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Immune Responses of Bivalves to Environmental Pollution and Abiotic Stress

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ABSTRACT

Bivalves are important for ecosystems providing food security for humans. Unfortunately, many bivalve fisheries are declining due to overexploitation, and attempts to culture important bivalve species are underway. However, the growth and survival of cultured bivalves are constantly challenged by various biotic and abiotic factors affecting their immune system. Hence, this paper reviews the current information about the immune response of bivalves to environmental pollution and abiotic stress. The review on environmental pollution was focused on heavy metals and harmful algae, whereas bivalves' responses to abiotic stress were focused on temperature and salinity stress. In this review, relevant scientific articles were examined to gain insights into the immune system of bivalves toward pollutants and abiotic stress. It revealed that more studies have been conducted on the effect of heavy metals and harmful algal blooms on the immune response of bivalves. In contrast, the information on temperature and salinity stress is scarce. Different bivalve species differed in their immune responses to these stressors. Total haemocyte count (THC), phagocytosis, apoptosis, lysozyme, and reactive oxygen species (ROS) were some of the immune factors activated during exposure to heavy metals, harmful algal blooms, and their biotoxins, as well as salinity and temperature stress. These factors play important roles in protecting bivalves from pollution and stress. Healthy bivalves showed an increase in THC, lysozyme, and phagocytosis and a decline in apoptosis and ROS. For successful conservation and aquaculture of bivalves, it is important to ensure that the habitats are not polluted by heavy metals or biotoxins. Although few studies were concerned about the effect of temperature and salinity stress on bivalve immune responses, they deserve attention as different bivalve species have different temperature and salinity preferences, given global warming and acidification.

INTRODUCTION

Bivalves (oysters, clams, mussels, scallops and cockles) are sedentary filter feeders that can concentrate bacteria, viruses, pesticides, industrial pollutants, toxic

metals, and petroleum derivatives (**Song *et al.*, 2010**). They are essential bio-monitoring pollutant indicators in aquatic ecosystems and suitable species for studying the impacts of environmental contaminants (**Song *et al.*, 2010**). These animals use their gills to capture particles from the water and select them according to size and density (**Gosling, 2003**). Ingested particles are processed in the gut and the waste is discharged as faeces, whereas rejected particles are expelled as pseudofaeces (**Beninger *et al.*, 1999; Alexander *et al.*, 2008**). Due to this biological process, the immunological capacities of bivalves are often at risk of exposure to a wide range of environmental consequences (**Renault, 2015**). Some bivalve species that are sources of seafood for humans have received more attention in recent years, especially for preventing stress from pollution and the weakening of the immune system. These are important considerations for successful bivalve aquaculture (**Griffith *et al.*, 2019)**.

The role of the bivalve immune system is to provide an effective defence against various invading pathogens. The cells forming the first line of resistance are haemocytes (**Cao *et al.*, 2007**) that are present in the open haemolymphatic circulatory system (**Renault, 2015**). These immune cells circulate throughout the haemolymph to protect the organism (**Cheng, 1996**) by serving as phagocytes, identifying, engulfing, and destroying pathogens and foreign particles (**Wootton *et al.*, 2003; De Vico & Carella, 2012**). They also secrete humoral and cytotoxic factors by producing reactive oxygen intermediates (**Pipe *et al.*, 1997**). In marine bivalves, there are two main haemocyte types; namely, granulocytes, which contain many granules within the cytoplasm and have the highest phagocytic activity (**Carballal *et al.*, 1997**), and agranulocytes, also known as hyalinocytes, which do not possess granules (**Hine, 1999**). The two main ways these haemocytes get rid of non-self substances and dead cells are through phagocytosis and encapsulation **(**Fig. 1) (**Takahashi & Muroga, 2008**). The internalization mechanism of phagocytosis is triggered by the recognition and binding of non-self material by receptors produced at the surface of haemocytes. Encapsulation is a typical immune defence response in invertebrates when the foreign item is too big to be phagocytised. Haemocytes form a capsule around the foreign organism and produce cytotoxic chemicals to eradicate the invader (**Song *et al.*, 2010**).

The first step in initiating an immune response is the recognition of the exogenous factor. It plays a critical role in the ability of the immune system to distinguish between non-self (not inherent in the body) and self-substances (intrinsic to the body). The initiation of immune response takes place when specialized, soluble, or cell-bound pattern recognition receptors (PRRs) detect and bind to the principal targets, known as pathogen-associated molecular patterns (PAMPs) (**Janeway & Medzhitov, 2002; Medzhitov & Janeway, 2002**). PAMPs, such as LPS or peptidoglycan (PGN) in bacterial cell walls and -1,3-glucan in fungal cell walls, are ubiquitous in microbes but rare or non-existent in host animals (**Medzhitov & Janeway, 2000**). Invertebrates acquire a complex system of PRRs and rely solely on innate immunity. There are seven types of PRRs found in

bivalves, mainly peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBPs), C-type lectins, galectins, thioester-containing proteins (TEPs), scavenger receptors (SRs), and Toll-like receptors (TLRs). These PRRs will initiate different signalling pathways to stimulate the systemic immune response and create response effectors when bivalves are challenged by the pathogen (**Song *et al.*, 2010**). The immunity signalling pathways include Toll-like receptors (TLRs), Janus kinase/signal transducer and activator of transcription (JAK-STAT), mitogen-activated protein kinase (MAPK), and NF-κB pathways. The immune effectors produced by these signalling pathways are antimicrobial peptides (AMPs), cytokines, complement components, antioxidant enzymes, and acute phase proteins. These immune effectors are used as executors to incapacitate and eradicate intruders through a broad array of defensive responses (**Song *et al.*, 2010**).

Immune responses of bivalves are greatly affected by both environmental pollution resulted from heavy metals (**Gagnaire *et al.*, 2004; Sokolova *et al.*, 2004; Thiagarajan *et al.*, 2006; Höher *et al.*, 2013; Ahmad *et al.*, 2011**), pharmaceutical products (**Canesi *et al.*, 2007a, b; Matozzo *et al.*, 2008a, b**), pesticides (**Canty *et al.*, 2007; Gagnaire *et al.*, 2007; Luna-Acosta *et al.*, 2012; Geret *et al.*, 2013; Moreau *et al.*, 2014**), polycyclic aromatic hydrocarbons (PAHs) (**Frouin *et al.*, 2007; Bado-Nilles *et al.*, 2008; Hannam *et al.*, 2009; Hannam *et al.*, 2010a, b**), nanoparticles (**Canesi *et al.*, 2008; Canesi *et al.*, 2010a, 2010b; Canesi *et al.*, 2012; Ciacci *et al.*, 2012**), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) (**Canesi *et al.*, 2003; Liu *et al.*, 2009; Ji *et al.*, 2013**), and harmful algal blooms (HABs) (**Silva *et al.*, 2008; Hégaret *et al.*, 2011; Prado-Alvarez *et al.*, 2013; Haberkorn *et al.*, 2014; Chi *et al.*, 2017**). The abiotic stress comprises temperature (**Chen *et al.*, 2007a, b; Cherkasov *et al.*, 2007; Yu *et al.*, 2009; Mosca *et al.*, 2013; Beaudry *et al.*, 2016**), salinity (**Malagoli *et al.*, 2007; Rodrick, 2008; Gajbhiye & Khandeparker, 2017; Nadirah *et al.*, 2018; Wu *et al.*, 2020**), and pH (**Malagoli & Ottaviani, 2005; Bibby *et al.*, 2008; Green & Barnes, 2010**) beyond the threshold limits of the bivalve. A survey in literature reveals more data on the effect of heavy metals, harmful algal blooms, and their biotoxins, as well as temperature and salinity. This review aimed to fill the knowledge gaps in the impact of other stressors and identify the sub-lethal and lethal concentrations of heavy metals and harmful algal blooms and their biotoxins on the immune system of bivalves besides the threshold of these animals to variations in salinity and temperature.

A total of 112 journal articles were reviewed for this study, including 34 on heavy metals, 35 on hazardous algal blooms and associated biotoxins, 24 on temperature, and 19 on salinity. Among the five commercially important bivalve groups, the least studied group is cockles, whereas the most studied groups are oysters and mussels.

