espinosa and Allam, 2018; Wright et al., 2013) (see also Section 2.2.1). This mucus generally allows bivalves to effectively capture particles as small as 3–4 µm and some smaller particles as well (Wright et al., 2013). Indeed, mussels (*M. edulis* and *Perna viridis*) are more likely to consume smaller MPs such as microbeads instead of larger microfibers (Qu et al., 2018). Besides, MPs can also adhere to muscle, mantle, and other bivalve organs (Kolandasamy et al., 2018). Many MPs are within the 1–4 µm range and therefore, bivalves are incredibly susceptible to ingesting them. Bivalves can't digest MPs easily, and they build up in tubules and the digestive organs, sometimes persisting for 48 h (Wright et al., 2013). The build-up of these MPs can cause clogging of the bivalve digestive tract and decrease the ability to feed (Wright et al., 2013). As the microplastics weaken these animals, they may make them more susceptible to predation, causing bioaccumulation of the toxins in the food web. In addition to the incorporation into the food web and the risk for human consumption (Van Cauwenberghe and Janssen, 2014), the evidence is building that MPs also affect the physiology of the bivalves.

Experimental exposure of sediment-dwelling bivalves to environmental relevant MPs results in changes of the energy reserve tissues directly (Bour et al., 2018) or indirectly (Xu et al., 2017). Exposures of (*Mytilus galloprovincialis*) to polycyclic aromatic hydrocarbons commonly found in MPs led to bioaccumulation in digestive glands and gills (Pittura et al., 2018). Prolonged exposure of oysters to polystyrene microspheres resulted in significant decreases in oocyte number and diameter, and sperm velocity as well (Sussarellu et al., 2016). The larval development of bivalves can also be impacted by leachate from MPs (Gandara e Silva et al., 2016). Nano polystyrene particles reduce the

filtering activity of mussels (Wegner et al., 2012). Interestingly, mussels exposed to PVC particles do not result in physiological effects (Santana et al., 2018). MPs can also affect the benthic communities associated with bivalves (Green, 2016). The literature on the effect of MPs on the immune system of marine macrofauna is very minimal. Corals under the acute MP exposure exhibit immune system and detoxification repression (Tang et al., 2018). When exposed to MPs, *M. galloprovincialis* had MPs in hemocyte cells that exhibited destabilized lysosomal membranes (Pittura et al., 2018). It has been shown that MPs (< 1 mm) translocated into the circulatory system of mussels where they enter in direct contact with hemocytes (Browne et al., 2008). MPs exposure of *M. galloprovincialis* results in disruption of the homeostasis associated with the production of stress and immune-related proteins (Detree and Gallardo-Escarate, 2018).

Microplastics have become prevalent not only in our environment but in the food we eat and even ourselves. As they accumulate in the ocean, they are ingested by bivalves and moved up through the food web. The effects of these toxins are still mostly unknown due to the highly variable compounds and concentrations found in these plastics. What we do know is that bivalves are one of the primary organisms that can transfer them into humans. As a result, further study of the toxicity of MPs in bivalves is critical to determine the risks to the bivalve community, marine ecosystems and humans. Finally, using bivalves as bioindicators can play an essential role in understanding pollution levels, bioavailability and the ecological risks of contaminants (Su et al., 2018).

2.5. Regeneration and stem cells

Regeneration is the replacement of lost tissue with new following damage or wounding (Wells and Watt, 2018). Although sharing similarities with developmental growth or asexual reproduction, regeneration is, therefore, a distinct process at both the cellular and molecular level (Bely and Nyberg, 2010; Erickson and Echeverri, 2018; Simoes and Riley, 2018; Wells and Watt, 2018). Regeneration within the body can occur at different, e.g., cellular, tissue, organ, and limb levels. However in the current context of regenerative medicine in humans its focus is usually on critical organs such as the brain, central nervous system, or heart, which if critically damaged or wounded do not regenerate and at very best repair with scarring that highly limits functional recovery (Bely and Nyberg, 2010; Erickson and Echeverri, 2018; Wells and Watt, 2018). Mammals and birds are largely incapable of larger organs/limb regeneration (Bely and Nyberg, 2010; Erickson and Echeverri, 2018). However, across the animal kingdom, there is high variability in a species ability to regenerate lost body parts (Bely and Nyberg, 2010; Erickson and Echeverri, 2018).

