1. SUMMARY

Marine organisms are exposed to a wide range of anthropogenic substances, many of them considered as emerging contaminants due to their growing production and not well-known environmental impact.

Among emerging contaminants, pharmaceuticals and personal care products are cause for increasing concern, being bioactive substances widely used in both human and veterinary medicine.

Climate changes, such as ocean acidification, could have a powerful effect on pharmaceuticals by altering their environmental behaviour and exposure pathways, thus resulting in an increased toxicity. Furthermore, shifts in environmental parameters could alter marine organism susceptibility to these compounds.

In this context, the combined effects of seawater acidification, as predicted in climate change scenarios, and emerging contaminants (the non-steroidal anti-inflammatory drug, NSAID, diclofenac and the metabolic activator caffeine) were investigated for the first time in the Mediterranean mussel *Mytilus galloprovincialis* and the Manila clam *Venerupis philippinarum.*

In adults of both species, we evaluated haemocyte and oxidative stress parameters in order to highlight potential effects due to the combination of seawater acidification and exposure to diclofenac.

Other two experiments were carried out to investigate whether the exposure to seawater acidification-diclofenac and acidification–

caffeine may affect physiological responses and haemocyte parameters, respectively, in adults of *M. galloprovincialis*.

A flow-through system was used to carry out a three-weeks exposure of the studied bivalves. In the first week, animals were exposed to three pH values (8.1, 7.7, 7.4) only. Thereafter, they were maintained for 7 and 14 days at the three experimental pH values and exposed simultaneously to environmentally relevant concentrations of diclofenac/caffeine (0, 0.05 and 0.5 µg/L). To analyzed haemocyte parameters [total haemocyte count (THC), haemocyte volume and diameter, Neutral Red uptake (NRU), haemocyte proliferation and lysozyme activity) and oxidative stress parameters [superoxide dismutase (SOD), catalase (CAT) and cyclooxygenase (COX) activities, lipid peroxidation (TBARS) and DNA strand-break formation), haemolymph, gills and digestive gland were collected after 7, 14 and 21 days of exposure to differing pH value or pH/diclofenac and pH/caffeine combinations. The same experimental set-up was used to study physiological responses in bivalves. After 7, 14 and 21 days, clearance rate, respiration rate and excretion rate were evaluated in individual mussels.

The results obtained showed that cellular, biochemical and physiological parameters measured in both mussels and clams were more influenced by pH than by the contaminants (diclofenac and caffeine). In both species, immune parameters were the most responsive to differing experimental conditions. In *V. philippinarum*, THC and haemocyte proliferation significantly increased with decreasing pH values, after one week of exposure. Interestingly, NRU

decreased significantly at pH 7.4, suggesting that clams were trying to compensate a weak pinocytotic capability, due to damaged cell membranes, by increasing the number of circulating haemocytes to maintain immunesurveillance. Despite COX is involved in inflammatory processes and it should be inhibited by NSAIDs, no significant effects due to diclofenac were revealed in both bivalve species, whereas pH significantly influenced this enzymatic activity. However, an opposite pattern of variation was observed in tissues of clams and mussels kept at low pH. In *M. galloprovincialis*, differing modulation patterns were found in physiological rates measured after 7, 14 and 21 days of exposure to the experimental conditions tested. Reduction in filtration and nitrogen excretion and increase in oxygen consumption highlighted stressful condition under reduced pH.

The combined effects of seawater acidification and diclofenac were also investigated in larvae of the clam *V. philippinarum*.

An experimental flow-through system was used to carry out a 96 hours exposure of clam larvae. Fertilized eggs were exposed to two different levels of pH (8.1 and 7.7) combined with two concentrations of diclofenac (0 and 0.5 µg/L). Throughout the experiment, larval mortality and growth were daily checked and recorded. At the end of the experiment, pools of larvae were constituted to assess oxidative stress (CAT activity) and lipid peroxidation.

About 50% mortality was observed in larvae exposed to pH 7.7, both with and without diclofenac, while mortality was lower than 10% in controls kept at pH 8.1. Low pH significantly reduced shell length and

height and altered their ratio. Diclofenac significantly decreased shell length only, the reduction being higher at pH 7.7 than at pH 8.1. A strong tissue prolapse outside the shell was observed in larvae kept at pH 7.7. pH significantly increased CAT activity in larvae kept at pH 7.7 for 96 hours, whereas no significant difference in lipid peroxidation was found among treatments. This study demonstrated that seawater acidification negatively influenced susceptibility of *V. philippinarum* larvae to environmentally relevant concentrations of diclofenac, mostly by altering shell growth.

2. RIASSUNTO

Gli organismi marini , soprattutto in ambienti costieri ed estuarini, sono continuamente esposti ad una vasta gamma di sostanze di origine antropica, tra cui molte vengono considerate “contaminanti emergenti” a causa della crescente produzione ed immissione in ambiente a fronte di una quasi completa assenza di informazioni relative al loro impatto sul biota. Tra questi composti, una crescente attenzione viene data ai principi attivi contenuti in farmaci per uso medico e veterinario e in prodotti per la cura e l’igiene personale di largo consumo.

Le problematiche derivanti dal continuo rilascio di nuovi contaminanti in ambiente si inseriscono in un quadro di cambiamenti climatici, riconducibili all’aumento delle emissioni di anidride carbonica in atmosfera, in particolare all’acidificazione delle acque marine. Una riduzione dei valori di pH in mare potrebbe incidere

pesantemente sulle caratteristiche chimico-fisiche dei farmaci rilasciati in ambiente, alterandone distribuzione, biodisponibilità e potenziale tossicità. D'altro canto, variazioni ambientali, come quelle previste in uno scenario di cambiamento climatico, potrebbero modificare la suscettibilità degli organismi marini nei confronti di questi composti.

In questo contesto, il presente studio ha voluto indagare gli effetti combinati dell'acidificazione e di contaminanti emergenti (il diclofenac, un antiinfiammatorio non steroideo, e la caffeina, un attivatore metabolico) in due bivalvi marini, il mitilo

Mytilus galloprovincialis e la vongola Venerupis philippinarum.

In organismi adulti di entrambe le specie, sono stati valutati parametri morfo-funzionali negli emociti e risposte legate allo stress ossidativo in condizioni di esposizione a pH ridotto e a diclofenac . Solo nel mitilo si sono indagati gli effetti delle medesime condizioni sperimentali sulle performance fisiologiche e, in un ulteriore esperimento, gli effetti combinati di acidificazione e caffeina su parametri cellulari negli emociti.

Per condurre queste diverse sperimentazioni, ognuna della durata di una settimana, è stato utilizzato un impianto a flusso continuo appositamente allestito. Durante la prima settimana gli animali sono stati esposti a tre diversi valori di pH (8.1, 7.7, 7.4), nelle successive due settimane alle stesse condizioni di pH si è associata un'esposizione a concentrazioni ambientali di diclofenac o caffeina (0, 0.05 and 0.5 µg/L). Dopo 7, 14 e 21 giorni di esposizione alle condizioni sperimentali sopra indicate, sono stati prelevati emolinfa, branchie e ghiandola digestiva e misurati i parametri emocitari

[numero di emociti circolanti (THC), volume e diametro degli emociti, saggio di assunzione del Rosso Neutro (NRU), saggio di proliferazione cellulare, attività del lisozima] e i parametri di stress ossidativo [attività della superossido dismutasi (SOD), della catalasi (CAT) e della cicloossigenasi (COX), perossidazione lipidica (TBARS) e rotture al DNA (DNA strand-breaks)]. Analogamente, con la stessa cadenza temporale, sono state misurate nei mitili le risposte fisiologiche (tasso di filtrazione, tasso di respirazione e tasso di escrezione).

Nel complesso i risultati ottenuti evidenziano che, sia nei mitili, sia nelle vongole, le risposte risposte biochimiche, cellulari e fisiologiche indagate sono state maggiormente influenzate dalla riduzione di pH che dall'esposizione ai contaminanti (diclofenac e caffeina). In entrambe le specie, i parametri emocitari hanno risposto in maniera più sensibile alle diverse condizioni sperimentali.

In V. philippinarum, THC e proliferazione cellulare sono aumentati significativamente al diminuire del pH dopo una settimana di esposizione. I valori di NRU invece hanno mostrato una significativa diminuzione a pH 7.4, suggerendo quindi la messa in atto di meccanismo di compensazione dell'indebolita capacità di pinocitosi, dovuta ad un possibile danno alla membrana cellulare, attraverso un aumento dei valori di THC, in modo da mantenere adeguate condizioni di immunosorveglianza. Nonostante la COX sia coinvolta nei processi infiammatori e venga normalmente inibita dagli antiinfiammatori non steroidei, in nessuna delle due specie la sua attività è risultata significativamente influenzata dall'esposizione a diclofenac. Viceversa si è evidenziato un effetto significativo del pH, anche se con un opposto pattern di variazione in mitili e vongole

dopo esposizione a pH ridotto. In M. galloprovincialis sono stati rilevati differenti pattern di modulazione nelle risposte fisiologiche misurate dopo 7, 14 and 21 giorni di esposizione alle condizioni sperimentali saggiate. In particolare, una riduzione della filtrazione e dell'escrezione azotata ed un concomitante aumento del consumo di ossigeno hanno messo in luce condizioni di stress associate ad una riduzione del pH.

Gli effetti combinati di acidificazione dell'acqua di mare e di esposizione a diclofenac sono stati indagati anche durante la fase larvale della vongola V. philippinarum. In un impianto sperimentale a flusso continuo, uova fecondate sono state esposte per 96 ore a due diversi valori di (8.1 and 7.7) in combinazione con due concentrazioni di diclofenac (0 and 0.5 µg/L). Durante l'esperimento è stata rilevata ogni giorno la mortalità delle larve ed è stata misurata la crescita della conchiglia. Alla fine dell'esperimento sono stati raccolti campioni di larve per la determinazione dell'attività della CAT e della perossidazione lipidica. Una mortalità intorno al 50% è stata registrata a pH 7.7, sia in presenza che in assenza di diclofenac, ma tale valore è sceso a meno del 10% a pH 8.1. A pH 7.7 si è osservata una riduzione significativa delle dimensioni della conchiglia (lunghezza e altezza) e un'alterazione del loro rapporto. L'esposizione a diclofenac ha portato ad una significativa riduzione della sola lunghezza, più marcata a pH 7.7 che a pH 8.1. A pH ridotto sono state osservate larve anomale con un prolasso dei tessuti molli che fuoriuscivano dalla conchiglia. Nelle larve mantenute a pH ridotto si è riscontrato un aumento significativo dell'attività della CAT, ma nessuna variazione nei livelli di perossidazione lipidica rispetto ai

controlli. Questo studio ha fornito i primi risultati sui possibili effetti dell'acidificazione nello sviluppo larvale di V. philippinarum , evidenziando come una riduzione di pH possa influire negativamente sulla sensibilità delle larve di vongola al diclofenac, con ripercussioni soprattutto sulla crescita della conchiglia.