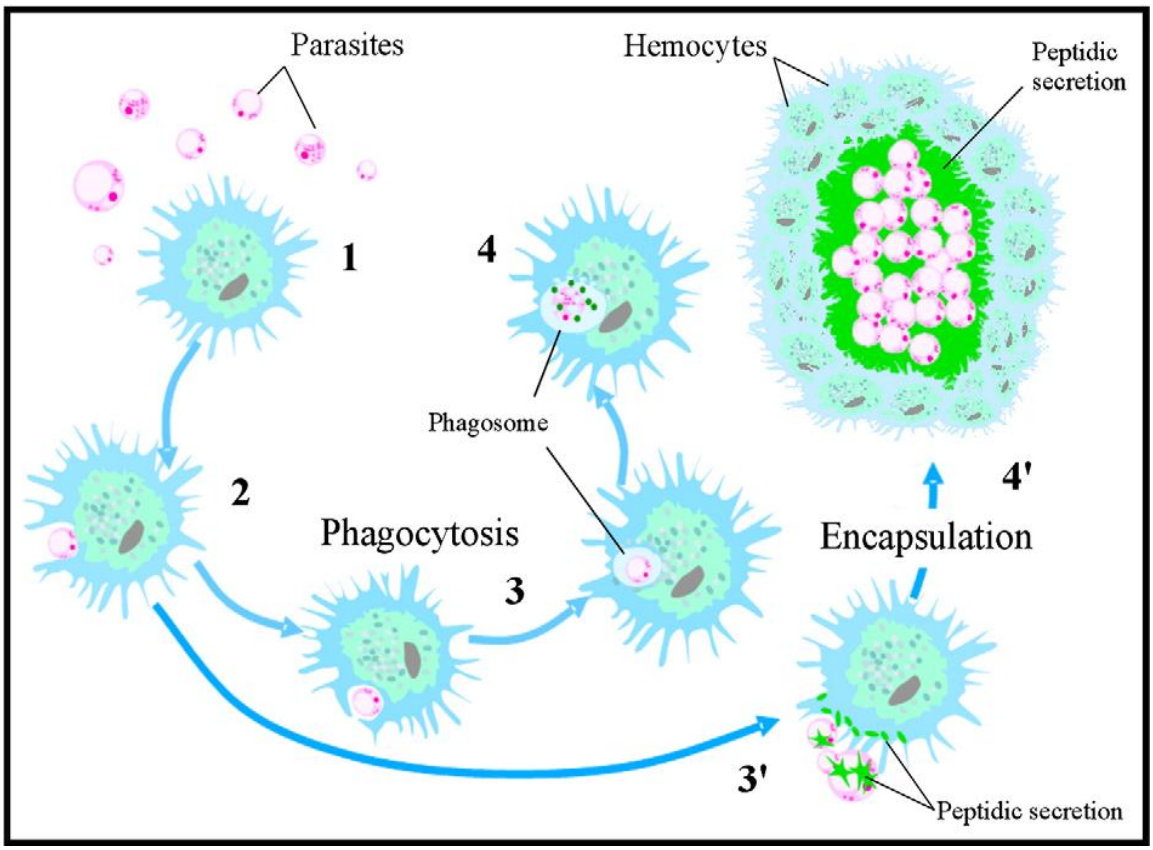

Fig. 1. Schematic presentation of putative cellular response in clam defence mechanisms upon microbial infection and parasitism **(Soudant *et al.*, 2008)**

EFFECTS OF HEAVY METALS ON BIVALVE IMMUNE SYSTEM

Pollution of estuarine areas is caused by the discharge of industrial wastes containing heavy metals. Filter-feeding species such as marine bivalves have been reported to accumulate metals (**Renault, 2015**). Thus, several aspects of their immune systems can be altered since heavy metals are known for their immunotoxicity (**Fournier *et al.*, 2001**).

As mentioned earlier, mercury is a highly toxic heavy metal to the immune system of bivalves. The strong reactivity of many mercury species toward thiol-groups and other functional groups, particularly proteins, is thought to cause most of the harmful effects of mercury (**Schwenk *et al.*, 2009**). There may be cross-reactions between functional groups and conformational alterations. Although other metals can display this sort of reactivity, mercury appears to have the most diversified effects on the immune system of all metals, making it an appealing chemical model for studying metal-immune interactions. Earlier reviews have documented some elements of these relationships (**Fournier *et al.*, 2001;**

Sauvé *et al.*, 2002; Gagnaire *et al.*, 2004; Ahmad *et al.*, 2011; Parisi *et al.*, 2021). **Gagnaire *et al.* (2004)** conducted a study to test the effects of cadmium and mercury (methylmercury and mercury chloride) on *Crassostrea gigas* haemocytes. They recorded haemocyte mortality upon mercury exposure; however, these cells were unaffected by cadmium. Mercury is well-known for its severe toxicity and bioaccumulation in various organisms, making it a significant cause of mortality in aquatic animals (**Cossa & Fichet, 1999**). In addition, the methyl group increases penetration into cells and binding to proteins, making methylmercury the deadliest form of mercury, compared to mercury chloride. Concordant views have been expressed in the study of **Parisi *et al.* (2021)** who emphasized that, methylmercury reduced the phagocytic activity and cell viability of *Mytilus galloprovincialis* haemocytes. Phagocytosis is well conserved in both invertebrates and vertebrates. It is susceptible to environmental xenobiotics in several animal species, highlighting the utility of this approach in pollution monitoring (**Wong *et al.*, 1992; Voccia *et al.*, 1994; Cooper *et al.*, 1995**). Mercury affects the intracellular Ca^{2+} content in marine mussels (**Marchi *et al.*, 2004**), a key regulator of the cytoskeleton structure and chemical dynamics that they entail for maintenance. Ca^{2+} regulates cytoskeletal alterations by influencing the activities of several contractile proteins (**Nunes & Demaurex, 2010**). It serves as a cofactor for the activation of several intracellular signalling proteins that are known to be involved in phagocytosis (**Nunes & Demaurex, 2010**). Mercury inhibits tubulin polymerization and promotes microtubule disassembly **(Pendergrass *et al.*, 1997)** by modifying tubulin SH groups, decreasing cellular F-actin content, and converting phosphorylated to non-phosphorylated forms of cofilin **(Vendrell *et al.*, 2010)**. These processes regulate actin dynamics and promote actin filament turnover **(Vendrell *et al.*, 2010)**. When phagocytic activity is diminished, the number of viable cells decreases, resulting in a weakened immune response and a decline in cell viability.

Ironically, despite mercury being the most damaging heavy metal, most of the investigations have given attention to cadmium and copper. According to **Roesijadi (1996)**, cadmium receives a great interest since it is likely to influence coastal and estuarine ecosystems, as well as being generally considered as a potent immunosuppressant in bivalves (**Auffret *et al.*, 2002; Hemdan *et al.*, 2006; Paul-Pont *et al.*, 2010b; Chandurvelan *et al.*, 2013; Ivanina *et al.*, 2014**). The oxidative damage caused by cadium can lead to cell death and reduce THC (**Ittoop *et al.*, 2009**). **Evariste *et al.* (2017)** reported that phagocytic activity of haemocytes in *Dreissena polymorpha* was reduced when exposed to different concentrations of cadmium (10^{-6}M, 10^{-5}M, 10^{-4}M, $5x10^{-4}$M, 10^{-3}M). Cadmium disrupts haemocyte phagocytosis, resulting in cytoskeleton disruption (**Evariste *et al.*, 2017**). The actin cytoskeleton has been demonstrated to change the haemocyte count in the *M. galloprovincial*s following exposure to cadmium, resulting in conspicuously rounded cells without pseudopods **(Olabarrieta *et al.*, 2001; Gómez-Mendikute & Cajaraville, 2003**). Cadmium-induced cell shape alterations were

also detected in haemocytes of *Haliotis tuberculata* (**Latire *et al.*, 2012; Ladhar-Chaabouni *et al.*, 2015**).

Regarding copper, the immunological effects of this metal appear to be more varied, as it has been documented that copper may both hinder and stimulate immune activity (**Parry & Pipe, 2004; Jing *et al.*, 2007; Foster *et al.*, 2011; Silva-Aciares *et al.*, 2013**). **Ittoop *et al.* (2009)** reported reduced total haemocyte count (THC) and proportion of granulocytes in *Crassostrea madrasensis* when exposed to three different concentrations of copper (0.1 ppm, 0.5 ppm, 1.0 ppm). Similar results were reported in the studies of **Parry and Pipe (2004**) and **Huang *et al.* (2018)** on *Mytilus edulis* and *Crassostrea rivularis*, respectively, when exposed to higher concentrations of copper. These results contradict most studies for total haemocyte count has been reported to increase by the exposure of bivalve to heavy metals (**Höher *et al.*, 2013; Haberkorn *et al.*, 2014; Shi *et al.*, 2018; Ivanina *et al.*, 2016**). According to **Donaghy *et al.* (2009)**, the increase in THC levels is due to cell multiplication. The authors hypothesized that these haemocytes are 'young' cells less prone to death, lowering the proportion of dead haemocytes in the total haemocyte population. The effects of heavy metals on immune system of bivalves are summarized in Table (1).

Table 1. Summary of immune responses of bivalve species upon exposure to different levels of heavy metals

Bivalves Species	Heavy Metal	Duration	Effect	Reference
Pacific oyster, *Crassostrea gigas*	Cadmium chloride, $CdCl_2$: $3x10^{-11}$M, $3x10^{-10}$M, $3x10^{-9}$M, $3x10^{-8}$M, $3x10^{-7}$M, $3x10^{-6}$M, $3x10^{-5}$M, $3x10^{-4}$M ;	4h 24h	(=) Haemocyte mortality	**Gagnaire *et al.* (2004)**
	Methylmercury, MeHg: $2x10^{-11}$M,$2x10^{-10}$M, $2x10^{-9}$M, $2x10^{-8}$M, $2x10^{-7}$M, $2x10^{-6}$M, $4x10^{-10}$M, $4x10^{-7}$M		Haemocyte mortalities present	
	Mercury chloride, $HgCl_2$: $2x10^{-6}$M, $2x10^{-5}$M, $2x10^{-4}$M		Haemocyte mortalities present, (↑) haemocyte aminopeptidase (All concentrations, 4h), (=) esterase, peroxidase and phagocytosis, (↓) phenoloxidase (PO)	