In evolutionary terms, the ability to regenerate all body parts is common to metazoan lineages and some of lophotrochozoan and deuterostomes (Bely and Nyberg, 2010). Platyhelminths and tunicates can regenerate from small body fragments (Bely and Nyberg, 2010; Gehrke and Srivastava, 2016). A number of lizards can regenerate their tail but not limbs (Alibardi and Toni, 2005; Bely and Nyberg, 2010), while amphibians like salamanders can regenerate their tails, limbs, and jaws (Alibardi and Toni, 2005; Bely and Nyberg, 2010; Erickson and Echeverri, 2018; Yin et al., 2015). The reasons why some animals are capable of regenerating while others not, combined with an explosion in research on stem cells; cells capable of differentiating into any tissue, has led to a renaissance in research in regenerative medicine.

Although they represent one of the most abundant phyla, little research has been done in bivalves concerning regenerative pathways (Bely and Nyberg, 2010; Rinkevich, 2011). Bivalves including oysters do appear to be very capable of repair and regenerate at least shell and shell and mantle (Acosta-Salmon and Southgate, 2006; Huning et al., 2016; Mamangkey and Southgate, 2009; Takahashi et al., 2012). Although there is no evidence that regeneration of the whole body can occur from simple small fragments (Bely and Nyberg, 2010). Working

with vertebrate research models requires to follow the policy on institutional care and use of laboratory animals (e.g., Institutional Animal Care and Use Committee, IACUC in the USA; Directive, 2010/63/EU of the European Parliament). Given the availability and ethical use of invertebrates over vertebrates such as bivalves, we would argue these represent not only a hugely underdeveloped phylum for this research but also a less restricted phylum for animal experimentation since with the exception of cephalopods in Europe, no IACUC is yet required. It is also worth noting that although not studied directly for regenerative capacity the unique qualities of some marine bivalve products, most notably from the mussel, have inspired polymers that are being trialed for potential uses in regenerative studies in mammals (Kaushik et al., 2015). Mussels secrete a sticky glue that allows them to stick to rocks and solid surfaces (Kaushik et al., 2015). This protein glue, made up of proteins called mussel adhesive proteins rapidly hardens into a water-resistant adhesive (Kaushik et al., 2015). Products based upon mussel adhesive proteins have been tested for diverse applications such as tissue glue, a suture free wound closure glue (Mehdizadeh et al., 2012), which may reduce tissue scarring (Jeon et al., 2017), as well as structurally inspired microspheres, nanofiber and 3D printed matrices that can be developed as drug delivery systems for bone repair (Wei et al., 2018b), wound healing (Kim et al., 2017; Wang et al., 2016b), and cancer drug delivery (Ma et al., 2016). Similarly, nacre (mother of pearl) is being tested as a biodegradable material for bone grafting, bone scaffolding, and surgical screws (Gerhard et al., 2017; Libouban et al., 2016). Powdered extract nacre has also been shown to stimulate mammalian fibroblast migration (Lopez et al., 2000). Since bivalves represent approximately 23% of all the named marine organisms, there is undoubtedly a considerable potential for novel marine or marine-inspired products to form the basis of therapies that translate into human medicine.

Since regeneration in bivalves is highly likely to share similarities with other species, it is possible to learn about this potential, from looking at these common pathways shared in animal regeneration (Agata et al., 2007; Bely and Nyberg, 2010; Erickson and Echeverri, 2018; Wells and Watt, 2018). Moreover, with advances in genome sequencing and RNA-Seq technology, it is likely that species-specific and regeneration-specific gene expression pathways will only become easier to study. Unsurprisingly, when comparing events occurring in tissue regeneration, there is considerable tissue remodeling associated with an early immune response (Kimura et al., 2003; Li et al., 2016a; Ramírez-Gómez et al., 2008); growth factor up-regulation and signaling e.g. fibroblast growth factor (FGF) and Wnt (Lengfeld et al., 2009; Lin and Slack, 2008), and the production of matrix metalloproteinases (Quinones et al., 2002; Yokoyama, 2008), along with re-epithelialization (Rousselle et al., 2018; Takeo et al., 2015). The Wnt signaling pathways, in particular, appear critical for early blastema (the regenerative seed) development (Lengfeld et al., 2009; Lin and Slack, 2008). Searching the recently published genomic data from *C. gigas*, for example, indicates that homologs for all these pathways are very likely to be present in this and other related bivalve species. Interestingly, the cloned *C. gigas* epithelial growth factor receptor transfected into mouse myoblast cells was able to stimulate proliferation showing a remarkable level of conservation between molluscan and mammalian growth factor signaling systems (Sun and Irvine, 2014). Additionally, innervation of the regenerating tissue also appears a relatively common early step driving the process (Kumar et al., 2007; Monaghan et al., 2009). One caveat that must be noted from these studies is that many, if not all, of these events, are also shared by wound healing processes in mammals that do not exhibit regeneration (Fathke et al., 2006; Houschyar et al., 2015). An important question, therefore, is what distinguishes wound healing (or even scar-free wound healing) from regeneration? One answer may lie in the presence, numbers, and location of different types of stem cells. Regeneration is initiated by the formation of a blastema (Satoh et al., 2015; Stocum, 2017). This blastema can be formed of adult stem cells, de-differentiated cells of mature tissue (vertebrate limb

regeneration; repair in jellyfish), or by a combination of both (Satoh et al., 2015; Stocum, 2017). One factor that appears particularly important in this trans-differentiation process and achieving stem-like biology is the P-Element induced wimpy testis protein (Piwi) and its homolog Cnivi (van Wolfswinkel, 2014).