3. INTRODUCTION

Global climate changes (GCCs)

Human activities always had an influence on terrestrial and aquatic environments. From the Industrial Revolution, this influence has progressively increased resulting in the global climate changes (GCCs) as documented in several studies (Petit et al., 1999; Houghton et al., 2001; Augustin et al., 2004; Feely et al., 2004; Sabine et al., 2004; Siegenthaler et al., 2005; The Royal Society, 2005; IPCC, 2007). Increased emission of greenhouse gasses, like CO_2, has been indicated as the main cause for GCCs (IPCC, 2001) (Fig. 1).

Consequences of GCCs have already been recorded, such as the increment of ocean temperature (Roemmich, 1992), changes in thermohaline circulation (Bryden et al., 2005; Roether et al., 1996), ice malting (Curran et al., 2003), ocean acidification and variation in ocean salinity (IPCC, 2007).

To better understand these phenomena and to predict their evolution for the future, we have to know the history of CO_2 emissions during the last two centuries. Indeed, from the beginning of the Industrial Revolution (1760-1780) CO_2 levels in the atmosphere have increased from 280 ppm of the pre-industrial age (Petit et al., 1999; Augustin et al., 2004; Siegenthaler et al., 2005) to the modern 380 ppm (Feely et al., 2004). Main cause of this phenomenon is the growing use of fossil fuel related to industrialization (Kleypas et al., 2006), like carbon and petrol, and deforestation with emissions of

CO_2 that increase about 0.5% every year (Petit et al., 1999; Houghton et al., 2001; Augustin et al., 2004; Siegenthaler et al., 2005).

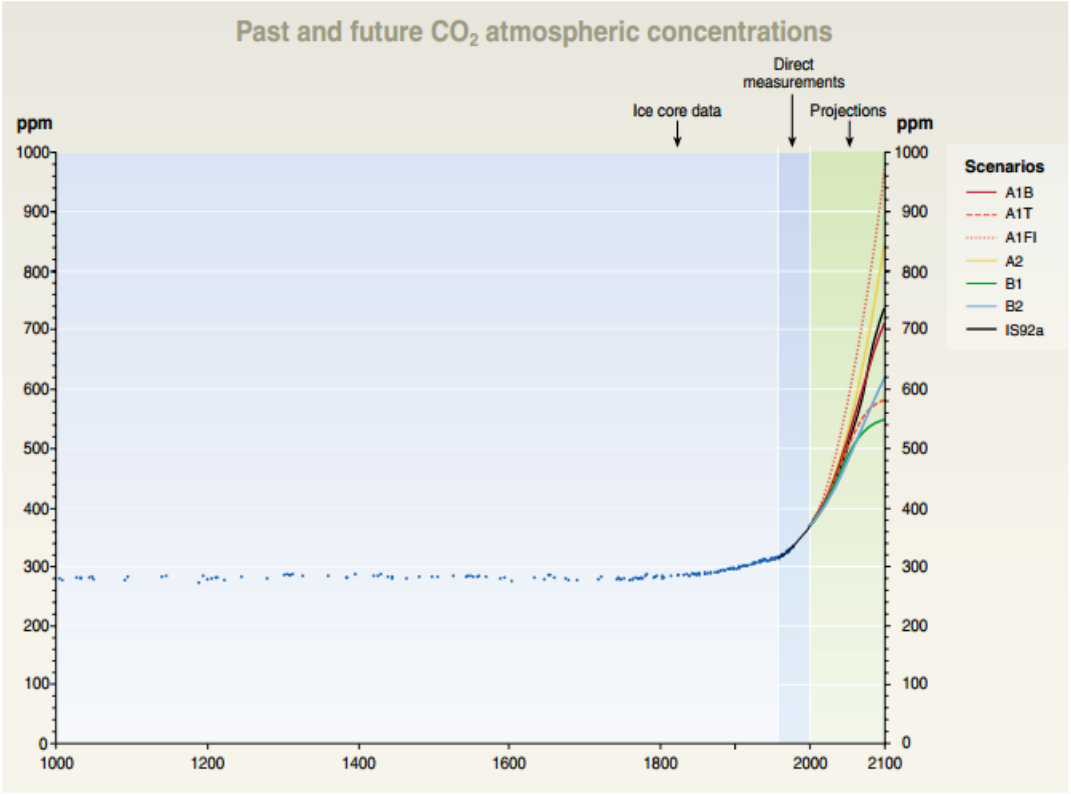

Fig. 1 – Atmospheric CO_2 concentration from the 1000 A.D. to the 2000 A.D. measured from ice carrots and direct atmospheric from the last decades (IPCC, 2001).

As a result of this emission rate we are going to experience a CO_2 atmospheric levels around 800 ppm by the end of this century (Feely *et al*., 2004). According to literature, levels of CO_2 in the atmosphere have been 100 times lower during the 650,000 years before the Industrial Revolution (Siegenthaler et al., 2005) and the time needed to restore pre-industrial values is difficult to predict (the Royal Society, 2005).

Oceans play a fundamental role in the exchange of CO_2 with the atmosphere. Over the past 200 years, since pre-industrial times, the

oceans have absorbed about a half of the CO_2 emissions produced from burning fossil fuels and cement manufacture (Feely *et al*., 2004; Sabine *et al*., 2004). This demonstrates the role played by the oceans within the natural processes of cycling carbon on a global scale.

The phenomenon of ocean acidification

Carbon dioxide, like other gases, obeys to Henry's law, which means that higher levels of atmospheric CO_2 increase the concentration of CO_2 in the surface of oceans (Raven *et al*., 2005).

Carbon dioxide in the atmosphere is an nonreactive gas but, when dissolved in seawater it takes part in several chemical, physical, biological and geological reactions. One of the overall effects of CO_2 dissolving in seawater is to increase the concentration of hydrogen ions ($[H^+]$). This is the result of an initial reaction between water (H_2O) and CO_2 to form carbonic acid (H_2CO_3). This weak acid readily releases the hydrogen ions to form the other types of dissolved inorganic carbon.

$$CO_2(atm) \rightleftharpoons CO_2(aq) + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$$

The amount of CO_2 that dissolves in seawater has a strong influence on the resultant acidity/alkalinity and pH of the oceans.

In the oceans, dissolved CO_2 exists in three main inorganic forms commonly known as dissolved inorganic carbon (DIC):

1. aqueous CO_2 (about 1% of the total) which include also H_2CO_3;
2. bicarbonate ion HCO_3^- (about the 91%);

3. carbonate ion CO_3^{2-} (about 8%).

Thus under current ocean conditions, bicarbonate is the most abundant form of CO_2 dissolved in seawater followed by carbonate and then aqueous CO_2 (The Royal Society, 2005). But the three forms of DIC (CO_2, HCO_3^- and CO_3^{2-}) and their proportions have a role in the regulation of sea water pH, and maintain its values within relatively narrow limits, between 7.5 and 8.4 (Nybakken, 2004), thus acting as a 'carbonate buffer'. If an acid (such as CO_2) is added to seawater, the additional hydrogen ions react with carbonate (CO_3^{2-}) ions and convert them to bicarbonate (HCO_3^-).

When atmospheric CO_2 dissolves in seawater, the oceans increase in acidity but, because of the carbonate buffer, the resultant solution is still slightly alkaline. Since the emission rate of anthropogenic CO_2 in the atmosphere is increasing, the capacity of the carbonate buffer to restrict pH changes is diminishing. For this reason, when CO_2 dissolves, the chemical processes that take place reduce some carbonate ions, which are required for the ocean pH buffer (Skirrow & Whitfield, 1975; Broecker &Takahashi, 1977; Broecker et al., 1979; Feely & Chen, 1982; Caldeira & Wickett, 2003; Feely et al., 2004; Orr et al., 2005).

During the last 250 years, an increase in hydrogen ions (H^+) of about 30% has been recorded. As a consequence, the surface water pH of oceans has decreased from 8.16 to 8.05 unit of pH. As for the atmospheric concentration of CO_2, such variation in sea water pH values has never been experienced during the last 20 million of years on Earth (Feely et al., 2004). An additional reduction between 0.3 and 0.5 units of pH is expected for the end of the century (Caldeira &

Wickett, 2005; Raven et al., 2005; IPCC, 2007; Caldeira et al., 2007; Feely et al., 2008) and a reduction of 0.7 units of pH for the 2300 (Caldeira & Wickett, 2003).

A reduction in pH values could be negative for marine organisms, in particular for those with calcareous structures which could not adapt to the new environment conditions (Guinotte & Fabry, 2008). Only recently it has been demonstrated that ocean acidification can cause significant alterations in cellular parameters related to the immune response (Bibby et al., 2008; Matozzo et al. 2012b), in antioxidant responses (Matozzo et al, 2013; Vehmaa et al., 2013; Matoo et al., 2013) in growth rates (Hiebenthal et al., 2013) and reproduction (Vehmaa et al., 2013; Uthicke et al., 2013) of marine invertebrates. But GCCs are not the only threat that marine organisms have to cope with.

Pharmaceuticals as emerging contaminants

Pharmaceuticals are bioactive substances used in human and veterinary medicine to cure or prevent different pathologies. Together with personal care products they lay in a great category of chemicals collectively identified as “pharmaceutical and personal care products” (PPCPs).

Pharmaceuticals are designed to have a specific mode of action, and many of them to have some persistence in the body. According to their mode of action, they are divided into different classes , which are relevant for their potential toxicity, high consumption and environmental persistence: non-steroidal anti-inflammatory drugs

(NSAIDs), beta-blockers, neuroactive compounds, steroidal hormones and antibiotics (Fent *et al*., 2006).

Public and scientific concern about these compounds has born in the '90s, since then many studies have focused only on the presence and the behaviour of PPCPs in the aquatic ecosystems, but very little is known about their possible effects on non-target organisms.

Aquatic organisms are continuously exposed to a wide range of PPCPs which come into the environment through wastewater treatment plants or because directly discharged.

It is currently known that residues of pharmaceuticals at trace quantities are widespread in aquatic systems suggesting a low risk for acute exposure, while for chronic effects the situation might be different (Fent et al., 2006). There is a need to focus on long-term exposure assessment aimed at evaluating specific modes of action and effects of pharmaceuticals on different life-stages of aquatic organisms.

Environmental sources, fate and levels of pharmaceuticals

Consumption of pharmaceuticals, in particular of antibiotics and anti-inflammatory drugs, is constantly growing, both in human and veterinary medicine.

In Germany for instance, 75 tons of diclofenac, one of the most common and wide spread NSAIDs, are sold every year (Ternes, 2001) (Tab. 1).