			activity ($2x10^{-6}$M and $2x10^{-5}$M,3h)	
	Cadmium, Cd: 10µg/L	31 days	(↑) Reactive oxygen species (ROS) and haemocyte apoptosis (pH 7.80 and 7.60), (↓) phagocytosis (pH 7.60)	**Cao *et al.* (2018)**
	Cadmium,Cd: 50µg/L	4 weeks	Hemocyte counts (↑), phagocytosis (↓) (800 µatm P_{CO2}), adhesion capacity (↓) (2000 µatm), lysozyme activity (↓) (800 µatm)	**Ivanina *et al.* (2014)**
	Copper,Cu: 50µg/L	4 weeks	Circulating haemocyte, adhesion capacity, phagocytic activity, lysozyme activity (400 µatm) (↑), cysteine proteases (=)	**Ivanina *et al.* (2016)**
	Zinc,Zn Chronic: 0.53±0.04 mg/L	10 weeks	THC (↓), non-specific esterase activity (↑) 4 weeks, (↓) 3 weeks, PO (↓) 1 week then (↑), phagocytosis (1,2,4 weeks) and ROS (1,5,10 weeks) (↑)	**Mottin *et al.* (2012)**
	Acute: 10.2±1.2 mg/L	1 week	THC, phagocytosis, ROS and non-specific esterase activity (↓), PO (↑) (96h) then (↓)	
	Cadmium,Cd: 50 ng/L, 500 ng/L	66 days Time intervals: 0,4,21,39,66 days	50 ng/L: haemocyte mortality, esterase and peroxidase positive cells (↑) (21 days) then (↓), phagocytosis (↓) 500 ng/L: esterase and peroxidase (↑) (21 days), PO (↑)	**Bouilly *et al.* (2006)**
	Cadmium,Cd and Copper,Cu	4 days	THC, dead haemocytes and PO (↑), phagocytic, ROS (granulocytes and hyalinocytes) (↓)	**Haberkorn *et al.* (2014)**
Crassostrea	Zinc,Zn: 0µg/L,	*In vivo*: 7	Granulocytes (50 and	**Luo and Wang**

hongkongensis	50µg/L, 300µg/L	and 14 days *In vitro*: 1 and 3 hours	300µg/L, 14d), granulocytes mortality (300µg/L, 7d), agranulocytes (50 and 300µg/L, 14d), phagocytosis in granulocytes and all haemocytes (300µg/L, 14d), lysosomal content granulocytes and haemocytes (300µg/L, 14d) (↑) Semigranulocytes and agranulocytes (50 & 300µg/L, 14d), ROS (300µg/L, 7d), granulocytes phagocytosis (300µg/L, 14d) (↓) Granulocyte mortality (300µg/L, 1h; 50 and 300µg/L, 3h), ROS (300µg/L, 3h), phagocytic activity (50 and 300µg/L, 3h) (↑)	**(2022)**
Crassostrea rivularis	Copper,Cu: 0µg/L (control), 10µg/L (medium), 50µg/L (high)	*In vivo*: 2 weeks *In vitro*: 2h and 5h	High Cu: (↓) THC, esterase and lysosomal content, (↑) haemocyte mortality, phagocytosis and ROS	**Huang *et al.* (2018)**
Crassostrea madrasensis	Copper,Cu: 0.1 ppm, 0.5 ppm, 1.0 ppm	4 weeks	THC (↓), DHC granulocytes (↓), semigranulocytes and hyalinocytes (↑), phagocytosis (↑) 0.1 ppm, (↓) 0.5 ppm and 1.0 ppm	**Ittoop *et al.* (2009)**
Crassostrea virginica	*In vivo:* $CuCl_2$, 318 µg/L *In vitro:* 3.18 mg/L to 63.55 mg/L	14 days	*In vivo:* THC (n.s.), apoptosis (↑) (1-2 days), (↓) (7-14 days) *In vitro:* Apoptosis (↑) > 9.53 mg/L	**Foster *et al.* (2011)**
	Cadmium,Cd: 50 µg/L	10,20 or 45 days	Apoptosis (=)	**Cherkasov *et al.* (2007)**
Blood clam, *Tegillarca granosa*	Cd^{2+}: 25µg/L Cd2+-spiked	10 days	Total haemocyte counts (THC) (↓), phagocytosis (↓)	**Shi *et al.* (2018)**

	seawater with 10% elevated Ca^{2+} Cd2+-spiked seawater with 20% elevated Ca^{2+}		THC (↑), phagocytosis (↑) THC (↑), phagocytosis (↑)	
Hard shell clam, *Mercenaria mercenaria*	Cadmium,Cd: 50µg/L	4 weeks	Hemocyte counts (↑), phagocytosis (↓) (elevated CO_2 level), adhesion capacity (n.s.), lysozyme activity (↓) (elevated CO_2 level)	**Ivanina *et al.* (2014)**
	Copper,Cu: 50µg/L	4 weeks	Circulating haemocyte, adhesion capacity, cysteine proteases (↑), phagocytic activity (↓) (2000 µatm), lysozyme activity (=)	**Ivanina *et al.* (2016)**
Tapes philippinarum	Copper, Cu: 0µg/L, 10µg/L, 60µg/L, 110µg/L	7 days	Phagocytosis and superoxide dismutase (SOD) (↓), neutral red retention (NRR) and cytochrome oxidase (60µg/L) (↑)	**Matozzo *et al.* (2001)**
	Cadmium, Cd: 0µg/L, 150µg/L, 300µg/L, 450µg/L		Phagocytosis and SOD (=), NRR and cytochrome oxidase (300µg/L) (↑)	
Ruditapes philippinarum	Cd, 133nM (15 µg/L $CdCl_2$)	7 days	Phagocytosis, THC, oxidative burst and hyalinocyte concentration (n.s.)	**Paul-Pont *et al.* (2010b)**
Mya arenaria	Mercuric chloride, $HgCl_2$, and methylmercury chloride (CH_3HgCl) 10^{-9}M to 10^{-5}M	28 days	Phagocytic activity: (↓) 10^{-6}M $HgCl_2$ (D-28) and 10^{-6}M CH_3HgCl (D-7) Hemocytes viability: (↓) 7 days CH_3HgCl, 28 days $HgCl_2$	**Fournier *et al.* (2001)**
Mya arenaria *Mactromeris polynyma*	Cadmium chloride, $CdCl_2$ Mercuric chloride, $HgCl_2$ Silver nitrate, $AgNO_3$ Zinc chloride, $ZnCl_2$ Methylmercury, CH_3HgCl	18h	Cell viability: (n.s.) any metals 10^{-9}M to 10^{-5}M, (↓) 10^{-4}M CH_3HgCl, 10^{-3}M $HgCl_2$, 10^{-4}M $AgNO_3$, *M.polynyma* Phagocytosis: (↑) 10^{-9}M to 10^{-8}M $HgCl_2$, CH_3HgCl, $CdCl_2$, $ZnCl_2$,	**Sauvé *et al.* (2002)**

	10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M, 10^{-4}M, 10^{-3}M		*M.polynyma* and *M.arenaria*	
Scrobicularia plana	Mercury, Hg (On-Site): -Highly contaminated (Hi) -Moderately contaminated (Mo)	-	Hemocytes density (↑) Hi, haemocytes phagocytosis (↓) Hi, haemocytes oxidative burst (↑) Mo, haemocytes and haemolymph plasma lipid peroxidation (LPO) (↑) Mo, agglutination titre (↓) (Mo and Hi)	**Ahmad *et al.* (2011)**
Elliptio complanata	Cadmium chloride, $CdCl_2$ Mercuric chloride, $HgCl_2$ Silver nitrate, $AgNO_3$ Zinc chloride, $ZnCl_2$ Methylmercury, CH_3HgCl 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M, 10^{-4}M, 10^{-3}M	18h	Phagocytosis: (↑) 10^{-9}M to 10^{-8}M $HgCl_2$, CH_3HgCl, $CdCl_2$, $ZnCl_2$	**Sauvé *et al.* (2002)**
Dreissena polymorpha	Cd: 10^{-6}M, 10^{-5}M, 10^{-4}M, $5x10^{-4}$M, 10^{-3}M	21h	(↓) haemocyte viability (10^{-3}M), (↓) phagocytic activity, ROS granulocytes (=), hyalinocytes (↓) (10^{-3}M and 10^{-4}M), blast-like haemocytes (↓) (10^{-3}M), (↓) lysosomal hyalinocytes ($5x10^{-4}$M), granulocytes (10^{-4}M), blast-like haemocytes (10^{-3}M)	**Evariste *et al.* (2017)**
	Cadmium chloride, $CdCl_2$ Mercuric chloride, $HgCl_2$ Silver nitrate, $AgNO_3$ Zinc chloride, $ZnCl_2$ Methylmercury, CH_3HgCl 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M,	18h	Phagocytosis: (↑) 10^{-9}M to 10^{-8}M $HgCl_2$, CH_3HgCl, $CdCl_2$, $ZnCl_2$	**Sauvé *et al.* (2002)**

	10^{-4}M, 10^{-3}M			
Mytilus edulis	Cadmium chloride, $CdCl_2$: 20µg/L and 50µg/L	11 days	NRR: 20µg/L (D-4 and D-8) (↑), 50µg/L (=) Phagocytosis and THC (=)	**Sheir and Handy (2010)**
	Copper,Cu: 0.02 ppm, 0.05 ppm	7 days	THC: 0.02 ppm (↑), 0.05 ppm (↓) DHC: 0.02 ppm (↓) (basophilic), 0.05 ppm (↑) Phagocytosis: 0.02 ppm (↑), 0.05 ppm (↓)	**Parry and Pipe (2004)**
	Copper,Cu: 5µg/L, 9µg/L, 16µg/L	1,7,13 days	16µg/L: THC and DHC granulocyte (↑) (D-1), hyalinocytes and phagocytic activity (↓) 9µg/L: phagocytic (↓) (D-7 and D-13)	**Höher *et al.* (2013)**
	Cadmium chloride, $CdCl_2$ Mercuric chloride, $HgCl_2$ Silver nitrate, $AgNO_3$ Lead chloride, $PbCl_2$ 10^{-3}M to 10^{-9}M	3h	Viability: (↓) Ag and Hg (10^{-5}M), Cd and Pb (10^{-3}M) Phagocytosis: (↓) Ag (10^{-5}M-10^{-3}M), Hg (10^{-6}M-10^{-3}M), Cd and Pb (10^{-3}M), (↑) Ag (10^{-7}M), Hg (10^{-9}M-10^{-7})	**Rault *et al.* (2013)**
	Lab: $CdCl_2$, 20 µg/L On-Site Unpolluted- Port Quin (reference) Polluted- Mount Batten (poll) Cd,Cu,Fe,Pb,Zn,Na,Ca,K	Lab: 0,1,4,8 days	Lab: Neutral red uptake (NRU): (↑) ref+Cd, poll+Cd, (↓) poll+Cd (D-4) Phagocytosis (↓) poll+Cd (D-4_ On-Site: Poll. site: (↑) NRU, (↓) phagocytosis	**Sheir and Handy (2013)**
	On-Site: Roskilde Fjord: Zn,Cu,Pb	-	THC and haemolytic activity (↓), phagocytic activity (↑), caspase activity (=)	**Höher *et al.* (2012)**
	Cadmium chloride, $CdCl_2$ Mercuric chloride, $HgCl_2$ Silver nitrate, $AgNO_3$ Zinc chloride, $ZnCl_2$	18h	Cell viability: (n.s.) any metals 10^{-9}M to 10^{-5}M, (↓) 10^{-4}M CH_3HgCl, 10^{-3}M $HgCl_2$, 10^{-4}M $AgNO_3$ Phagocytosis: (↑) 10^{-9}M to 10^{-8}M $HgCl_2$,	**Sauvé *et al.* (2002)**