Ever since the cloning of the first mammal “Dolly the sheep” (Gurdon, 2017) there has been a huge interest and development in stem cell research for regenerative medicine. The “stem cells” are self-replicating, undifferentiated master cells that are capable of differentiating and producing daughter cells that make up every different cellular and tissue type in the body (Rognoni and Watt, 2018). One focus has been on embryonic stem cells, but these do not account for regenerative capacity once an animal is developed and born. Hence there has been interest in the stem cell populations that may exist in juveniles and adults (adult stem cells), that allow for regeneration of tissue after wounding. A number of adult stem cell populations, although relatively rare, have been found in different tissue in mammals, with most demonstrating a more limited ability to produce cells along certain lineages such as the hematopoietic stem cells (Kiel and Morrison, 2008; Rognoni and Watt, 2018). These mammalian adult ‘stem cells’ do not exhibit true stem cell totipotency; i.e., the ability to differentiate into all cell types of the ectoderm, mesoderm, and endoderm embryonic germ layers. These adult stem/progenitor cell populations appear to have more of a role in tissue homeostasis and minor repairs rather than large-scale regenerative capacity (Abnave and Ghigo, 2018; Rognoni and Watt, 2018). These hurdles have not stopped researchers trying to isolate and expand these cell types so that they can be used in larger quantities to test for regenerative ability (Abnave and Ghigo, 2018; Rognoni and Watt, 2018).

Although the presence and activity of adult stem cells in bivalves has not been investigated in depth, many marine invertebrates, such as sponges are known to contain relatively large pools of adult stem cells, that are involved in their ability to regenerate through their lifetime (Bode et al., 2006; Rinkevich, 2011; Sun et al., 2007). Since they also represent some of the longest-lived animals, we believe represent a vast area of untapped stem cell research.

One of the limitations for studying stem cells outside of mammalian biology is the ability to identifying stem cells by either transcription factor or cell surface markers. Mammalian researchers have access to a substantial increasing resource of well-characterized specific antibodies that allow identification, isolation, and characterization (both in terms of stem cells and their lineages) of stem cells. At this moment in time, more resources are required to identify both the conserved proteins and antibodies that cross-react with these in bivalves to allow identification and isolation of these stem cell populations. Recent data does, however, allow a degree of hope for this, as there appears a degree of conservation between mammalian and at least oyster adult stem cells. *C. gigas* germline cells exhibit intense alkaline phosphatase activity, the transcription factor Sox2 and a cytoplasmic marker for germline tissue Vasa (Cavelier et al., 2017). *C. gigas* adult hemocytes were recently shown to be derived from Sox2 positive stem cells found in an irregularly folded structure of the gill epithelium (Jemaà et al., 2014). Moreover, recent evidence also indicates roles for the conserved hematopoietic transcription factors Tal-1/Scl (Stem Cell Leukemia), GATA3, C-Myb, and c-kit (Song et al., 2016) in this hemocyte generation. Tal-1/Scl, GATA3, C-Myb, and c-kit are all transcription factors associated with leukocyte development in mammals (Labastie et al., 1998).

Another experimental approach to generate and investigate stem cells, and now routinely used in mammalian systems, but not yet bivalves, is the reprogramming of adult cells into induced pluripotent stem cells iPSc (Di Baldassarre et al., 2018; Rinkevich, 2011). Here adult cells are forced to either permanently or transiently express a combination of transcription factors that dedifferentiate the cells back to ones that closely resemble embryonic stem cells. Interestingly, one of the factors commonly used to induce iPSc is Sox2 (along with others

such as Oct4, KLF4, cMyc and Nanog) (Di Baldassarre et al., 2018). To our knowledge, this has yet to be reported on in marine invertebrates, so it is not known whether these exact combinations will induce iPSc, but the approach using these transcription factors or similar homologs, offers a considerable potential to understand the biology and regenerative capacity of marine bivalves. With current technology it should be feasible as molluscan cells can be transfected with plasmid DNA and with the recent advances in genomic sequencing, species-specific homologs can be identified and cloned if necessary. One limitation to note does remain, in the lack of ability to grow long-term cell line cultures from marine bivalves. Although cells can be isolated and can be maintained alive for extended periods, they stop dividing and become quiescent relatively quickly (Rinkevich, 2011). As yet only one molluscan cell line Bge exists from the land snail (Yoshino et al., 2013), so advances also need to be made here to allow the long-term culture of any derived cells. It is possible that understanding multipotency of cells and the ability to culture them may go hand in hand to potentiate both fields. Understanding, cell growth and differentiation in bivalves, will not only help understand processes of regeneration that may be applied to species such as humans that have poor regenerative capacity but ultimately inform the physiology and biology of bivalves themselves greatly.