Annual consumption of different classes of prescribed drugs for different countries

Compounds	Germany 1999[a]	Germany 2000[a]	Germany 2001[a]	Austria 1997[b]	Denmark 1997[c]	Australia 1998[d]	England 2000[e]	Italy 2001[f]	Switzerland 2004[g]
Analgesics, antipyretics and anti-inflammatory									
Acetylsalicylic acid	902.27 (1)	862.60 (1)	836.26 (1)	78.45 (1)	0.21 (7)	20.4 (9)			43.80 (3)
Salicylic acid	89.70 (12)	76.98 (17)	71.67 (17)	9.57 (11)					5.30 (6)
Paracetamol	654.42 (2)	641.86 (2)	621.65 (2)	35.08 (2)	0.24 (6)	295.9 (1)	390.9 (1)		95.20 (1)
Naproxen				4.63 (16)		22.8 (7)	35.07 (12)		1.70 (12)
Ibuprofen	259.85 (5)	300.09 (5)	344.89 (5)	6.7 (13)	0.03 (19)	14.2 (13)	162.2 (3)	1.9 (15)	25.00 (4)
Diclofenac	81.79 (16)	82.20 (14)	85.80 (14)	6.14 (15)			26.12 (16)		4.50 (7)
β-Blocker									
Atenolol							28.98 (13)	22.07 (4)	3.20 (9)
Metoprolol	67.66 (18)	79.15 (16)	92.97 (11)	2.44 (20)					3.20 (10)
Antilipidemic									
Gemfibrazol						20 (10)			0.399 (18)
Bezafibrate				4.47 (17)				7.60 (8)	0.757 (15)
Neuroactive									
Carbamazepine	86.92 (13)	87.71 (13)	87.60 (12)	6.33 (14)		9.97 (18)	40.35 (8)		4.40 (8)
Diazepam					0.21 (8)				0.051 (21)
Antiacidic									
Ranitidine	85.41 (15)	89.29 (12)	85.81 (13)			33.7 (5)	36.32 (10)	26.67 (3)	1.60 (13)
Cimetidine							35.65 (11)		0.063 (20)
Diuretics									
Furosemide					3.74 (1)			6.40 (19)	1.00 (14)
Sympatomimetika									
Terbutalin					0.46 (3)				0.0099 (23)
Salbutamol					0.17 (9)				0.035 (22)
Various									
Metformin	368.01 (4)	433.46 (4)	516.91 (3)	26.38 (3)		90.9 (2)	205.8 (2)		51.40 (2)
Estradiol					0.12 (13)				
Iopromide	64.93 (19)	63.26 (19)	64.06 (19)						6.90 (5)

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Tab. 1 - Annual consumption of different classes of prescribed drugs for different countries. For every country a top 20 sold-list is taken into account. Data in bracket represent the position in the ranking list within a country. Data are in t/year.

After their application, pharmaceuticals can be excreted in their native form or as metabolites and enter aquatic systems via different ways (Fig. 2).

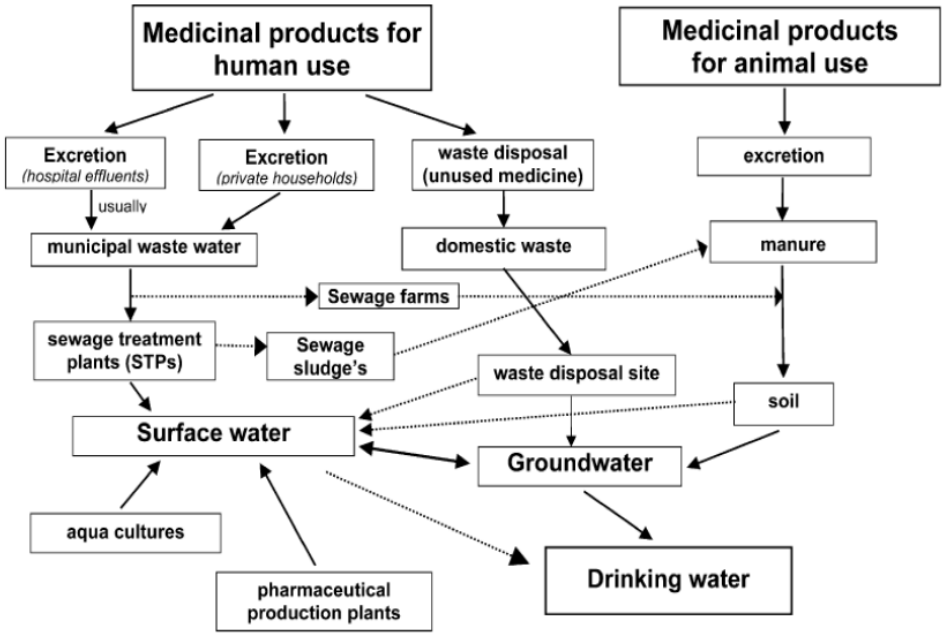

Fig. 2 - Scheme showing possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment. (Heberer, 2002).

Municipal wastewater is therefore the main pathway that brings human PPCPs -after normal use and disposal of unused medicines - into the environment. Hospital, industrial and animal farms' wastewaters may contain significant concentrations of pharmaceuticals (Holm *et al.*, 1995).

Pharmaceuticals that are not readily degraded in the sewage treatment plants (STPs) are discharged in treated effluents resulting in the contamination of rivers, lakes, estuaries and also groundwater and then drinking water.

In addition, veterinary pharmaceuticals may enter aquatic systems via manure application to fields and subsequent runoff, but also via direct application in aquaculture (fish farming).

Of environmental concern is not necessarily a high production volume of a certain pharmaceuticals per se, but the environmental persistence and critical biological activity (e.g. high toxicity, high potency for effects on biological key functions such as reproduction). Pharmaceuticals having environmental relevance share the following properties: often, but not always, high production volume combined with environmental persistence and biological activity, mainly after long-term exposure (Fent et al., 2006).

As soon as they are released into the aquatic environment, the behaviour and fate of pharmaceuticals and their metabolites are not well known. In wastewater treatments two elimination processes are important: adsorption to suspended solids (sewage sludge) and biodegradation. Absorption depends on both hydrophobic and electrostatic interactions of the pharmaceutical with particulates and microorganisms. Non steroidal anti-inflammatory drugs (NSAIDs),

such as ibuprofen, acetylsalicylic acid and diclofenac, are considered acid pharmaceuticals with pK_a values between 4.9 to 4.1 and they occur as ions at neutral pH with a low tendency to be absorbed by sludge. In general, levels of pharmaceuticals in digested sludge and sediments are suggested to be relatively low, as demonstrated in several monitoring studies (Ternes et al., 1998; Urase and Kikuta, 2005).

Irrespective of the chemical characteristics of the compounds, the efficiencies of various STPs also vary for the same compound because their equipment and treatment steps are not equivalent. In addition, other factors, such as water temperature and atmospheric parameters, can influence contaminant persistence and release in STP effluents. For instance, diclofenac showed largely different elimination rates in STPs, from 17% (Heberer, 2002), to 69% (Ternes, 1998), and even 100% (Thomas and Foster, 2004).

Then, in surface waters, other processes like biodegradation and abiotic transformation reactions can occur. One of the main degradation processes for many compounds is photodegradation (Buser *et al.,* 1998; Andreozzi *et al.,* 2003), but its efficiency depends, besides substance properties, on the strength of the solar irradiation, and therefore on latitude and season, and on constituents present in the water that may act as photosensitizers generating hydroxyl radicals and singlet oxygen (i.e. nitrates, humic acids).

First evidences of pharmaceuticals' release into the environment were recorded in treated wastewaters from the U.K. (Richardson and Bowron, 1985). Concentrations range from ng/L to µg/L in superficial

waters and they can reach values in mg/L in wastewater effluents (Fig. 3).

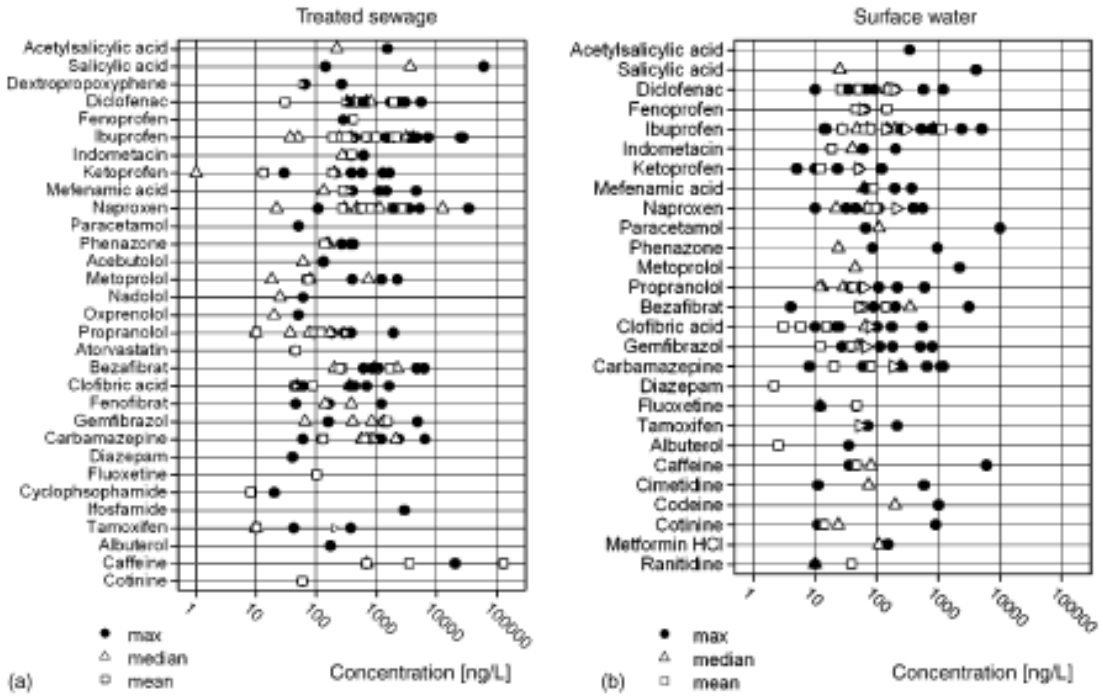

Fig. 3 - Concentration of pharmaceuticals in treated wastewater (a) and urface water (b).(Fent et al., 2006).

These generally low concentrations could seem harmless to aquatic organisms, still many PPCPs can display their toxicity even at environmental concentrations. As an example, the steroidal hormone 17α-etinilestradiol, used in contraceptive pills, has been found to be estrogenic in fish at concentrations between 1 and 4 ng/L (Fent et al., 2006).

The widely used NSAIDs ibuprofen, naproxen, diclofenac and some of their metabolites (e.g. hydroxyl-ibuprofen and carboxy-ibuprofen) are very often detected in sewage and surface water. Ternes (1998) reported levels in sewage exceeding 1 μg/L, and in effluents of

conventional STP (mechanical clarification and biological treatment) concentrations often approach or exceed 0.1 µg/L in the U.S.A. (Gross et al., 2004). In many countries diclofenac was frequently detected in wastewater in the ng/L-µg/L range, and in surface water at lower levels (Stan & Heberer, 1997; Buser et al., 1998b; Ternes, 1998; Stumpf et al., 1999; Farre et al., 2001; Heberer, 2002).