	Methylmercury, CH_3HgCl 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M, 10^{-4}M, 10^{-3}M		CH_3HgCl, $CdCl_2$, $ZnCl_2$	
Perna viridis	$CuSO_4$: 50µg/L, 100µg/L, 150µg/L, 200µg/L	72h	NRR and phagocytosis (↓)	**Nicholson, 2003**
	On-Site: San Antonio del Golfo (SAG): Cu and Fe Chacopata-Guayacán (CG): Zn,Cr,Cd,Pb Río Caribe (RC): N/A	-	THC (↓) CG and RC, phagocytes (↑) CG and RC, lysozyme (↑) RC, NRR (↑) CG	**Zapata-Vívenes *et al.* (2012)**
	Cu and Hg: 10µg/L and 20µg/L	25 days	PO (↑), phagocytosis (↓) after 5 days but (↑) to control level (25 days), superoxide anion generation (↓) Cu 20µg/L (5 days) then (↑) (15 and 25 days), (↓) Hg 10µg/L (5 and 10 days) then (↑) (25 days)	**Thiagarajan *et al.* (2006)**
Mytilus galloprovincialis	Methylmercury,MeHg 10^{-7}M, 10^{-6}M, 10^{-5}M, 10^{-4}M	30 minutes	Viability (↓) 10^{-4}M, phagocytic activity (↓) 10^{-7}M and 10^{-6}M, NRR (↓) 10^{-6}M and 10^{-5}M	**Parisi *et al.*, 2021**
	Hexavalent chromium: 0.1 µM, 1 µM, 10 µM, 100 µM	In vitro: 60 minutes	NRRT (↓), lysozyme (↓) (all concentrations), phagocytic activity (↓) (low concentrations)	**Ciacci *et al.* (2011)**
		In vivo: 96h	NRRT (↓) (high concentrations), serum lysozyme (↓), phagocytic activity (↓) (low concentrations), THC (↓) (low concentrations)	
Perna canaliculus	Copper,Cu: 25 µM, 62.5 µM, 125 µM, 187.5 µM	3h	(↑) haemocyte mortality (all concentrations except 25 µM), ROS (all concentrations), apoptosis	**Nguyen *et al.* (2018)**
Cerastoderma edule	Cadmium,Cd: 15µg/L	7 days	Phagocytosis (=), ROS and THC (↑) (interaction with *H.elongata*),	**Paul-Pont *et al.* (2010a)**

			haemocyte viability (↓) (interaction with *H.elongata* and *V.tapetis*)	
Zhikong scallop, *Chlamys farreri* Bay scallop, *Argopecten irradians*	$PbCl_2$: 0.2 mg/L, 0.3 mg/L. 0.5 mg/L	10 days	Hemocyte mortality: Zhikong (↑), Bay (↑) (0.2 and 0.5 mg/L) Phagocytic activity: Zhikong (↓) (0.2 and 0.3 mg/L), Bay (↓) (0.2 mg/L) (↑) phagocytosis in Bay than Zhikong Respiratory burst: (↑) both species SOD: Bay (↑) than Zhikong (0.2 mg/L) Acid phosphatase (ACP): Bay (↑) than Zhikong	**Wang *et al.* (2013)**

Notes: * (=) No change, (↑) Increase, (↓) Decrease, (n.s.) Non-significant, (N/A) Not available

EFFECTS OF HARMFUL ALGAL BLOOMS ON BIVALVE IMMUNE SYSTEM

Harmful algal blooms (HABs), sometimes called red tides, are worldwide occurrences generated by various microalgal species, mostly dinoflagellates and diatoms, which release a variety of biotoxins (**Hallegraeff, 1993; Smayda, 1997**). Most dinoflagellate species are naturally ingested by suspension-feeding bivalves (**Gainey & Shumway, 1988; Lesser & Shumway, 1993**), exposing them to harmful components. Toxicity accumulation and persistence in bivalves varies by species and depends on the bloom concentration. feeding intensity and toxin elimination rates in the shellfish (**Shumway, 1990**).

A review in the literature suggested that *Ostreopsis cf. ovata* appeared to harm the immunological responses of bivalves. For instance, it has been experimentally demonstrated by **Faustino *et al.* (2021)** that *Ostreopsis cf. ovata* caused cell mortality of *Crassostrea gasar*. The evidence strongly links it to the poisonous chemicals, putative palytoxin, and ovatoxins, produced by this harmful alga (**Moore & Scheuer, 1971; Ciminiello *et al.*, 2010; Ramos & Vasconcelos, 2010; Rossi *et al.*, 2010; Ciminiello *et al.*, 2011**). Palytoxin is the most potent nonprotein toxin ever discovered (**Botana *et al.*, 2009**), and it has been interpreted in the studies of **Pelin *et al.* (2016**) and **Nascimento *et al.* (2020)** that its analogues generated by *O. cf. ovata* exposure may be responsible for the cytotoxic impact on haemocytes. The mechanism of the action of these toxins involves disrupting the Na^+/K^+ pump function, which after binding to palytoxin (**Bellocci *et al.*, 2011**), acts as a cationic channel, eliminating the ion gradient and triggering deleterious biological consequences (**Pelin *et al.*, 2011**).

Athough *Prorocentrum* sp. and *Alexandrium* sp. do not have the same negative effects on the immune systems as *O. cf. ovata* did, there is some evidence to support the notion that these two species may weaken immune systems (**Galimany *et al.*, 2008c; Hégaret *et al.*, 2011; Medhioub *et al.*, 2013; Neves *et al.*, 2019**). In their study on *Mytilus edulis*, **Galimany *et al.* (2008a)** postulated that *Prorocentrum minimum* produced no discernible impact on the immunological parameters (phagocytic capability, ROS, haemocyte mortality, and apoptosis), except for haemocyte complexity, which was reduced from the 1st day of the experiment till the 6th day. The reduction in haemocyte complexity may be explained by degranulation as a component of the immune response or by dilution of existing granules when cells divide more quickly than new granules are produced **(Carballal *et al.*, 1997)**. The lack of immunosuppression in mussels exposed to *P. minimum*, rather than the activation of a defensive immunological response, suggests that mussels perceive this dinoflagellate as a probable intruder instead of being harmed by the chemical toxin of this planktonic organism.

For *Alexandrium* sp., immunosuppression is produced when bivalve haemocytes are exposed *in vitro* to the dinoflagellates; namely, *Alexandrium fundyense* and *A. minutum* (**Hégaret *et al.*, 2011**). These authors have noticed that *Alexandrium* species inhibited the quahog and soft-shell clam phagocytosis and quahog haemocyte adhesion. In a few oyster species (examples, *Crassostrea virginica* and *C. gigas*), both the *Alexandriu*m species enhanced haemocyte mortality. Paralytic shellfish toxins (PST), which are dangerous and lethal to animals, are known to be produced by *Alexandrium* spp. On the other hand, **Ford *et al.* (2008)** documented that soft-shell clam haemocytes treated *in vitro* with a non-PST, generating strain of *Alexandrium tamarense* inhibited adhesion and phagocytosis. However, a strain of *A. tamarense* that produces PST had no effect on these immunological functions. A report published by **Tang *et al.* (2007)** pointed out that *Alexandrium leei* could release soluble polar ichthyotoxin(s) that are unrelated to PST, which can cause fish lesions and death. *In vitro* actions of *Alexandrium* sp. on bivalve haemocytes are most likely linked to the synthesis and release of chemical compounds.

The effects of biotoxins on bivalve immune responses were addressed in limited research works, where saxitoxin (STX) appeared to be the most damaging toxin. Previous research showed increased apoptosis in *C. gigas* haemocytes when exposed to saxitoxin, which ultimately resulted in cell death (**Abi-Khalil *et al.*, 2017)**. Haemocytes displayed chromatin condensation, a classic marker of apoptosis, at the nuclear periphery with no obvious membrane damage. Results demonstrating that STX exposure reduced the amount of circulating haemocytes in the gigantic lions-paw scallop are also consistent with the fact that STX induced mortality of oyster haemocytes (**Estrada *et al.*, 2010**). In vertebrates, STX inhibits voltage-gated Na+ channels in nerve and muscle cells, preventing neuronal transmission at low nano-molar concentrations **(Kao, 1972; Narahashi, 1972; Cestele & Catterall, 2000)**. It is unknown, though, if STX binds to specific targets or receptors in molluscs.