2.6. Bivalve transmissible neoplasia (BTN)

Disseminated neoplasia, or hemic neoplasia, is a leukemia-like proliferation of cells (presumably of hemocyte origin) in the hemolymph of bivalves that eventually disseminates through the tissues of the organism, leading to death, and it has been reported in at least 15 different bivalve species (Barber, 2004; Carballal et al., 2015). The disease can occur at stable enzootic levels, but outbreaks have been reported in multiple species, including soft-shell clams (*Mya arenaria*), in which massive mortality has been observed (Muttray et al., 2012). In at least four affected species (*M. arenaria*, *Mytilus trossulus*, *Cerastoderma edule*, and *Politiapes aureus*), the disease has been shown to be a transmissible clonal cancer—a lineage of cancer in which the cells themselves jump from one animal to the next as a natural allograft (Metzger et al., 2015, 2016). Transmissible cancer had only been observed in the wild previously in Tasmanian devils (Pearse and Swift, 2006) and dogs (Murgia et al., 2006; Rebbeck et al., 2009). Transmissible cancer models can be used to investigate some oncogenic mechanisms, including cancer evolution, metastasis, and the role of transposable elements in oncogenesis, as well as mechanisms of resistance of the host itself to cancer engraftment.

Transmissible cancer cells have been used to investigate classical oncogenic proteins such as p53 and myc. The presence of a LINE1 insertion immediately upstream of c-myc in all known cases of the canine transmissible venereal tumor (CTVT) provides evidence that transposons can generate oncogenic mutations that are selected for in cancer (Katzir et al., 1985, 1987). The role of many other well-known cancer proteins (including p53 and telomerase) have been studied in CTVT (Murchison, 2008) and devil facial tumor disease (DFTD) (Deakin and Belov, 2012). In bivalves, the clam neoplasia model has been used to investigate the conserved role of p53 in invertebrate cancers, showing that mortalin sequesters p53 in the cytoplasm of neoplastic cells (Power et al., 2006; Walker et al., 2011). These models may also allow us to identify oncogenes or tumor suppressors that are either conserved in human cancers or are novel players in independent pathways.

Bivalve transmissible neoplasia (BTN) offers a novel model to examine the process of cancer evolution. These cancers represent extreme cases of metastasis (Lazebnik and Parris, 2015) in which cancers have acquired the ability to outlive their hosts and continue to evolve as they repeatedly engraft into new hosts. Their evolution, therefore, represents exaggerated versions of adaptations that occur in common primary tumors as well as adaptations to unique challenges (Greaves and Maley, 2012; Nowell, 1976; Ujvari et al., 2016). While investigation of a single

Table 1

Published molluscan genome assemblies. The access to the latest sequences technologies together with the continuous decrease in the price is resulting in an increasing number of genomes publicly available. Modified from (Takeuchi, 2017).

Species	Common name	Genome size (Mb)	Total scaffold length (Mb)	Scaffolds	Scaffold N50 (Kb)	Reference
<i>Crassostrea virginica</i>	Eastern oyster	578–675	669	10 + 1 ^a	1900	(https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Crassostrea_virginica/100/)
<i>Saccostrea glomerata</i>	Sydney Rock oyster	788.1	746.5	10,101	804.2	Powell et al. (2018)
<i>Bathymodiolus platifrons</i>	Deep sea mussel	1630	1660	65,664	343.4	Sun et al. (2017)
<i>Modiolus philippinarum</i>	Mussel	2210	2630	74,575	100.2	Sun et al. (2017)
<i>Patinopecten yessoensis</i>	Scallop	1440	987.6	82,731	803.6	Wang et al. (2017a)
<i>Argopecten irradians</i>	Bay scallop	990	700.3	217,310	6.8	Du et al. (2017)
<i>Ruditapes philippinarum</i>	Manila clam	1370	2571	223,851	48.4	Mun et al. (2017)
<i>Mytilus galloprovincialis</i>	Mediterranean mussel	1600	1599	1,746,447	2.6	Murgarella et al. (2016b)
<i>Pinctada fucata martensii</i>	Pearl oyster	–	990.6	8621	324.3	Du et al. (2017)
<i>Pinctada fucata</i>	Pearl oyster	1150	815.3	29,306	167.3	(Takeuchi et al., 2012; Takeuchi et al., 2016)
<i>Crassostrea gigas</i>	Pacific oyster	637	558.6	11,969	401.3	Zhang et al. (2012)

^a 10 chromosomes and mitochondrial DNA.

cancer lineage may give us some insight into possible effects of retrotransposons (as in the case of LINE-c-myc in CTVT), the finding of multiple independent lineages of BTN increases our ability to understand the conserved processes involved in the multiple steps from healthy cell to neoplasia, to metastasis, to transmission to another organism, and finally to a widespread successful transmissible cancer lineage.