Caffeine was generally found in U.S. streams at maximal levels of 6.0 µg/L (median 0.1 µg/L) (Kolpin et al., 2002) and this compound can even serve as an anthropogenic marker in aquatic systems due to its ubiquity in surface water, seawater (Weigel et al., 2004), and also in groundwater.

Effects of climate change on contaminants' environmental fate and behaviour

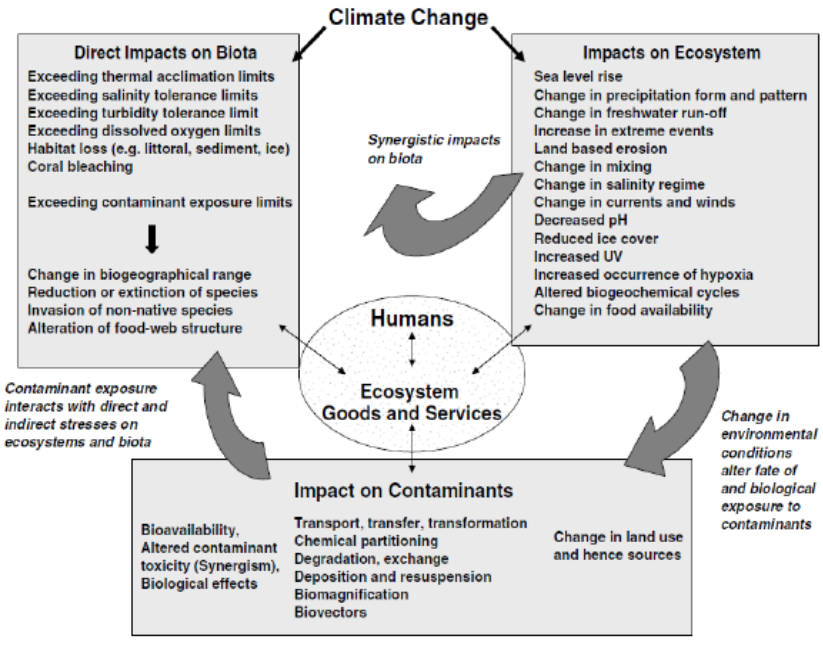

Fig. 4 - Overview of climate change impacts on ecosystem and biota, and how they interact with contaminants, and their fate and effects. (Schiedek et al., 2007).

Climate change may affect the environmental distribution of chemicals and their biological effects (Noyes et al., 2009) (Fig. 4). GCCs modify temperature,

salinity, precipitation rate and sea water pH. Such variations may act on contaminants altering their bioavailability and mode of action.

Consequences of this interaction could affect different biological compartments, both increasing the susceptibility of aquatic organisms to contaminants and compromising environmental health.

The major part of data available are about the most known toxicants, in particular pesticides and POPs (persistent organic pollutants), but their interaction with environmental parameters is dependent on certain dynamics shared among different classes of contaminants, suitable also for PPCPs.

The first aspect to be taken into account is the distribution of a chemical in the aquatic environment. Transport can occur in solution or in association with suspended particles. Diffusion would depend on hydrologic characteristics of the aquatic environment (streams, tides, wind and atmospheric perturbations). These characteristics are also influenced by temperature and salinity which modify oceanic circulation (IPCC, 2007). A contaminant can be transported very far away from its original source and then exhibits its effect on biota.

Transport from an environmental compartment to another depends on several environmental parameters. For instance, global warming may reduce concentration of pesticides in water and soil increasing volatility of these substances. Conversely, higher precipitation rates facilitate the exchange between air and water or land (Bollmhor et al., 2007). Modifying distribution and availability of a contaminant often results in an increased toxicity. Variations in seawater salinity influence bioavailability of many classes of contaminants, facilitating bioaccumulation and biomagnifications. Several studies have

demonstrated that under reduced salinity assumption of heavy metals increases in marine organisms (Lee et al., 1998). Other studies showed that solubility of polycyclic aromatic hydrocarbons (PAH) depends on salinity in estuarine and coastal waters (Ramachandraan et al., 2006).

Recently, an experiment performed to test the effects of increased seawater concentrations of CO_2 combined with heavy metals, demonstrated that in vivo Cd-, Pb-, or Cu pretreatment under reduced pH decreased metallothionein production in soft tissues suggesting that acidification can aggravate heavy metals pollution and toxicity for marine organisms (Han et al., 2014). Furthermore, in the same study, the mortality of *Mytilus edulis* increased with reduced pH in all heavy metals (Cd, Pb, Cu) treatments compared to control group.

In another study, the exposure to different levels of pH (pH 8.1, 7.85 and 7.6) and temperature (16 and 19°C), resulted in a significant increase of the absorption of the radionuclide ^{110m}Ag (1 kBq/L) in the eggs of the common cuttlefish, *Sepia officinalis*, kept under reduced pH and high temperature (Lacoue-Labarthe et al., 2009).

Patra et al. (2007) demonstrated that exposure to sublethal concentrations of pesticides (endosulfan and chlorpyrifos) decreased maximum thermal tolerance in four freshwater fish species: at higher temperature the organisms may have dealt with greater amount of toxicants because of increased diffusion or more active uptake, thus enhancing toxic effects. Buckman et al. (2007) found that in rainbow trout the transformation of PCBs (polychlorinated biphenyls) in their

active and toxic forms was facilitated by an increment of environmental temperature.

Then, some populations, particularly those living at the edge of their homeostatic or physiological tolerance range, may become more vulnerable to the dual stress of climate change and contaminant exposure (Cranford, 2003; Heath et al., 1994; Patra et al., 2007).

In spite of the majority of studies in this field concerns well known substances (POPs, pesticides and heavy metals), there are very few published data about emerging contaminants such as pharmaceutical products.

In a recent study, the effects of environmental parameter variations on the toxicity of different pharmaceuticals were evaluated in *Daphnia magna*. Among pharmaceuticals tested, paracetamol (an analgesic), enrofloxacin and sulfatiazol (both antibacterics) increased their acute toxicity with decreasing pH up to level around 7.4 pH units. Also the increment of temperature increased the toxicity of the three pharmaceuticals (Kim et al., 2010).

Data reported above represent an example of how toxicological stress interact with environmental stress factors in aquatic organisms (Hummel et al., 2011). Animals living under conditions close to their environmental tolerance limits appear more vulnerable to additional chemical stress, and exposure to sublethal concentrations of a contaminant may result in more detrimental or even lethal effects. As a consequence of synergistic interactions between stressful natural factors and pollutants the ecological niche of a species and its distribution may be sensibly altered. Since coping with environmental changes will be a pressing challenge for marine organisms in the near

future, further insights into potential interactions of multiple stressors are urgently needed.

AIM OF THE STUDY

The aim of this PhD project was to evaluate the combined effects of seawater acidification, as predicted by climate change scenarios, and two emerging contaminants, the non-steroidal anti-inflammatory drug diclofenac and the metabolism activator caffeine on different biological parameters, from molecular to organism level, in two marine bivalve species, the Mediterranean mussel *Mytilus galloprovincialis* and the Manila clam *Venerupis philippinarum*.

M. galloprovincialis is widely distributed both in the Lagoon of Venice and along the coast of the North Adriatic Sea, while *V. philippinarum* is widespread in the Lagoon basin only. Furthermore, mussels live on hard substrates in the water column, more subject to fluctuating conditions, while clams live buried in sandy-muddy sediments which shelter them and mitigate variations in environmental parameters.

It is very important to consider the ecological repercussions that GCCs may have in narrow marine environments, such as the North Adriatic Sea and the Lagoon of Venice. The limited extension, the shallow waters and a big input of fresh waters from rivers may make them more susceptible to climate changes. Furthermore, because of the presence of several river estuaries, the high urbanization and the variety of industrial activities along the coast and in the mainland, the North Adriatic Sea and the Lagoon of Venice, are characterized by the presence of a wide range of contaminants. Of the benthic

populations, potentially more affected by environmental changes, marine bivalves represent a relevant component and they play fundamental ecological and economical roles, since they are fished and farmed.

The use of marine bivalves represents an useful tool to predict and to understand the impact of seawater acidification and emerging contaminants.

One of the main goals in this project was to highlight the interactive effects of seawater acidification and contaminants. This approach allow a better insight into responses of marine animals to environmental changes, contrary to many studies which examine the effects of multiple stressors using separate experiments for each stressor or do not directly analyze interactive terms. As clearly stated by Byrne & Przeslawski (2013), this condition limits the ability to directly measure the relationships between stressors.

Experiments involving multistressors should consider ecologically realistic conditions (e.g. environmentally relevant concentrations of contaminants) that include current mean-ambient and extreme conditions in the habitat, and increases in acidification based on the predicted climate change scenarios.

For this reason we chose to expose adult animals for the first week, just to different levels of pH, including current mean-ambient and predicted conditions, in order to acclimatize the animals and then verify whether or not the previous exposure to reduced pH may increase (or decrease) the susceptibility to the contaminants.

Moreover, intertidal and shallow water species that routinely experience fluctuations in temperature, salinity and pH, and species

that naturally experience low pH water due to upwelling or infaunal species inhabiting low pH sediments, may have some resilience to warming and/or acidification (Matson et al., 2012; Talmage and Gobler, 2009, 2011; Wolfe et al., 2013). In contrast, species from relatively invariable habitats may be more vulnerable (Melzner et al., 2009).

Although the two species used in this study have proven to be relatively tolerant to environmental stressors, they are representative of different habitats with different characteristics. Bearing in mind the ecological features of the studied species, mussels could be expected to be more tolerant to shifts in environmental parameters.

A further issue addressed in this study was the potential different sensitivity of clams to changing environmental conditions, when experienced in different stages of their life cycle.

The choice of investigating the combined effects of seawater acidification and emerging contaminants, both in adults and early-life stages, was made to understand which stage may be the most vulnerable to this kind of stressful conditions.

The planktonic embryos and larvae of benthic marine invertebrates are likely to be very sensitive to environmental changes (Pechenik, 1987), but we have a limited understanding about potential synergistic effects of multiple stressors. This study tried to fill the gap. In addition, first evidence will be provided to verify the hypothesis that higher sensitivity to environmental stressors makes planktonic stages of clams a population bottleneck in a changing environment (Byrne, 2012).

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4. RESULTS

4.1. Research article I: Combined effects of seawater acidification and diclofenac on haemocyte parameters of two marine bivalve species

4.1.1. Introduction

Marine organisms are exposed to a wide range of anthropogenic substances, many of them considered as emerging contaminants due to their growing production and not well-known environmental impact.

In this regard, increasing concern arises from the release of pharmaceutical products into the aquatic environments.

Pharmaceuticals are designed to target specific metabolic and molecular pathways in humans and animals, but they often have important side effects as well. Recently, acute toxicity of pharmaceuticals has been tested on organisms belonging to different taxa, such as algae (Yang et al., 2008), cnidarians (Quinn et al., 2007), crustaceans (Haap et al., 2008) mollusks (Canesi et al., 2007) and fish (van den Brandhof & Montforts, 2010), and their effects have been displayed at different levels of biological organization.