Okadaic acid is another typical biotoxin being investigated in recent years in addition to STX. Okadaic acid (OA) was evaluated in *Ruditapes decussatus* at four different concentrations of 10, 50, 100, and 500 nM for a period of four hours in the study of **Prado-Alvarez *et al.* (2013)**. Apoptosis and cell death increased at higher concentrations (100 and 500 nM), although esterase activity appeared to decrease. OA causes chromosomal loss, apoptosis, DNA damage, and inhibited phosphatases in *in vitro* trials **(Fernández *et al.*, 1991; Nuydens *et al.*, 1998; Traoré *et al.*, 2001; Lago *et al.*, 2005; Valdiglesias *et al.*, 2010)**. Additionally, this biotoxin is a well-known phosphatase inhibitor with tumorigenic and apoptotic effects even at low concentrations **(Prego-Faraldo *et al.*, 2015)**. Notably, long-term exposure to OA can result in genotoxic and cytotoxic damage, which produces lethal results **(Prego-Faraldo *et al.*, 2013; Valdiglesias *et al.*, 2013)**. The effects of harmful algal blooms are summarized in Table (2).

Table 2. Summary of immune responses of bivalve species upon exposure to different species of harmful algal blooms or their biotoxins

Bivalves species	HABs/Biotoxins	Duration	Effect	Reference
Crassostrea gigas	*Alexandrium minutum* $5x10^3$ cells/mL	4 days	Phagocytosis and PO (↑)	**Haberkorn *et al.* (2014)**
Crassostrea gasar	*In vitro:* *Prorocentrum lima:* 10^3 *and* 10^2 *cells/mL* *Ostreopsis cf. ovata*: 10^4, 10^3 and 10^2 cells/mL	3h	*P.lima*: (=) haemocyte mortality, phagocytosis rate *O.cf.ovata:* (↑) haemocyte mortality, (↓) phagocytosis (=) ROS	**Faustino *et al.* (2021)**
	In vivo: *Ostreopsis cf. ovata*: 60-200 cells/mL	4 days	(↑) haemocyte mortality, (↓) ROS, (=) THC, phagocytosis, ABC proteins	
Ruditapes philippinarum	*Karenia selliformis* 10^2 cells/mL	First 2 weeks and last 3 weeks	Hemocyte size and apoptosis (↓), agglutination activity (↑) Exposure time (↓) haemocyte size, complexity, dead and phagocytic haemocytes after 3 weeks then (↑) Apoptosis (↑) 3 weeks ROS (↑) at the beginning then (↓)	**Silva *et al.* (2008)**
Mercenaria mercenaria	*Prorocentrum minimum*	5 days	(↑) mean size of haemocytes and ROS, (↓)	**Hégaret *et al.* (2010)**

	$2x10^4$ cells/mL		phagocytic haemocytes	
Perna perna	*Prorocentrum lima* 900 cells/mL	96h	THC (↓), ROS, size and phagocytic (↑)	**Neves *et al.* (2019)**
Mytilus edulis	*Prorocentrum minimum* $1x10^4$ cells/mL	9 days	(=) phagocytic, respiratory burst, haemocyte mortality, apoptosis (↓) complexity of haemocytes 6 days	**Galimany *et al.* (2008b)**
Mytilus edulis	*Alexandrium fundyense* $4x10^3$ cells/mL	9 days	Size of basophilic haemocytes, complexity of eosinophilic haemocytes, THC (↓), apoptosis (↑), small size of haemocytes	**Galimany *et al.* (2008c)**
Mytilus galloprovincialis	*O.cf.ovata* $1x10^5$ cells/mL	7 and 14 days	Granulocytes, phagocytosis and lysosomal (↓), hyalinocytes (↑)	**Gorbi *et al.* (2013)**
Mytilus edulis	*Pseudo-nitzschia Multiseries* 1400 cells/mL *Prorocentrum lima* 150 cells/mL	48h	(↑) larval PO for first 22h	**Rijcke *et al.* (2015)**
Argopecten irradians	Okadaic acid 50,100,500 nM	48h	SOD: (↓) 100-500 nM at 3h, (↑) 6h, (↓) 12-48h ACP: (↓) 500 nM at 6h and 12-48h at all concentrations Alkaline phosphatase (ALP): (↑) 500 nM at 12-24h then (↓) 48h, 100 nM Lysozyme: (↓) low concentration at 3-6h (↑) 12h, (↑) 100 nM at 6 and 12h, and 500 nM at 3-12h Total protein content (TPC): (↑) 100 nM and 500 nM at 12h, (=) 50 nM	**Chi *et al.* (2017)**
Crassostrea virginica *Argopecten irradians irradians*	*Prorocentrum minimum* 10^5 cells/mL	7 days	Oysters (↓) THC and hyalinocytes (↑) granulocytes, small granulocytes, haemocytes mortality (=) aggregation, oxidative	**Hégaret and Wikfors (2005)**

			burst,phagocytosis Scallops (↑) granulocyte, dead haemocyte, THC, hyalinocytes mortality and aggregation (↓) mortality of granulocytes, phagocytosis, respiratory burst	
Crassostrea gigas	*Alexandrium minutum* $2x10^4$ cells/mL	4h	(=) cellular complexity of hyaline and granular, viability (↑) granular cell size (↓) Phagocytic of granular and hyaline, ROS	**Mello *et al.* (2013)**
Ruditapes decussatus	Okadaic acid 10,50,100,500 nM	4h	100 and 500 nM: (↑) apoptosis and cell death (↓) esterase and phagocytosis	**Prado-Alvarez *et al.* (2013)**
	Prorocentrum lima $2x10^2$ and $2x10^4$ cells/mL	48h	(↑) apoptosis, cell death and esterase	
Mytilus chilensis	Saxitoxin 1,10,100 nM	4 and 16h	(↓) phagocytosis and ROS (1 and 10 nM) (↑) ROS (100 nM)	**Astuya *et al.* (2015)**
Argopecten irradians	Domoic acid 0,10,50,100 nM	48h	ACP (↑) 10 nM, 3-24h; 50 nM, 3-12h; 100 nM, 3-48h ALP (↑) 10 nM, 3-48h; 50 nM, all time; 100 nM, 3-24h Lactate dehydrogenase (LDH) (↑) 10 nM, 3-12h; 50 and 100 nM, 3-24h LPO level (↑), NO level (↓) TPC (↑) 50 and 100 nM; 10 nM, 3-12h	**Chi *et al.* (2019)**
Crassostrea gigas	Brevetoxin 3,30,100,300,1000 µg/L	12h	Cell viability (n.s.), apoptosis (=)	**Mello *et al.* (2012)**
Argopecten irradians	Palmitoleic acid 20,40,80 mg/L	48h 3,6,12,24 hour-post-exposure (hpe)	SOD: (↑) 20 mg/L, 12,24,48 hpe Malondialdehyde (MDA): all concentrations, 3 and 6	**Chi *et al.* (2016)**

			hpe; 40 and 80 mg/L, 12 to 48 hpe ACP: (↑) 80 mg/L, 6 hpe, (↑) all concentrations, 12,24,48 hpe Lysozyme: (↓) 80 mg/L, 12,24,48 hpe Phagocytic: (↑) 40 and 80 mg/L, 6 and 12 hpe; (↓) 24 and 48 hpe ROS: (↑) all concentrations, 12,24,48 hpe Protein levels: (↑) 80 mg/L, all time intervals; 40 mg/L, 3 hpe	
Argopecten irradians	Okadaic acid 50,100,500 nM	48h 3,6,12,24,48 h	THC: (↓) 100-500 nM, 12-48h; 50 nM, 48h ROS: (↑) at all-time intervals MDA: (↑) all concentrations, 6-48h; 500 nM, 3h NO level (↑) Glutathione (GSH): (=), 3-6h; (↑) 100 and 500 nM, 12h, (↓) 24-48h LDH: (↑) 500 nM, all time intervals; 50 and 100 nM, 12-48h	**Chi *et al.* (2016)**
Crassostrea gigas *Perna perna* *Anomalocardia brasiliana*	On-Site: *Dinophysis acuminata* 17,600 cells/mL	-	THC: (↑) *P.perna* and *A.brasiliana*, (=) C.gigas DHC: (↓) *P.perna*, granular (↓) exposed mussels, (=) *C.gigas* and *A.brasiliana* (↓) apoptosis Hemagglutinating activity, PO and TPC: (↑) *P.perna*	**Mello *et al.* (2010)**
Crassostrea gigas	Saxitoxin 0.8, 1, 3.3 µM	3.5h	Apoptosis (↑)	**Abi-Khalil *et al.* (2017)**
Crassostrea gigas	*Alexandrium minutum* $5x10^3$ cells/mL	4 days	THC (↑), granulocytes (↑), viability, agglutination titre and phagocytosis (=) ROS (↑ April granulocytes, ↓ May granulocytes and hyalinocytes) PO (↓ April, ↑ May)	**Haberkorn *et al.* (2010)**