The transmissible cancer lineage in soft-shell clams is marked by a massive amplification of a retrotransposon called *Steamer*, from about 2 to 10 copies per haploid genome in healthy animals to > 150 in neoplastic cells (Arriagada et al., 2014). By definition, retrotransposon activity induces chromosomal mutations, but the extent of their role in cancer mutations and the mechanisms and pathways that they affect are not fully known. Development of genomic resources will allow us to identify the locations of these neoplasia-associated transposon insertion sites in the soft-shell clam neoplasia and will allow for testing whether different transposable elements are amplified in the other BTN lineages. Additionally, in soft-shell clams, BrdU injection has been reported to induce new transmissible cancers experimentally (Oprandy and Chang, 1983; Taraska and Anne Bottger, 2013), and this may provide many new oncogenic events for study. The soft-shell clam is, therefore, a model organism that would allow for direct testing of the role of transposable elements in oncogenesis and transmission.

In addition to the standard hallmarks of cancer (Hanahan and Weinberg, 2000), transmissible cancer cells also evade the host immune system, which could otherwise identify them as coming from a foreign individual. Normal cancers have mutations that can form neoantigens, so they too must escape immune recognition and destruction, and the extreme phenotype of transmissible cancers allows for investigation of the mechanisms by which hosts allow or reject cancer cells. Bivalves do not possess an MHC-based histocompatibility system, but BTN does not appear to cross species barriers readily. Multiple experiments have shown that disseminated neoplasia can be transplanted from one individual to another within the same species (McLaughlin et al., 1992; Taraska and Anne Bottger, 2013; Weinberg et al., 1997), but cross-species transplantation has been unsuccessful. In three of the four bivalve species analyzed, each is affected by one or more cancer independent lineages derived from within the same species (Metzger et al., 2015, 2016). In the one exception (*Politiitapes aureus*) cancer appears to be a transmissible lineage derived from a closely related species from the same family (*Venerupis corrugata*), which, interestingly, may itself have evolved resistance to cancer. These observations suggest that there is some mechanism of resistance to cancer lineages in bivalves, which may be a type of self/non-self-recognition system that clears cancer or some host factor required for cancer cell engraftment which can be mutated in the host, or some other unknown pathway. These contagious cancers are essentially acting as massive natural

experiments in which outbred organisms are being challenged with a clonal cancer. Understanding the mechanisms of resistance that have been selected for may have profound impacts on human health.

Also, primary cell cultures from marine bivalves have been used in basic research for decades; still, the attempts to establish a cell line have been unsuccessful (Rinkevich, 2011; Yoshino et al., 2013). Most efforts to generate cell lines from bivalves were based on explants transferred into culture media for spontaneous immortalization derived from stochastic genetic events. Most invertebrate cells stop dividing 24–72 h after isolation (Rinkevich, 2011). BTN may provide a useful immortalized cell line itself; understanding the mechanisms behind BTN's survival and continued growth may provide the clues for immortalizing cells from marine bivalves. The development of bivalve cell lines would provide a simple model for studying numerous aspects of complex biological systems.

While the study on the transmissible nature of bivalve disseminated neoplasia is new, and many questions remain to be answered, the research on this cancer model has the potential to yield new insights into human cancer development and evolution as well as mechanisms by which humans can block cancer engraftment and growth.

3. Resources and tools

Over the last decade, there has been an enormous sequencing effort in species of interest in the aquatic environment; early advances focused on finfish with zebrafish (*Danio rerio*) becoming a model organism for numerous human diseases and conditions (Sarasamma et al., 2017). Bivalve species are also benefiting from the latest technologies for genome sequencing (Abdelrahman et al., 2017) (Table 1). *P. fucata* and *C. gigas* genomes encode for 23,257 and 28,027 predicted genes respectively (Takeuchi et al., 2012; Zhang et al., 2012). Initial insights into the Mediterranean mussel (*M. galloprovincialis*) genome indicate a gene enrichment of gene ontology categories related to multixenobiotic resistance, glutamate biosynthetic process, and the maintenance of ciliary structures (Murgarella et al., 2016a). The number of predicted genes (39,493 protein-coding genes, 4230 non-coding) for eastern oyster genome (684 Mb; C.virginica-3.0; GCF.002022765.2) is much larger than for *P. fucata* and *C. gigas* genomes (Gómez-Chiarri et al., 2015b). In addition to the genome, the eastern oyster research consortium is now focusing in comparing *C. virginica* genomes from multiple sites in the USA. Next-generation sequencing offers an unique opportunity not only to understand the general process of innate defenses in invertebrates but also as a essential tool for comparative immunology of vertebrates and invertebrates (Schultz and Adema, 2017).