Among pharmaceuticals, the non-steroidal anti-inflammatory drug NSAID diclofenac is very often detected in sewage and surface water since it is widely used in both human and veterinary medicine (Fent et al., 2006).

Diclofenac acts by inhibiting either reversibly or irreversibly one or both of the two isoforms of the cyclooxygenase enzyme (COX-1 and COX-2), which catalyze the synthesis of prostaglandins from arachidonic acid (Vane and Botting, 1998) in mammals. In other organisms diclofenac can cause different effects such as renal lesion and gill damage in rainbow trout (Schwaiger et al., 2004), or kidney damage in vultures (Oaks et al., 2004). In the freshwater bivalve *Dreissena polimorpha* the cytogenotoxicity of three common NSAIDs (diclofenac, ibuprofen and paracetamol) was evaluated using an in vitro biomarker approach on the haemocytes (Parolini et al., 2009), revealing that each tested drug was able to compromise haemocytes' functionality starting from the lowest dose (0.2 μM). Despite this, very little is known about the effects of NSAIDs on immunomarkers in bivalves.

Besides environmental contaminants, marine organisms have to face another issue that is cause of concerning, global climate changes (GCCs).

The shift of environmental parameters can affect marine organisms by altering many biological pathways. Haemocyte-mediated immune parameters have been shown to be particularly affected by environmental factors such as temperature and salinity (Monari et al., 2007; Matozzo et al., 2007) and pH (Bibby et al., 2008). More recently, Matozzo et al. (2012) showed that in the mussel *Mytilus galloprovincialis* and the clam *Chamelea gallina* the combined exposure to different levels of temperature, salinity and reduced pH can significantly alter haemocyte functionality.

But climate changes not only will influence aquatic organisms, they will have a powerful effect on the environmental fate and behaviour of chemical toxicants as well, by altering physical, chemical, and biological drivers of partitioning between atmosphere, water, soil/sediment, and biota, including: air-surface exchange, wet/dry deposition, and reaction rates (e.g., photolysis, biodegradation, oxidation in air) (Noyes et al., 2009). Furthermore, shifts in environmental parameters could alter marine organisms' susceptibility to these compounds. It has been demonstrated that lysosomal and cell membranes of haemocytes from the mussel, *Mytilus edulis*, were destabilised at 0°C compared with those of haemocytes from mussels acclimated at 10°C when animals were exposed to 500 µg/L of phenanthrene (a polycyclic aromatic hydrocarbon) for four days (Camus et al., 2000). Recently, Lewis et al. (2013), have observed that the exposure to copper (0.002 µM) in acidified condition (pH 7.2) significantly reduced larval survival in the intertidal polychaete *Pomatoceros lamarckii*. However the knowledge about the possible effects of anthropogenic pollutants, in particular emerging contaminants such as pharmaceutical products, and GCCs is still lacking.

Thus, with the aim of filling the lack of information on these issues, in this study the combined effects of low pH, as predicted by climate change scenarios, and diclofenac, on haemocyte parameters, such as total haemocyte count (THC), Neutral Red (NR) uptake, lysozyme activity in haemocyte lysate (HL) and cell free haemolymph (CFH), and haemocyte proliferation, were investigated for the first time in

two important bivalve species, the mussel *Mytilus galloprovincialis* and the clam *Venerupis philippinarum*.

4.1.2. Materials and Methods

Animals

Specimens of *M. galloprovincialis* (4.0 ± 0.5 cm shell length) and *V. philippinarum* (3.5 ± 0.5 cm shell length) were collected along the west coast of the Northern Adriatic Sea (near Cavallino Treporti, Italy) and in the Lagoon of Venice (near Chioggia, Italy), respectively, and immediately transferred to the laboratory. Bivalves were carefully checked for shell damage (damaged animals were not used for experiments), and epibionts (such as barnacles and algae) were removed from the mussels. Prior to starting exposure, bivalves were acclimatised to the experimental conditions by gradually increasing or decreasing the seawater temperature (2°C per day) to 22°C and by gradually decreasing pH values from 8.1 to 7.7 and 7.4.

Experimental setup for bivalve exposure

The experimental flow-through system (Fig. 1) used for both mussel and clam experiments was built inside the Idrobiological Station 'Umberto D'Ancona' at Chioggia. It consisted of a main outside reservoir filled with natural seawater (about 1000 L of capacity). The seawater was then pumped into two tanks (about 300 L each) inside the laboratory and equilibrated to the controlled laboratory temperature (20°C). The seawater next moved on to three tanks of approximately 120 L capacity, where the pH was adjusted to

experimental values by bubbling CO_2 using an automatic control system (ACQ110 Aquarium Controller Evolution by Aquatronica) connected with pH electrodes (ACQ310N-PH by Aquatronica). The seawater was finally pumped (25 ml min^{-1}, using ACQ450 Dosing pumps by Aquatronica) into the experimental tanks (A, B and C, approximately 50 L each) containing the bivalves.

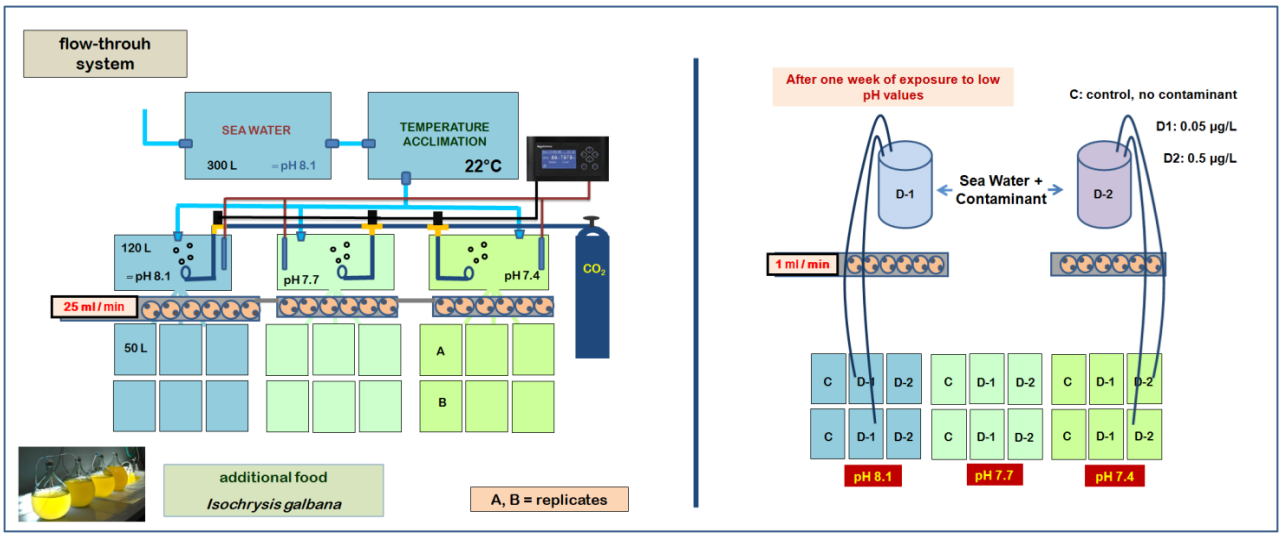

Fig. 1 - Experimental flow-through plant.

During the first week of exposure, animals were subjected to three pH values (8.1, as control, 7.7 and 7.4). During the second and the third week, they were exposed to three concentrations of diclofenac (0, 0.05 and 0.5 µg/L) for each pH value. For each concentration, two replicates (with 70 bivalves each) were constituted (control without contaminant: C-A, C-B; diclofenac 0.05 µg/L: D1-A, D1-B; diclofenac 0.5 µg/L: D2-A, D2-B).

The experimental concentrations of diclofenac were maintained by distributing in continuous with peristaltic pumps stock solutions of the contaminant (1 ml min^{-1}, using a MCP Process Pump, mod.

ISM915A, provided with a MS/CA pumphead, mod. ISM724A, by ISMATEC) from two 15 L glass jars. Taking into account the two combined flows (25 ml min^{-1} for seawater and 1 ml min^{-1} for the contaminant solution, respectively), the concentrations of diclofenac inside the two glass jars were calculated to be 25 times higher than the concentrations needed inside the respective tanks with bivalves.

Of the experimental conditions tested, the control without diclofenac at 8.1 pH was considered as a reference.

During the experiments, animals were fed with microalgae (*Isochrysis galbana*). Twice a day, 250 ml of algae (3.5 x 10^6 cells ml^{-1}) were added in each 120 L tank, in order to keep algae concentration of about 2000 cells/ml in the experimental tanks with animals. Bivalve mortality was checked daily. At the end of the first (T0), the second (T1) and the third (T2) week of exposure, samples of haemolymph were collected for each condition tested (3 pools per tank, 7 animals per pool) to measure haemocyte parameters.

Haemolymph collection

Haemolymph (approximately 300 µl per animal) was collected from the anterior adductor muscle with a 1-ml plastic syringe and stored in ice. An equal volume of 0.38% sodium citrate (Sigma) in 0.45 µm-filtered sea water (FSW) with a pH of 7.5 was added to haemolymph samples to prevent clotting. One hundred µl of pooled haemolymph were used to determine the THC; 500 µl and 400 µl of haemolymph were used to assay the NR uptake and the haemocyte proliferation, respectively. One hundred µl of pooled haemolymph (without

sodium citrate) were used to measure both lysozyme-like activity and total protein concentration in HL and CFH.

THC, haemocyte volume and diameter determination

A Scepter^{TM} Handheld Automated Cell Counter (Millipore) was used to determine THC and haemocyte volume and diameter. 20 µl of pooled haemolymph were added to 2 ml of isotonic solution (COULTER ISOTON II Diluent). THC results were expressed as the number of haemocytes (x10^6) ml haemolymph^{-1} . The haemocyte diameter and volume were expressed in µm, and in picolitres (pl), respectively.

Neutral Red (NR) uptake assay

NR uptake assay was performed according to the procedure reported in previous studies (Cajaraville et al., 1996; Matozzo et al., 2002). Pooled haemolymph was centrifuged at 780 g for 10 min. Haemocytes (at a final concentration of 10^6 cells ml^1) were resuspended in an equal volume of 8 mg l^{-1} NR dye (Merck) solution in FSW, and incubated at room temperature for 30 min. They were then centrifuged at 780 g for 10 min, re-suspended in distilled water, sonicated at 4°C for 30 s with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12000 g for 15 min at 4°C. The supernatant, corresponding to HL was collected for the NR uptake assay. Absorbance at 550 nm was recorded on a Beckman 730 spectrophotometer.

The results were expressed as optical density per ml haemolymph (OD ml haemolymph^{-1}).