Crassostrea gigas	*Alexandrium minutum* $5x10^3$ cells/mL	4 days	THC, ROS, PO (=) Phagocytosis (↑)	**Haberkorn *et al.* (2014)**
Crassostrea gigas *Crassostrea virginica*	*Alexandrium fundyense* 1, 2.5, $5x10^2$ cells/mL *Alexandrium catenella* 75 cells/mL $1.5x10^2$ cells/mL	1 and 7 days 14h 10h, 4 days	THC, size, complexity, viability, phagocytosis, ROS (=) both species	**Hégaret *et al.* (2007)**
Mytilus edulis	*Prorocentrum minimum* 10^4 cells/mL	9 days	Viability, phagocytosis, ROS, adhesion, apoptosis (=)	**Galimany *et al.* (2008b)**
Mytilus galloprovincialis	*Prorocentrum lima* 10^3, 10^5 cells/mL	24 and 48h	Apoptosis (↑) (48h, both concentrations)	**Prego-Faraldo *et al.* (2016)**
Mytilus galloprovincialis	Natural bloom: *Prorocentrum/Dinophysis* In vitro, Okadaic acid (OA) 10,50,100 nM	In vitro: 2 and 4h	Natural: apoptosis (↓) OA: apoptosis ↓ 100nM,4h	**Prado-Alvarez *et al.* (2012)**
Mytilus galloprovincialis	*In vitro:* Okadaic acid 10,50,100,200, 500 nM	1h and 2h	Apoptosis (↑) 500 nM,2h	**Prego-Faraldo *et al.* (2015)**
Perna perna *Crassostrea gigas*	Natural bloom: *Dinophysis acuminata* Praia Alegre (PA: 2950 cells/L) Praia de Zimbros (PZ: 4150 cells/L)	-	*P.perna*: THC (↓ PA, ↑ PZ), DHC and hemagglutination titre (=), PO (↑ PA, = PZ) *C.gigas*: THC, DHC, hemagglutination titre (=), PO (↓ PZ)	**Simões *et al.* (2015)**
Mercenaria mercenaria (M.m.) *Mya arenaria* (M.a.) *Crassostrea virginica* (C.v.) *Crassostrea gigas* (C.g.)	*In vitro:* -Whole culture (WC) -Media spent (S) *Heterosigma akashiwo* (H.a.), *Prorocentrum minimum* (P.m.): 10^5 cells/mL *Alexandrium fundyense* (A.f.): 10^4 cells/mL *Alexandrium minutum*	4h	H.a.: M.m.: viability (↓ WC), phagocytosis and ROS (=), adhesion (↓S); M.a.: phagocytosis and adhesion (=), ROS (↑ WC); C.v.: viability (↓ WC), phagocytosis and adhesion (=) P.m.: M.m.: size, complexity and phagocytosis (↓ WC), adhesion (↑ S); M.a.:	**Hégaret *et al.* (2011)**

Ruditapes philippinarum (R.p.)	(A.m.): $5x10^4$ cells/mL *Karenia selliformis* (K.s.), *Karenia mikimotoi* (K.m.): 4-$7x10^3$ cells/mL		ROS and adhesion (=), phagocytosis (↓ WC); C.v.: all parameters (=) A.f.: M.m.: phagocytosis (↓ WC and S), ROS (=), adhesion (↓ WC); M.a.: phagocytosis (↓ WC), ROS and adhesion (=); C.v.: viability (↓ WC), remaining parameters (=) A.m.: C.g.: viability (↓ WC and S), ROS (↓ WC), remaining parameters (=) K.s.: R.p.: size, phagocytosis and adhesion (↓ WC), viability and ROS (↓ WC and S) K.m.: R.p.: ROS and adhesion (↓ WC), other parameters (=)	
Mytilus edulis	Domoic acid 1,10,100,500 ng/g	48h, 7 days	THC (↑ 48h, 500 ng), viability (all concentrations, (=) 48h, ↑ 7 days), phagocytosis ↑ (48h, 100 ng)	**Dizer *et al.* (2001)**
Ruditapes philippinarum	*Heterocapsa circularisquama* 10^3 cells/mL	24 and 48h	THC (↑) 24h	**Basti *et al.* (2011)**
Crassostrea gigas	*Alexandrium catenella* (PST), and *Alexandrium tamarense* (non-PST) 10^3 cells/mL	48h	Apoptosis (PST>non-PST), 29h	**Medhioub *et al.* (2013)**
Nodipecten subnodosus	Saxitoxin 140 µg STX eq per 0.2 mL *In vitro:* 10-20 STX eq per. mL^{-1}	24h	Apoptosis (↑)	**Estrada *et al.* (2014)**
Nodipecten subnodosus	Saxitoxin Low dose: 6.25 MU High dose: 100 MU	12-72h 40 days	THC (↓) low dose, 12-24h; high dose, 10-40 days	**Estrada *et al.* (2010)**
Mytilus edulis	*Karlodinium*	3 and 6 days	Granulocytes (↓ 3 days, =	**Galimany *et***

	veneficum $6.25x10^4$ cells/mL	6 days), hyalinocytes (↑ 3 days, = 6 days) ROS and phagocytosis ↑	***al.* (2008a)**

* (=) No change, (↑) Increase, (↓) Decrease, (n.s.) Non-significant, (N/A) Not available

EFFECTS OF TEMPERATURE ON THE BIVALVE IMMUNE SYSTEM

In mollusks, changes in water temperature can have a major impact on immunological activities **(Mitta *et al.*, 2000; Monari *et al.*, 2007; Yu *et al.*, 2009)**. Besides, temperature affects the rates of biological, chemical, and enzymatic processes **(Pernet *et al.*, 2007)**. Supporting this view, **Rahman *et al.* (2019)** reported that temperature affected the survival of all molluscan species (oyster, mussel and cockle) with the highest mortality occurring at the temperature of 25°C. When the temperature was altered, the total haemocyte count (THC) increased significantly across all species. THC in *Crassostrea gigas*, *Mytilus galloprovincialis*, and *Katelysia rhytiphora* was temperature sensitive, increasing from 15°C to 20°C or 20°C to 25°C after a 14-day exposure, indicating that temperature variations could influence mollusk haemocyte functional responses. The findings were also consistent with extensive data which found that haemocyte numbers in *C. gigas*, *M. galloprovincialis*, and *Ruditapes philippinarum* are positively associated with rising water temperature **(Carballal *et al.*, 1997; Paillard *et al.*, 2004; Gagnaire *et al.*, 2006)**. Cell mobilization or cell proliferation from tissues into the haemolymph circulation is thought to cause higher THC in organisms at different temperatures **(Chen *et al.*, 2007a, b; Monari *et al.*, 2007)**.

Haemocyte phagocytic activity increases dramatically in response to heat stress, following a pattern similar to THC alterations in *C. gigas*, *M. galloprovincialis*, and *K. rhytiphora*. Such results have previously been observed in *M. galloprovincialis*, where the capacity of haemocytes to ingest foreign particles is reduced at 10°C than at 20°C and 30°C **(Carballal *et al.*, 1997)**. Similarly, **Monari *et al.* (2007)** found that the phagocytic activity of clam, *Chamelea gallina*, was significantly inhibited when held at 30°C for 7 days. While, **Hégaret *et al.* (2003)** noted a substantial decrease in phagocytic activity in *Crassostrea virginica* kept at 28°C for 7 days.

Reactive oxygen species (ROS) production increases with temperature, and this elevates the risk of oxidative damage. The raised level of ROS varied depending on the species. The increasing or decreasing temperature considerably impacted *M. galloprovincialis* and *K. rhytiphora*. However, for *C. gigas*, despite the increase in ROS levels, throughout temperature treatments ranging from 20°C to 25°C, no mortality was recorded, indicating that the species is more resistant to temperature stress. Only high-

temperature settings (40°C, 50°C and 60°C) significantly altered the function of *C. gigas* haemocyte activities **(Gagnaire *et al.*, 2006)**.

SOD activity increased considerably in these marine bivalves from 15°C to 25°C, indicating that the enzyme has defensive mechanisms to catalyse dangerous free radicals. Changes in environmental variables can activate the functional response of antioxidant enzymes. *M. galloprovincialis* and *K. rhytiphora* demonstrated higher SOD activity than *C. gigas* **(Pipe & Coles, 1995)**. Catalases (CAT) activity differed substantially in all three molluscan species, compared to SOD responses. The enhanced CAT activity seen in the haemocytes of *M. galloprovincialis* and *K. rhytiphora* during temperature stress indicates that oxidative stress is responsive to peroxide radicals. Other molluscan species with increased CAT activity include *M. edulis* and *M. galloprovincialis* **(Cancio & Cajaraville, 1999; Gonzalez *et al.*, 2005)**, and *Crassostrea* sp. **(Orbea *et al.*, 2002)**. The effects of temperature exposure to the immune system of bivalves are summarized in Table (3).

Table 3. Summary of immune responses of bivalve species upon exposure to different levels of temperatures

Bivalves species	Temperature (℃)	Duration	Effect	Reference
Mactra veneriformis	10^0C, 20^0C, 30^0C	24h	THC (↓) 10^0C, (↑) 30^0C Phagocytic: (↓) 30^0C, (=) 10^0C and 20^0C Lysosomal: (↓) 30^0C, (↑) 20^0C NRR: (↑) 20^0C SOD (=)	**Yu *et al.* (2009)**
Perna viridis	20^0C, 25^0C, 30^0C	24,48,96,168 ,196h	Hemocyte mortality (=) except 48h, phagocytosis (↑) 20^0C, esterase (48h) and ROS (24h) ↓ higher temperatures, lysosomal ↑ lower temperatures (24 and 96h)	**Wang *et al.* (2011)**
Mytilus edulis	10^0C, 15^0C	7 days Experiment 1: with *V. tubiashii* Experiment 2: with *V.tubiashii* and copper	Exp 1 DHC basophils: (↓) 10^0C THC: (↓) 10^0C SOD: (↑) 10^0C Phagocytosis: (↑) 15^0C Exp 2 DHC: (↓) 15^0C SOD: (↑) 15^0C Phagocytosis (=)	**Parry and Pipe (2004)**
Crassostrea virginica	Temperature elevation	1 week	Aggregation haemocyte (n.s.), granulocytes and	**Hégaret *et al.* (2003)**