Significant economic losses derived from infectious diseases in the aquaculture industry, as well as interest in the development of selected strains with faster growth, drove pioneering efforts in the 1990s for

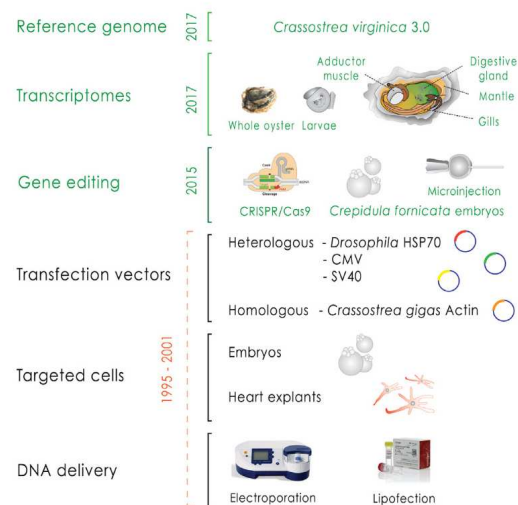


Fig. 5. Overview of available resources towards a genetically tractable system in bivalves. Most attempts to introducing genetic material in oysters date from two decades ago and focused on the Pacific oyster. With the genomes of commercial and non-commercial bivalves being sequenced, the minimum tools and resources for building a genetically tractable system are already available. Indeed, CRISPR/Cas9 has been recently achieved in the gastropod *Crepidula fornicata* (common slipper shell).

developing systems for delivering genetic material into bivalves of commercial interest (Boulo et al., 1996; Buchanan, 1999; Buchanan et al., 2001; Cadoret et al., 1999; Powers et al., 1994–1997). These efforts, however, were hindered by the low efficiency of the transfection methods (1% for embryos, and not reported for primary cultures), as well as the lack of information on the molecular basis for the traits of interest. Also, the lack of transcriptomes and a draft genome precluded the placement of the genes in context to define promoter regions to build homologous transfection vectors. Therefore, in following decades, the oyster research community focused efforts on the development of genomic tools needed to identify traits of commercial, biological, and ecological interest (Abdelrahman et al., 2017). Taking full advantage of the oyster genome and transcriptomes and other approaches (Powers et al., 1995; Tsai et al., 1997) requires to build and validate the genetic toolbox for making the oyster a genetically tractable system for both basic and applied research (Fig. 5). Along the same lines, immortalized cell lines provide the simplest model for studying numerous aspects of complex biological systems. Without cell lines, the analysis of cell biology is limited by the availability and lifetime of cells and primary cultures, the access to tissue donors or animals, and the genetic background variability from sample to sample. Clones can be grown indefinitely in culture, providing unlimited and standardized material that allows for reproducibility on genetically identical cells and in tightly controlled experimental conditions and datasets that are comparable across laboratories. Growing cells *in vitro* is also a cost-effective way to produce proteins of interest similar to those found in multicellular organisms. Despite decades of extensive research efforts by multiple laboratories around the world and the urgent need to create new cell lines from recalcitrant species in phyla underrepresented or not represented, there is not yet a single cell line available from marine invertebrates (reviewed in (Rinkevich, 2005; Yoshino et al., 2013)).

Primary cell cultures from marine bivalves have been used in basic research for decades; still, the attempts to establish a cell line have been unsuccessful (Rinkevich, 2011; Yoshino et al., 2013). Most attempts to