Haemocyte proliferation

Hemocyte proliferation was evaluated using a colorimetric method with a commercial kit (Cell proliferation Kit II, Roche). The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye in metabolically active (viable) cells. Briefly, the XTT labelling reagent and electron-coupling reagent were thawed at 37°C and mixed immediately before use to obtain the XTT labelling mixture. A total of 200 µl of the mixture was added to 400 µl of pooled hemolymph and incubated for 4 h in a dark humidified chamber. The absorbance at 450 nm was subsequently recorded using a Beckman 730 spectrophotometer. The data were normalized to THC values that were recorded in clams from each experimental condition and expressed as optical density (OD) values at 450 nm.

Haemolymph lysozyme activity assay

Lysozyme activity was quantified in both HL and CFH. Pooled haemolymph was centrifuged at 780 g for 10 min. The supernatant, corresponding to CFH, was collected, whereas the haemocytes were resuspended in distilled water, sonicated at 4°C for 1 min, and then centrifuged at 780 g for 30 min to obtain HL. CFH and HL were frozen and stored at -80°C before analyses. Fifty ml of CFH and HL were added to 950 ml of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer, pH 6.2, and the decrease in absorbance ($\Delta A\ min^{-1}$) was continuously recorded at 450 nm for 5 min at room temperature. Standard solutions containing 1, 2.5, 5 and 10 µg lysozyme per ml of 66 mM phosphate buffer, pH 6.2, were prepared from crystalline hen egg white lysozyme (Sigma). The

average decrease in absorbance per minute was determined for each enzyme solution, and a standard curve of enzyme concentration versus ΔA min^{-1} was drawn. One unit of lysozyme was defined as the amount of enzyme producing activity equivalent to 1 mg of lysozyme, in the conditions described above. Results were expressed as µg lysozyme mg $protein^{-1}$.

Haemolymph protein concentration

Protein concentrations in CFH and HL were quantified using a commercial kit (Quick Start Bradford protein assay, BIO-RAD, Hercules, CA, U.S.A.). Twenty microlitres of CFH and HL were incubated at room temperature for at least 5 minutes with 1 ml of 1x dye reagent. Bovine serum albumin was used as a standard (Bradford, 1976).The absorbance was measured at 595 nm, and the results were expressed as mg protein ml $haemolymph^{-1}$.

Statistical analysis

Haemocytes parameters (i.e. THC, haemocyte volume and diameter, NR uptake, haemocyte proliferation) were analysed with a permutation-based nonparametric MANOVA (Pesarin, 2001). For each parameter, a nonparametric full factorial ANOVA was set with factors pH, Contaminant and Time (the last as a repeated measure). To take in account the dependence of measures within the same tank, a mixed-model approach was used (Finos and Basso, 2012, 2013).

4.1.3. Results

Results from the MANOVA analysis for mussels and clams are reported in Tab. 1.

M. galloprovincialis

At T0, THC was not affected by pH, while at T1 a significant effect of pH ($p<0.002$) on THC values was observed. At T1, the effects of diclofenac and diclofenac/pH interaction were not statistically significant, even if a reduction in THC values was recorded in animals kept at pH 8.1 and 7.7 in the presence of the contaminant. At T2, THC was significantly affected by pH showing lower values in mussels kept at pH 7.4 compared to those maintained at pH 8.1 and 7.7; neither diclofenac nor diclofenac/pH interaction significantly influenced THC values, even if a slight reduction was observed in animals exposed to the contaminant both at pH 8.1 and at pH 7.7.

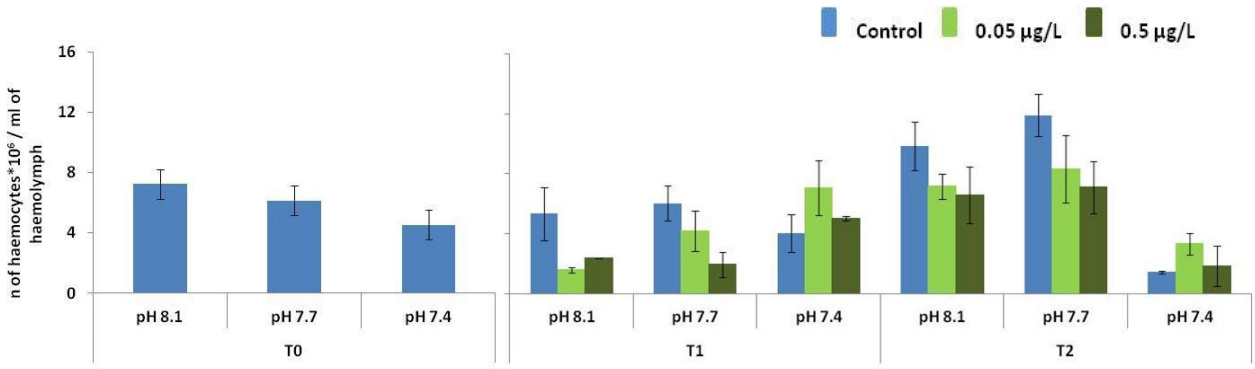

Fig. 2 - THC in *M. galloprovincialis*. Values are means ± SE.

At all the sampling times (T0, T1 and T2), the experimental conditions tested did not affect significantly haemocyte volume and diameter. At T1 and T2, for both parameters a slight increase was observed in the presence of diclofenac, at pH 7.7 and 7.4.

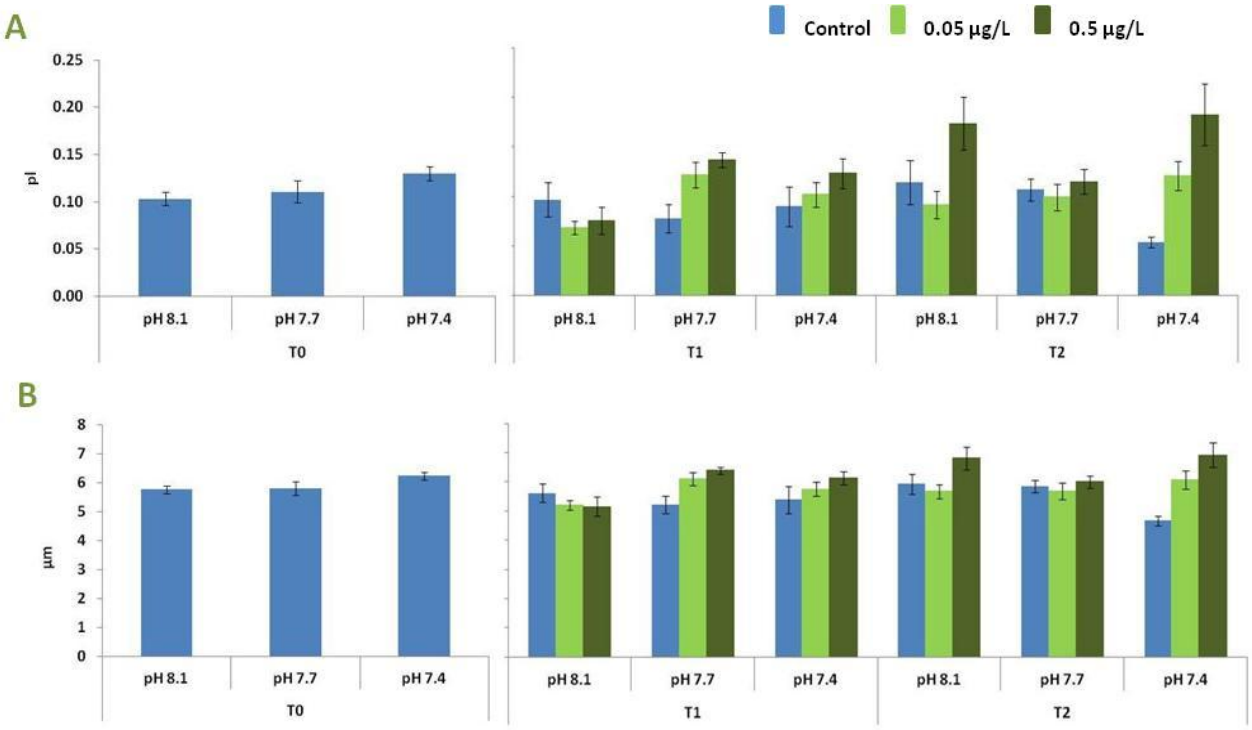

Fig. 3 – Haemocyte volume (A) and diameter (B) in *M. galloprovincialis*. Values are mean ±SE.

Only at T0, NR uptake significantly increased ($p<0.000$) in animals kept at pH 7.7 and 7.4 (Fig X). Despite increased NR uptake values were observed at T1 in animals kept at pH 7.4, no significant effects due to pH, diclofenac or their interaction were highlighted. At T2, NR uptake levels in animals kept at pH 7.7 and 7.4 were quite similar to those of animals maintained at pH 8.1, at all diclofenac concentrations tested. Indeed, no significant effects of experimental conditions were recorded by MANOVA.

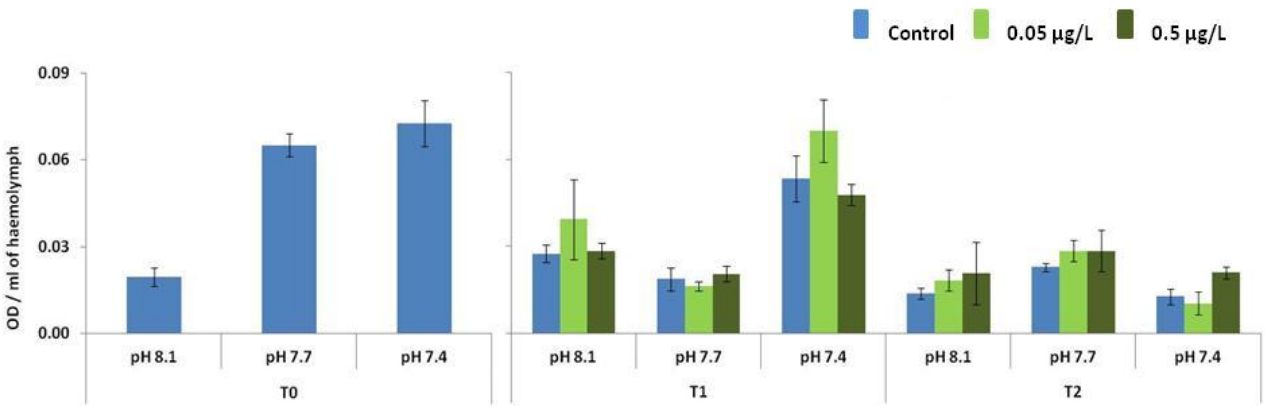

Fig. 4 – NR up-take in *M. galloprovincialis*. Values are mean ±SE.

Haemocyte proliferation was significantly influenced by pH at T1 ($p<0.049$), showing increased values in animals kept at pH 7.7 and 7.4 compared to controls. No significant effects due to the presence of diclofenac were highlighted, even if an increasing trend was observed in haemocyte proliferation of diclofenac-treated mussels at all pH tested, both at T1 and T2 (Fig. 5).