	20^0C to 28^0C		small granulocytes (↑), hyalinocytes (↓), hyalinocytes mortality and ROS (↑), phagocytosis (↓)	
Chlamys farreri	5^0C, 17^0C, 25^0C	2,6,12,24h	Hemocyte mortality ↑ (5^0C and 25^0C, 2h; 17^0C ,6h), phagocytosis ↓ (all treatments,2h; 5^0C and 17^0C, 24h), ROS ↑ (25^0C ,2h; 17^0C,24h) n.s. (5^0C), SOD (n.s.), ACP (5^0C and 17^0C, 2h)	**Chen *et al.* (2007)**
Mercenaria mercenaria	13^0C, 21^0C, 27^0C	4 months	Phagocytosis ↓ (13^0C) ↑ (21^0C), ROS, dead cells, lysozyme ↑ (13^0C), THC ↓ (13^0C)	**Perrigault *et al.*, 2011**
Mytilus galloprovincialis	40^0C	1 and 4h	Oxidative and spreading ↓	**Mosca *et al.*, 2013**
Crassostrea virginica	Elevated temperature 20^0C to 28^0C	2 days	Respiratory burst and mortality ↑, phagocytosis and aggregation ↓	**Hégaret *et al.*, 2004**
Chlamys farreri	11^0C, 23^0C, 28^0C	72h	THC ↑ (1h, all treatments; 11^0C,72h), phagocytosis ↓ (28^0C) = (11^0C, 23^0C), ROS ↓ (23^0C, 1h; 11^0C,72h) ↑ (28^0C), ACP ↓ cell-free haemolymph,CFH(28^0C; 23^0C,72h) = (11^0C), haemocyte lysate, HL (↑ 28^0C,1h; ↓ 72h), (↓ 11^0C,1h and 24h; ↑ 72h), SOD n.s. CFH, HL (↑ 23^0C and 28^0C, 1h; ↓ 28^0C,72h; ↑ 11^0C,72h)	**Chen *et al.*, 2007**
Crassostrea virginica	12^0C, 20^0C, 28^0C	45 days	Apoptotic haemocytes ↑ (28^0C), TPC = (12^0C and 20^0C) ↓ (28^0C)	**Cherkasov *et al.*, 2007**
Crassostrea gigas	4^0C, 11^0C, 20^0C, 25^0C, 35^0C, 40^0C, 50^0C, 60^0C	*In vitro:* 2 and 4h *In vivo:* 4h	*In vitro:* Cell mortality ↑ (2h,40^0C; 4h,50^0C and 60^0C) Aminopeptidase ↓ (50^0C and 60^0C) Esterase ↓ (2h,50^0C; 4h,50^0C and 60^0C) *In vivo:* Cell mortality ↑ (40^0C,	**Gagnaire *et al.*, 2006**

			50^0C and 60^0C) Esterase ↓ (4℃ and 60^0C) Phagocytosis ↓ (60^0C)	
Chamelea gallina	20^0C, 25^0C, 30^0C	7 days	THC ↑ (30^0C), phagocytosis ↓ (30^0C), lysozyme (25^0C) ↑ HL and ↓ CFH, SOD (25^0C) ↑ HL and CFH (30^0C) ↓ HL ↑ CFH	**Monari *et al.*, 2007**
Mytilus galloprovincialis	25±2^0C	24h	Circulating immunocytes and phagocytic immunocytes (=)	**Malagoli *et al.*, 2007**
Ruditapes philippinarum	5^0C, 15^0C, 30^0C	7 days	THC ↓ (30^0C, 18 psu; 5^0C and 30^0C, 38 psu), NRU ↑ (15^0C, 28 and 38 psu), HL enzyme activity ↑ (5^0C) lysozyme ↓ (30^0C), CFH enzyme ↑ (5^0C, 38 psu) ↓ (15^0C, 18 psu), TPC n.s.	**Munari *et al.*, 2011**
Chlamys farreri	29^0C	96h 0,3,6,12,24,48,96h	Survival rate ↓ (6 and 12h) ↑ (96h), O_2^- level, SOD, MDA ↑ (6h), ACP ↑ (12h)	**Wang *et al.*,2012**
Mytilus coruscus	25^0C (normal), 30^0C (high)	14 days	30^0C: Hemocyte mortality and ROS ↑, THC, phagocytosis, esterase, lysosomal ↓	**Wu *et al.*, 2016**
Ruditapes decussatus	20^0C, 30^0C	24h	THC, PO, lysozyme, esterase (n.s.), dead cell and ALP ↑ (20^0C), agglutinating activity ↑ (30^0C)	**Mansour *et al.*, 2017**
Crassostrea gigas *Mytilus galloprovincialis* *Katelysia rhytiphora*	15^0C, 20^0C, 25^0C	14 days	All species: THC ↓ (15^0C), ↑ (25^0C) and phagocytosis SOD, catalase (CAT), ROS ↑ with temperature (*M.galloprovincialis* and *K.rhytiphora*) ↑ ROS and SOD (*C.gigas*, 20^0C -25^0C)	**Rahman *et al.*, 2019**
Crassostrea gigas	4^0C, 25^0C, 37^0C	0,3,5,7 days	Phagocytosis ↓ all temperatures (1‰,32‰), cell mortality ↓ all temperatures (15‰)	**Rodrick, 2008**
Mytilus	12^0C, 20^0C, 28^0C	6 weeks	Esterase ↓ (all	**Parisi *et al.*, 2017**

galloprovincialis		(2 weeks for each temperature)	temperatures), ALP and NRR ↑ (12^0C), PO ↓ (12 to 20^0C), lysosomal ↓ (20 to 28^0C)	
Barbatia decussate	On-Site: Seasonal water temperature	1 year	MDA, SOD, CAT, glutathione peroxidase (GPx) ↑ (Apr-Aug) ↓ (Sep-Feb) Phagocytosis ↓ (Dec-Feb) ↑ (Mar-Sept) THC ↓ (Oct-Mar) ↑ (Apr)	**Khoei, 2021**
Ruditapes philippinarum	8^0C, 14^0C, 21^0C	30 days	THC and leucine aminopeptidase ↑ (21^0C), PDC and lysozyme ↑ (8^0C), TPC (n.s.)	**Paillard *et al.*, 2004**
Mytilus edulis	5^0C, 10^0C, 20^0C	10^0C T0: 0 day T7: 7 days T35: 28 days 20^0C T42: A week T70: 28 days	Hemocyte viability ↓ (5^0C), ↑ (10^0C, T7 and T35), 20^0C (↓ T42, ↑ T70) Phagocytic ↑ (5^0C, T0; 20^0C, T42 and T70), ↓ (10^0C, T7 and T35)	**Beaudry *et al.*, 2016**
Mytilus galloprovincialis	On-Site: Seasonal water temperature	1 year	Lysosomal membrane stability (LMS) ↓ (winter, Jan-Feb and early autumn,Sept-Oct), ↑ (spring,May and Dec) Phagocytic ↑ (winter,Jan), ↓ (spring-early summer, June and Sept) Lysozyme ↓ (late winter-early spring, Feb to Apr), ↑ (late summer-autumn, Oct and Nov)	**Ciacci *et al.*, 2009**

Notes: * (=) No change, (↑) Increase, (↓) Decrease, (n.s.) Non-significant, (N/A) Not available

EFFECTS OF SALINITY ON THE BIVALVE IMMUNE SYSTEM

Salinity can alter a variety of metabolic and physiological variables in aquatic organisms, including molluscs **(Matozzo and Marin, 2011)**. **Wu *et al.* (2020)** investigated the impact of salinity changes on bivalve immune responses in *M. edulis* when subjected to three salinity variations: 15‰ (normal salinity), 5 to 15‰ (fluctuating salinity), and 5‰ (extreme salinity) (low salinity). During exposure to low and

fluctuating salinity, both phagocytosis and neutral red chemical uptake increased. Neutral red is a supravital dye that is exclusively accumulated in lysosomes via active (ATP-dependent) transport **(Repetto *et al.*, 2008)**. It is only taken up by living cells **(Repetto *et al.*, 2008)** and is dependent on the overall lysosomal volume of the cells **(Winckler, 1974)**. During low salinity, however, haemocytes abundance and adhesion were reduced, but their mortality increased. The number of circulating haemocytes is determined by the balance of haemocyte mortality, haematopoiesis, and haemocyte mobility to and from various body compartments **(Allam and Raftos, 2015; Pila *et al.*, 2016)**, which explains the findings described by **Wu *et al*. (2020)**.

Another study by **Xie *et al.* (2021)** highlighting that when *C. hongkongensis* was exposed to three different salinities (10‰, 25‰, and 35‰) for 14 days, haemocyte mortality increased at lower salinity (10‰), which explained the drop in THC and increased ROS. Changes in salinity reduced THC in the thick-shell mussel, *M. coruscus* **(Wu *et al.*, 2018)**, but high salinity increased THC in the green-lipped mussel, *Perna viridis* **(Wang *et al.*, 2012)** and Philippine clam, *R. philippinarum* **(Reid *et al.*, 2003; Wang *et al.*, 2012)**. There is evidence suggesting that lower salinity caused the increase in haemocyte abundance **(Matozzo *et al.*, 2007a; Matozzo *et al.*, 2012; Pérez-Velasco *et al.*, 2022)**. When the generation of ROS exceeds the antioxidant capacity of bivalves, cell oxidative damage occurs, resulting in a drop in THC **(Wu *et al.*, 2018)**, which may explain the findings of **Xie *et al.* (2021)**. The creation of ROS in haemocytes or the inhibition of ROS-producing enzymes can both explain the increase in ROS **(Lushchak, 2011).**