generate cell lines from bivalves were based on explants transferred into culture media for spontaneous immortalization derived from stochastic genetic events. However, invertebrate cells stop dividing 24–72 h after isolation (Rinkevich, 2011) although primary cell cultures can remain in culture for longer periods of time with no proliferation (2.5–22 mo (Chen and Wang, 1999; Daugavet and Blinova, 2015; Odintsova et al., 2010)). Targeted cells for deriving cell lines are variable (Table 1) with the heart being one of the first choices because it is easily accessible after cutting the pericardium which isolates it from the pallial liquid content—hence, reducing the risk of microbial contamination when transferred to culture medium. Using the mantle, a single individual can generate a large number of explants (Daugavet and Blinova, 2015), and there is some evidence of cell propagation (Fang et al., 2008). Cell lines from hemocytes are especially appealing since they are the first line of defense against pathogens. Hemocytes are also involved in transport and wound repair, and several protozoan parasites specifically residing in this cell type (e.g., *Perkinsus* spp., *Bonamia* spp. (Fernández et al., 2014)), and they are also used for testing for environmental contaminants (reviewed in (Barrick et al., 2018)). Also crucial, the knowledge of the bivalve innate immune system is still lagging behind vertebrates and arthropods even when dealing with similar variety of pathogens (Cunningham and Robledo, 2015). Over decades, research in numerous labs has resulted in an in-depth knowledge of the biology of *Perkinsus* spp. (Fernández et al., 2014). This genetically tractable protozoan parasite is becoming a model system for protozoan parasites in bivalves (Cold et al., 2016; Fernández-Robledo et al., 2008) and it has potential for medical applications (Cold et al., 2017; Wijayalath et al., 2014). Mechanistic studies at the cellular level would also benefit from cell lines and a genetically tractable system.

Primary cell cultures, derived from explants or circulating cells transferred into a culture medium, lead to cell lines when they lose their limited proliferative capability and start replicating. Interfering with mechanisms that trigger cellular senescence is a strategy for cell immortalization. One involves the critical shortening of the telomeres by interfering with the end-replication during chromosome replication (Baker et al., 2017; Campisi, 2005; Loaiza and Demaria, 2016). The second involves cell cycle arrest, which is controlled by two tumor suppressor pathways (Ahuja et al., 2005; Lundberg et al., 2000; Ozer, 2000). The over-expression of hTERT into the cells has been used to immortalize cells from multiple vertebrate species (He et al., 2016; Veitonmaki et al., 2003; Wang et al., 2017b), but to our knowledge, it has never been attempted in bivalves. Interestingly, the telomeric sequence (TTAGGG)_n target by the enzyme is present in numerous bivalves including *Crassostrea* spp. (Cross et al., 2005; Guo and Allen Jr, 1997; Huang et al., 2007; Koroleva et al., 2015; Perez-Garcia et al., 2010; Plohl et al., 2002). Simian Virus 40 T antigen (SV40 T) is a simple and reliable agent for the immortalization of many different cell types by interfering with the inhibition of the p53 and Rb-family of tumor suppressor. The small T antigen's action on the pp2A phosphatase p53 is a transcription factor and tumor suppressor involved in cell cycle arrest, apoptosis, or senescence in damaged cells; it is often mutated in human cancer (Chen and Wang, 1999), and it has been associated with cancers in bivalves (Barber, 2004; Farcy et al., 2008; Holbrook et al., 2009; Walker et al., 2011).

The pioneering efforts in the 1990's to develop systems to deliver genetic material into oyster primary cell cultures and embryos included lipofection, electroporation, and microinjection (Table 3, Fig. 5). Lipofection of *C. gigas* heart primary cell cultures with plasmids coding for the luciferase gene under transcriptional control of several heterologous promoters (*Drosophila hsp70* promoter, cytomegalovirus, CMV, and SV40 early promoters) resulted in enzymatic activity in the transfectants (Boulo et al., 1996). The *Drosophila hsp70* was the stronger promoter, and the expression was inducible in a similar way to that observed in *Drosophila* (Boulo et al., 1996). A promoter including a 1670 bp fragment upstream from the actin ORF from *C. gigas* was also validated (Cadoret et al., 1999). Transfection by electroporation of 3-h-

Table 2

Representative primary cell cultures derived from bivalves. DG: Digestive gland.

Species	Tissue origin	Base medium	Year	Reference
<i>Mytilus edulis</i>	Hemocytes	L-15	2018	Barrick et al. (2018)
<i>M. edulis</i>	Mantle edge	L-15	2015	Daugavet and Blinova (2015)
<i>Mytilus trossulus</i>	Embryos	L-15	2012	Odintsova et al. (2010)
<i>Dreissena polymorpha</i>	Hemocytes, heart, DG	L-15	2011	Parolini et al. (2011)
<i>Mya arenaria</i>	Disseminated Neoplasia	Eagle's MEM	2009	Walker et al. (2009)
<i>Crassostrea virginica</i>	Hemocytes	ASW/DME:HAMS F12	2009	Alavi et al. (2009)
<i>Dreissena polymorpha</i>	DG, gill, gonad	RPMI 1640, L-15	2009	Quinn et al. (2009)
<i>Crassostrea gigas</i>	Heart, mantle, DG	L-15	1999	Chen and Wang (1999)
<i>Pinctada fucata</i>	Mantle edge	Balanced salt solution	1998	Awaji and Suzuki (1998)
<i>C. gigas</i>	Heart	L-15	1996	Boulo et al. (1996)
<i>Ostrea edulis</i>	Heart	L-15	1995	Renault et al. (1995)
<i>C. gigas</i>	Heart	L-15	1993	Wen et al. (1993)