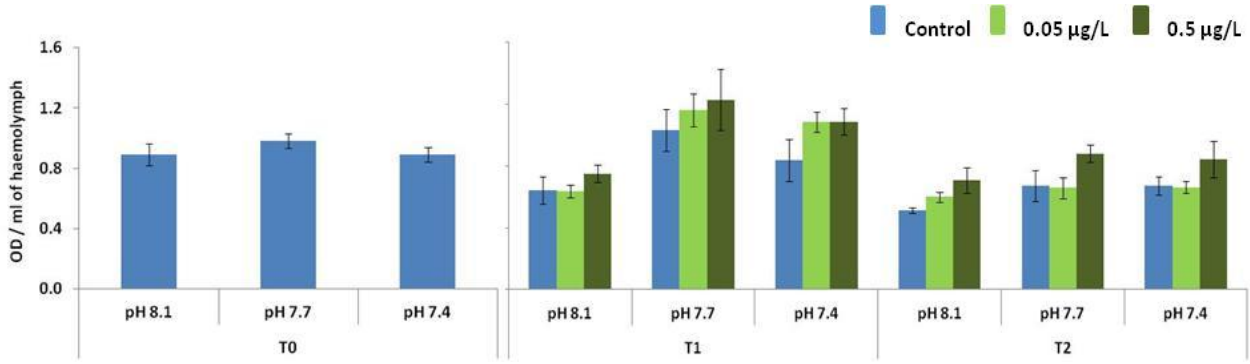

Fig. 5 – Haemolymph proliferation in *M. galloprovincialis*. Values are mean ± SE.

Lysozyme activity was not affected by the experimental conditions either in haemolymph or in haemocytes, at all haemolymph sampling times. Generally, at T0 and T1 a slight decrease in lysozyme activity

was observed in CFH for animals kept at pH 7.4 when compared to those kept at pH 8.1 and 7.7.

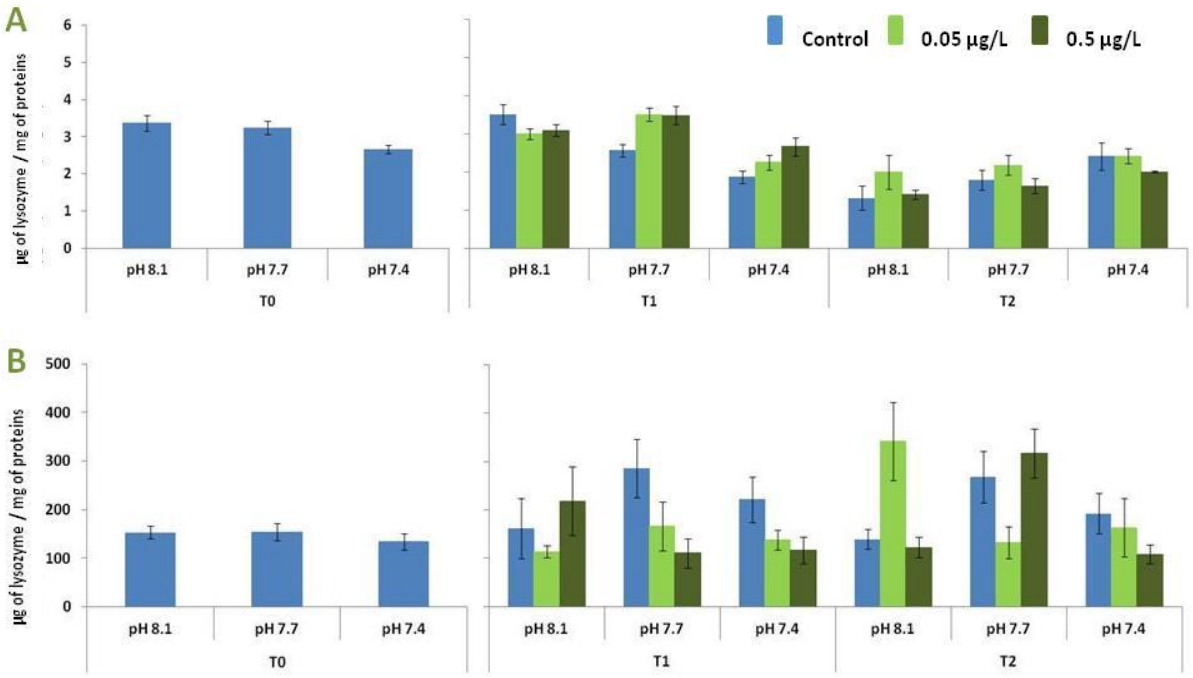

Fig. 6 – CFH lysozyme activity (A) and HL lysozyme activity (B) in *M. galloprovincialis*. Values are mean ± SE.

Venerupis philippinarum

At T0, THC was not significantly affected by pH even though increased values were observed with decreasing pH. At T1, THC was significantly influenced by pH ($p<0.026$) but not by diclofenac and diclofenac/pH interaction. Results showed that in animals kept at pH 7.7 THC levels were lower than those at pH 8.1 and 7.4.

Differences among THC levels in animals maintained with or without diclofenac were recorded at pH 8.1 and 7.4, but they were not significant (Fig. 7). At T2, THC was not influenced by anyone of the factors considered.

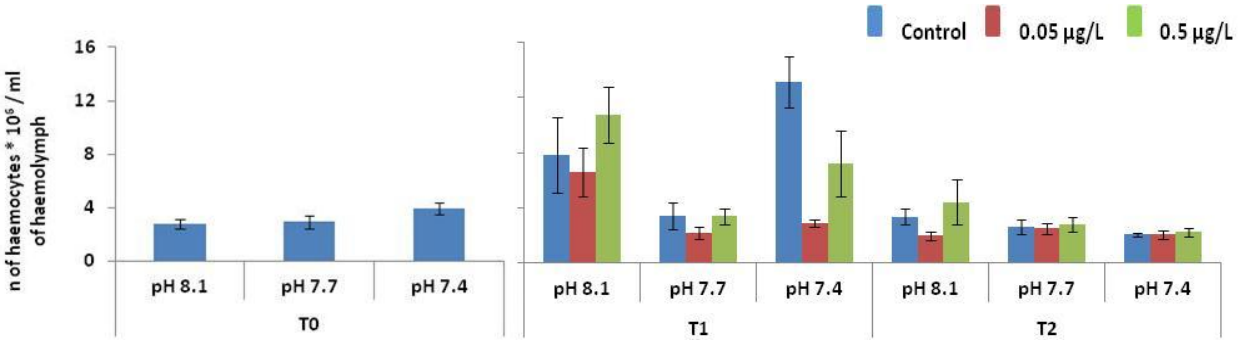

Fig. 7 – THC in *V. philippinarum*. Values are mean ± SE.

Haemocyte volume and diameter were not influenced by the experimental conditions tested at any sampling time. At T0, haemocytes of clams kept at low pH were slightly smaller than those of animals kept at pH 8.1, while at T1 and T2 there was not a clear trend of variation due to the maintenance at different pH values or to the exposure to diclofenac.

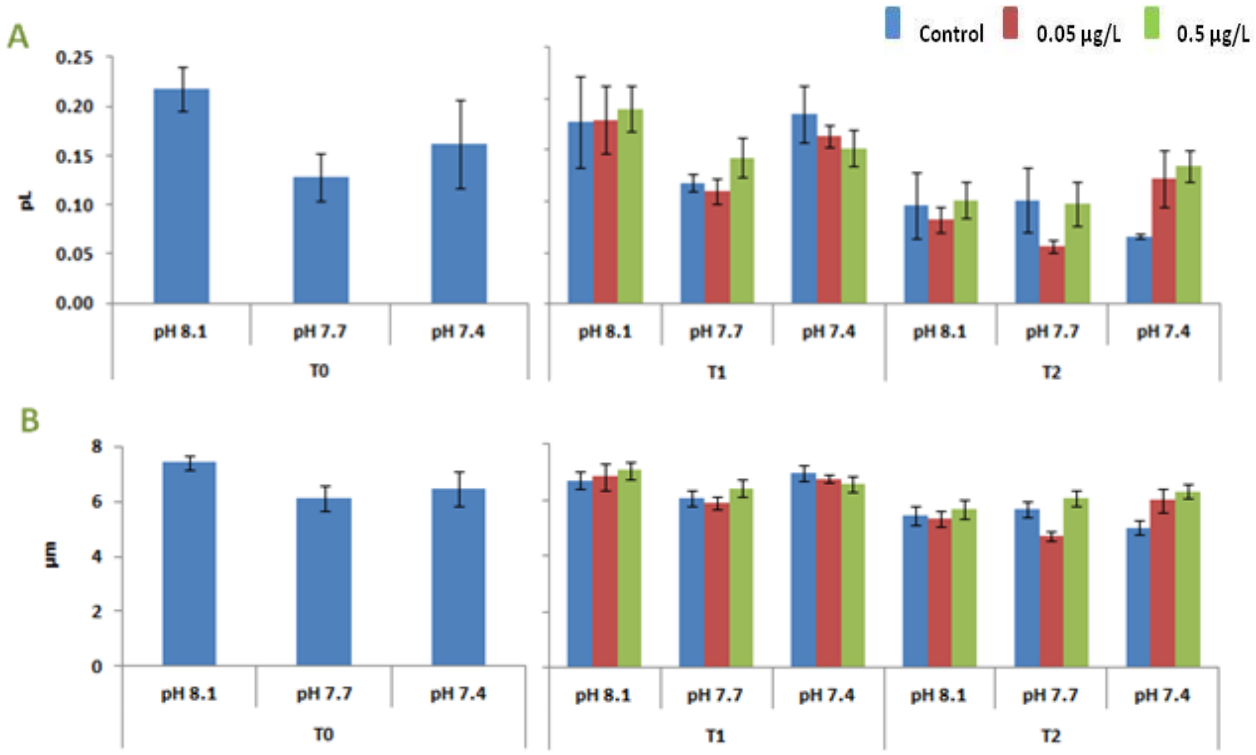

Fig. 8 – Haemocyte volume (A) and diameter (B) in *V. philippinarum*.

Values are mean ± SE.

At T0, MANOVA results show that NR uptake was negatively affected by pH ($p<0.000$) with a strong reduction in the values measured in animals kept at pH 7.7 and 7.4 (Fig. 9). NR uptake was also influenced by pH ($p<0.000$) both at T1 and T2. At T1 there was still a reduction in NR uptake values due to low pH, while at T2 an opposite trend was found, with a significant increase in animals kept at pH 7.7 and 7.4 respect to pH 8.1. No significant effects due to diclofenac and diclofenac/pH interaction were observed at T1 and T2.