Oysters exposed to 35‰ salinity showed no significant effect on haemocyte mortality, ROS, and THC but reduced the activity of esterase enzyme. This finding contradicts the observations of some past researchers. Low salinity reduced the esterase activity in oyster, *C. gigas* **(Gagnaire *et al.*, 2006)**, clam, *Paphia malabarica* **(Gajbhiye and Khandeparker, 2017)** and mussel, *P. viridis* **(Wang *et al.*, 2012)**. High salinity, on the other hand, had no effect on the esterase activity until long-term exposure caused in its decline. In haemocytes, the esterase is a hydrolytic enzyme involved in numerous processes of hydrolysis as well as intracellular degradation **(Pretti and Cognetti-Varriale, 2001; Mottin *et al.*, 2010)**. Factors modulating the activity of this enzyme, therefore, have consequences for the normal course of metabolism. The effects of salinity exposure to immune system of bivalves are summarized in **Table 4.**

Table 4. Summary of immune responses of bivalve species upon exposure to different levels of salinities

Bivalves Species	Salinity	Duration	Effects	Reference
Mytilus edulis	16‰, 32‰	2 days	16‰: ↓ no. of haemocytes,	**Bussell *et al.*, 2008**

			eosinophils, phagocytic activity	
Mytilus edulis	Normal (NS): 15‰ Fluctuating (FS): 5-15‰ Low (LS): 5‰	21 days	Hemocyte abundance (↑ LS), haemocyte mortality (↑ LS), adhesion (↓ LS), phagocytosis (↑ FS and LS), NRU (↑ FS and LS)	**Wu *et al.*, 2020**
Pinctada imbricata	25 ppt	24, 72, 120h	Granulocyte (↑ 24h,72h, 120h return to pre-treatment), phagocytosis (↓), THC (↓ 24h, ↑ 72h, return to pre-treatment 120h), TPC (↑ 24h, ↓ 72h, ↑ 120h), ACP (↓ 24h, ↑ 72h, ↓ 120h), PO (↓)	**Kuchel *et al.*, 2010**
Mytilus edulis	On-Site: Natural salinity Agersø (A) (11‰) Roskilde Fjord (R.F.), Roskilde Vig (R.V.) (13‰) Frederiksværk (F) (15‰) Isefjord (I) (19‰)	-	THC ↓ (R.F and I), phagocytic ↑ (R.F.) ↓ (other stations), caspase activity (n.s.), haemolytic activity ↓ (R.F. and R.V.) ↑ (A and I)	**Höher *et al.*, 2012**
Paphia malabarica	0,5,15,25,35‰	*In vitro:* 2 and 5h *In vivo:* 1,4,6 days	*In vitro:* Hemocytes mortality ↑ (0 and 5‰, 2 and 5h) n.s. (15,25,35‰) Lysosomal ↓ (0 and 5‰, 2h), ↑ (15, 25,35‰), ↓ (all except 0‰, 5h) Esterase ↓ (0 and 5‰, 2h), ↑ (15, 25,35‰), ↓ (all treatments, 5h) ROS ↓ (0,5,15‰, 2h) n.s. (25 and 35‰) ↓ (all except 0‰, 5h) Phagocytic ↓ (0 and 5‰, 2h) ↑ (35‰) n.s. (15 and 25‰) ↓ (all except 0‰, 5h) *In vivo:* D-1: haemocyte mortality (HM) (↑) 0 and 5‰, lysosomal (↓) 0,5,15‰ (=) 25 and 35‰, esterase (↓) 0 and 5‰ (↑) 15,25,35‰, ROS (↓) 0 and 5‰ (=) 15-	**Gajbhiye and Khandeparker, 2017**

			35‰, phagocytic (↓) 0,5 and 15‰ (=) 25 and 35‰, THC (=) D-4: HM (↑), lysosomal (↓), esterase (↓) all treatments except 25 and 35‰, ROS (↓) 0 and 5‰ (=) 25 and 35‰, phagocytic (↓) 0,5,15‰ (=) 25 and 35‰, THC (↓) 0 and 5‰ (=) 15-35‰ D-6: HM (↑) 15‰ (n.s.) 25 and 35‰, lysosomal (=) 15 and 25‰ (↑) 35‰, esterase (↓) 15-35‰, ROS (↓) 15‰, phagocytic (=) 15-35‰, THC (=) 15-35‰	
Ostrea edulis	16,25,32‰	7 days and 48h with L.anguillarum	No.of large granulocytes (↑) 32‰ Hydrogen peroxide (↓) 32‰ Lysozyme (↓) 25‰	**Hauton *et al.*, 2000**
Crassostrea gigas	*In vitro:* 29, 25.5, 22..5, 16, 6.5, 3, 0‰ *In vivo:* Exp.1: 15,35,45‰ Exp.2: 5,35,60‰	In vitro 2 and 18h In vivo Days 1,3,7	*In vitro:* 2h: (↑) cell mortality, (↓) esterase 6.5,3,0‰ 18h: (↑) cell mortality, (↓) esterase 3 and 0‰ *In vivo:* Exp.1: mortality (=), phagocytosis (↓) 15‰, D-1 and 35‰, D-3 and D-7 Exp.2: mortality (↑) 15 and 45‰, D-3 and D-6, phagocytosis (=) first 3 days	**Gagnaire *et al.*, 2006**
Mytilus galloprovincialis	25±1 psu 40±1 psu	24h	No.of circulating immunocytes (↑) 40 psu (=) 25 psu Phagocytic immunocytes (=)	**Malagoli *et al.*, 2007**
Crassostrea corteziensis	10 (hyposaline,HO), 35 (control,C), 50 psu (hypersaline,HP)	48h	THC, hyalinocytes count and granulocytes (↑) C and HO, (↓) HP Hemocyte viability (↑) HO (↓) HP Phagocytosis (=) Superoxide anion (SOA) (↑) HP and HO, (↓) C	**Pérez-Velasco *et al.*, 2022**
Crassostrea gigas	1,15,32‰	7 days	Hemocyte mortality (↑) 1 and 32‰ Phagocytosis (↑) 15‰	**Rodrick, 2008**
Chamelea	28,34,40‰	7 days	THC (↑) 28‰ (=) 34 and	**Matozzo *et al.*, 2007a**

gallina			40‰ Phagocytic (↑) 34‰ (↓) 28 and 40‰ Lysozyme (↑) 28 and 34‰	
Ruditapes philippinarum	20 (control),30,40 ‰	6 weeks	THC (↓) 20‰, (=) dead cells Granulocytes (↓) 30 and 40‰ Hyalinocytes (↑) 40‰ Lysozyme (↓) 30‰ (↑) 40‰ Phagocytic (↓) 40‰ PO (↑)	**Reid *et al.*, 2003**
Crassostrea iredalei	7,14,28,35ppt	2 weeks	THC (↓) 7 ppt (↑) 35 ppt Phagocytic (=) SOD (↑) 7 and 14 ppt TPC (↑) 28 and 35 ppt	**Nadirah *et al.*, 2018**
Mytilus coruscus	15 (low, LS),25,35‰ (high,HS)	In vivo 7 days In vitro 5h	*In vivo:* Hemocyte mortality, ROS (↑) LS and HS Phagocytosis (↓) all treatments Esterase and THC (↓) LS and HS Lysosomal (↓) LS (=) HS	**Wu *et al.*, 2018**
Crassostrea virginica	18.9 and 31.9‰	3 weeks	Hyalinocytes (↑) Apoptosis (↓) 18.9‰	**Goedken *et al.*, 2005**
Crassostrea hongkongensis	10,25,35‰	14 days	Hemocyte mortality (↑) 10‰ D-1- and D-14 (=) 35‰ ROS (↑) 10‰ (=) 35‰ THC (↓) 10‰ D-1, (=) 35‰ Esterase (↓) 35‰ D-1, (=) all treatments D-3 Lysosomal (↓) all treatments	**Xie *et al.*, 2021**
Chamelea gallina *Mytilus galloprovincia lis*	28,34,40 psu	7 days 8.1 pH, 22℃	*C.gallina* THC (↑) 28 and 40 psu (↓) 34 psu NR (↓) 28 and 34 psu (↑) 40 psu Lysozyme and TPC (↓) 28 and 34 psu (=) 40 psu *M.galloprovincialis* THC (=) 28 psu (↑) 34 and 40 psu NR (↑) 28, 34 and 40 psu Lysosomal (=) 28 and 34 psu (↑) 40 psu TPC (↑) 28 and 40 psu (=) 34 psu	**Matozzo *et al.*, 2012**
Anadara	Hyposaline: 8	2 days	ROS (↑) 8 ppm (↓) 35 and 45	**Kladchenko *et al.*,**

kagoshimensis	and 14 ppm Hypersaline: 35 and 45 ppm Control: 18 ppm		ppm (=) 14 ppm	**2021**
Ruditapes philippinarum	18,28,38 psu	7 days	THC (n.s.) NRU (↓) 18 psu,5℃ (↑) 28 psu,30℃ HL: enzyme activity (↑) 18 psu, lysozyme (↓) 28 psu CFH: enzyme activity (↑) 38 psu, lysozyme (↓) 18 psu TPC (n.s.)	**Munari *et al.*, 2011**

Notes: * (=) No change, (↑) Increase, (↓) Decrease, (n.s.) Non significant, (N/A) Not available

CONCLUSION

The common immune factors observed in most bivalves are total haemocyte count (THC), phagocytosis, lysozyme, apoptosis, and reactive oxygen species (ROS). Most healthy, but not all bivalve species, show higher THC, phagocytosis, and lysozyme activity, but lower apoptosis and ROS. These immune factors play important roles in the health and immune system of the bivalves. Among all the four factors affecting the immune responses of bivalves that have been discussed in this study, heavy metals deserve priority due to the high level of sensitivity of bivalves towards these environmental contaminants. Duration of exposure and concentration of heavy metals in the water are important factors in their toxicity to the bivalves and that depends on the threshold of different species. Further investigations are needed to establish the cause-and-effect relations related to the exposure of bivalves to different environmental pollutants.

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