postfertilization *C. virginica* embryos was achieved using the commercial plasmid pS65T-C1, which carries the genes for *neo*^r (a bacterial gene that confers Neomycin resistance) and *rsGFP* (red-shifted Green Fluorescence Protein) under the SV40 and the immediate-early promoter from human CMV (Buchanan et al., 2001). PCR and expression of green fluorescent protein were observed in transfected embryos (20% viability after transfection) (Buchanan et al., 2001). Interestingly, hemocytes expressed the same plasmid when adult oysters were injected with the plasmid mixed with dendrimers (Buchanan et al., 2001). Recent developments in genome editing are providing new opportunities in basic and applied biological research. Recently, the genome of the gastropod *Crepidula fornicata* (the Atlantic slipper snail) was edited by microinjecting CRISPR/Cas9 mRNA, donor DNA, and sgRNA in embryos; the targeted gene was endogenous β -catenin, and the knock-in was the *mCherry* coding sequence (Perry and Henry, 2015). From a total of 280 embryos injected, a total of 30 (11%) had visible *mCherry* fluorescence.

Ultimately, the generated cell lines, as well as the optimization of genetic tools and methodologies for manipulating the genome would make oysters a genetically tractable and appealing experimental system (Fig. 6). Achieving this goal would provide a critical resource for addressing multiple aspects of the oyster physiology, genetics, toxicology, virus infection dynamics, and host-parasite interactions. New cell lines could contribute to the prediction that “an ever-expanding breadth of model systems may be a hallmark of future cell biology” (Goldstein and King, 2016). (See Table 2)

4. Conclusions

The phylum Mollusca comprises around 200,000 extant species inhabiting many habitats. The bivalve diversity in size, anatomical structure, behavior, and habitat have fascinated scientists for centuries. Bivalves also are an important food source for humans. Here, we highlight several aspects of the bivalve biology that have the potential to impact human health. The last decade has seen the generation of draft genomes for most of the species with heavy aquaculture implantation and the field is now navigating the post-genomic era. Bivalve genomes are exceptionally rich in predicted genes, arguably derived from having evolved in an aquatic environment subjected to multiple environmental changes. In the review, we focus on aspects that have

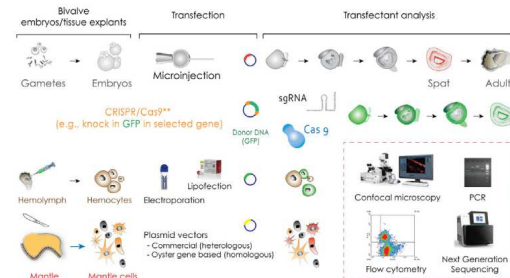


Fig. 6. Genetic material delivery approaches in bivalves. In the absence of cell lines of marine invertebrates, embryos are the only stage with proliferating cells necessary for testing knocking in genes using CRISPR/Cas9 approaches. Available all year round, hemocytes and mantle explants are useful to test plasmid constructs carrying fluorescence tags. For delivering the genetic material, microinjection, electroporation, and lipofection are techniques that have been successfully used in the past. Confocal microscopy, flow cytometry, enzymatic activity, and PCR/sequencing can be used to characterize the transfectants.

already received the attention of the scientific community; still, we believe that the biology and adaptation of these organisms hide clues to undercover mechanisms and strategies that have a direct application to human health. Bivalves as a model system is already a reality; however, to be able to attract students and junior scientists and engage funding agencies and foundations, we think that like in other well-established model organisms, developing a genetically tractable model system and cell lines are two much-needed tools.

Declarations of interest

None.

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Table 3Vectors and transfection methods used with bivalves. *Based on *Crassostrea gigas* actin gene.

Organism	Target	Vector	Transfection	Reference
<i>Crepidula fornicata</i> (Gastropoda)	Embryos	Cas9 RNA, sgRNA, donor DNA	Microinjection	Perry and Henry (2015)
<i>Crassostrea virginica</i>	Adults	pS65T-C1	Dendrimer	Buchanan et al. (2001)
<i>C. virginica</i>	Embryos	pS65T-C1	Electroporation	Buchanan et al. (2001)
<i>Crassostrea gigas</i>	Heart explants	pGL3-basic*	Lipofection	Cadoret et al. (1999)
<i>C. gigas</i>	Heart explants	pDr-luc, pC-luc, pCMV-L, pSV-luc	Lipofection	Boulo et al. (1996)

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