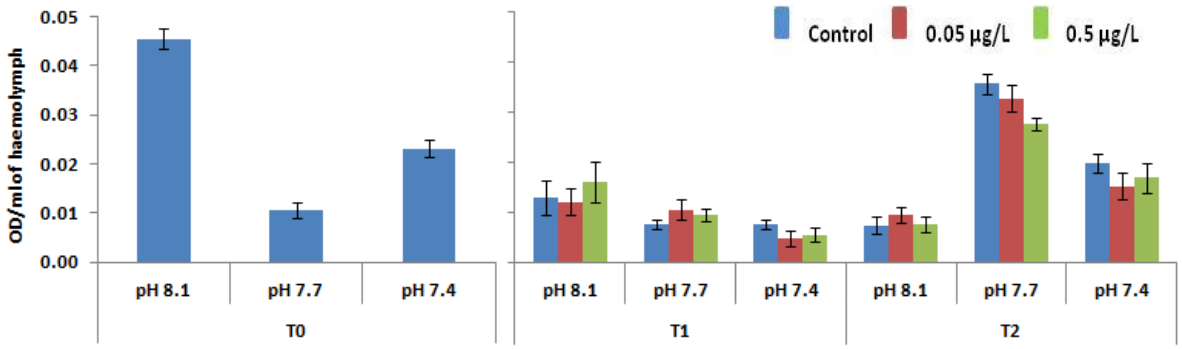

Fig. 9 – NR up-take in *V. philippinarum*. Values are mean ± SE.

Haemocyte proliferation was significantly influenced by pH at T0 ($p<0.000$). Although an increase in cell proliferation was observed at T1 in 7.4 pH-treated clams, no significant effects of pH, contaminant or their interaction were highlighted by MANOVA at both T1 and T2 (Fig. 10).

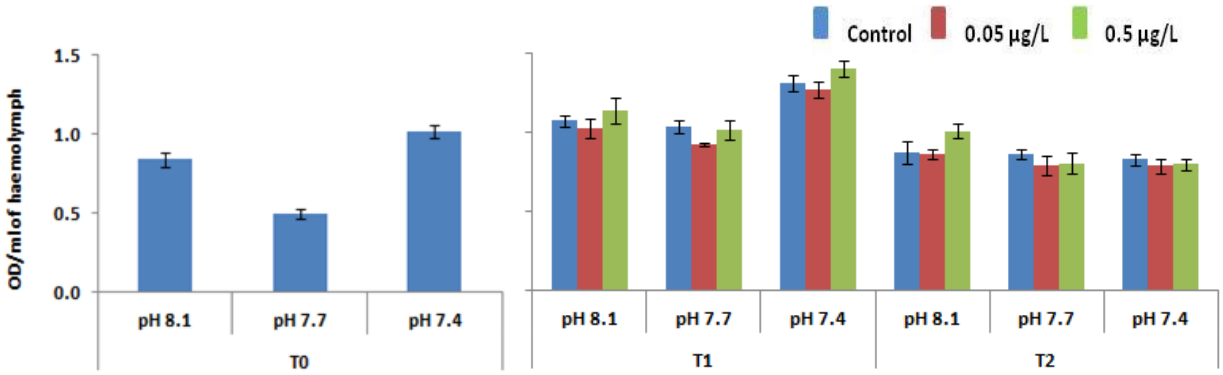

Fig. 10 – Haemocyte proliferation in *V. philippinarum*. Values are mean ± SE.

At T0, lysozyme both in CFH and HC was not affected by pH. MANOVA revealed that enzymatic activity was influenced by the presence of diclofenac both at T1 ($p<0.007$) and T2 ($p<0.018$) in haemocytes, but not in the haemolymph. In particular, both at T1 and T2 lysozyme activity of haemocytes was higher in animals kept at pH 8.1 with diclofenac than in controls (at T2 no activities were detected in controls, Fig. 11-B).

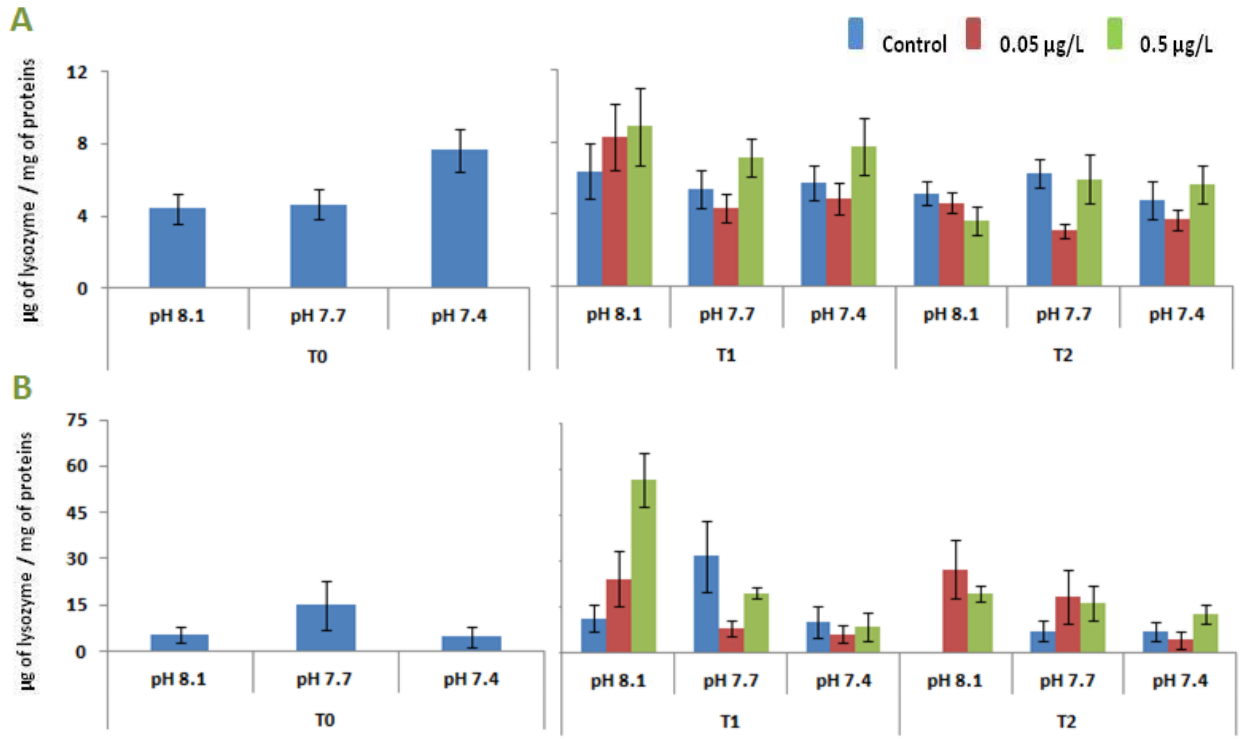

Fig. 11 – CFH lysozyme activity (A) and HL lysozyme activity (B) in *V. philippinarum*. Values are mean ± SE.

Sampling time	Species	Factor	THC	Haemocyte volume	Haemocyte diameter	NR up-take	Haemocyte proliferation	CFH Lysozyme	HL Lysozyme
T0	*M. galloprovincialis*	pH	ns	ns	ns	**0.000**	ns	ns	ns
	V. philippinarum		ns	ns	ns	**0.000**	**0.000**	ns	ns
T1	*M. galloprovincialis*	pH	**0.002**	ns	ns	ns	**0.049**	ns	ns
		diclofenac	ns	ns	ns	ns	ns	ns	ns
		pH*diclofenac	ns	ns	ns	ns	ns	ns	ns
	V. philippinarum	pH	**0.026**	ns	ns	**0.000**	ns	ns	ns
		diclofenac	ns	ns	ns	ns	ns	ns	**0.007**
		pH*diclofenac	ns	ns	ns	ns	ns	ns	ns
T2	*M. galloprovincialis*	pH	**0.010**	ns	ns	ns	ns	ns	ns
		diclofenac	ns	ns	ns	ns	ns	ns	ns
		pH*diclofenac	ns	ns	ns	ns	ns	ns	ns
	V. philippinarum	pH	ns	ns	ns	**0.000**	ns	ns	ns
		diclofenac	ns	ns	ns	ns	ns	ns	**0.018**
		pH*diclofenac	ns	ns	ns	ns	ns	ns	ns

Tab. 1 – MANOVA results for haemocytes parameters in *M. galloprovincialis* and *V. philippinarum*. Statistically significant effects are in red.

4.1.4. Discussion

In bivalve molluscs, circulating haemocytes are involved in important functions, such as immune defence (Cheng, 1981; Hine, 1999; Donaghy, 2009) and shell deposition (Mount, 2004). At an immunological level, non-self materials stimulate cell-mediated immune responses, which mainly include haemocytosis (increases in the circulating haemocyte number), phagocytosis or encapsulation of foreign particles (depending on their size), and the production of lysosomal hydrolytic enzymes (lysozyme in particular).

In bivalves, variations of environmental parameters can affect haemocyte functionality as highlighted in several studies, which reported effects of temperature (Monari et al., 2007), salinity (Matozzo et al., 2007) ,and pH alone (Bibby et al., 2008) or in combination with different temperature and salinity levels (Matozzo et al., 2012b).

Furthermore, several studies have pointed out as haemocyte parameters can be affected by the exposure to different contaminants, such as heavy metals (Pipe et al., 1999; Sauvè et al., 2002; Matozzo et al., 2001), organic xenobiotics (Beckmann et al., 1992; Coles et al., 1994) and more recently pharmaceutical products (Gagnè et al., 2006; Binelli et al., 2009; Matozzo et al.., 2012a; Parolini et al., 2011a, b). Variations in haemocyte parameters provide a warning signal on a potential reduction in immunosurveillance.

In this study, the combined effects of pH variations and diclofenac on immune parameters (the number of circulating haemocytes (THC), haemocyte volume and diameter, haemocyte proliferation, Neutral Red uptake, and lysozyme activity in the haemocytes and in the haemolymph) were investigated in two bivalves species, *M. galloprovincialis* and *V. philippinarum*.

THC is one of the most functional among haemocyte parameters in bivalves and it can show strong variations not only between different species but also among individuals of the same species (Matozzo et al., 2008).

Increased THC values are often associated with haemocyte proliferation or movements of haemocytes from peripheral tissues to haemolymph, while reductions in THC values can be caused by cellular lysis or movements from haemolymph to other tissues (Pipe & Coles, 1995).

In the present study, THC was significantly affected by pH in both mussels and clams. However, in mussels THC values were significantly influenced both at T1 and T2, with a strong reduction in circulating haemocytes in animals kept at pH 7.4 after 21 days of

exposure, while in clams THC was significantly influenced only at T1, with levels lower at pH 7.7 compared to pH 8.1 and pH 7.4. Both diclofenac and its interaction with pH did not affect THC, neither in clams nor in mussels, even if in both species negative trends of variation due to the presence of diclofenac were observed at pH 8.1 and 7.7 in mussels.

Data available from literature highlighted variable responses of this biomarker in molluscs exposed to different stressors.

In some studies a reduction in THC was observed following exposure to environmental contaminants. Such result was obtained for instance in *V. philippinarum* after a 7 day -exposure to triclosan (Matozzo et al., 2012a), and in the gastropod *Haliotis diversicolor* after a 7 day-exposure to benzo(a)pyrene (Gopalakrishnan et al., 2009). On the contrary, a significant increase in THC values was recorded in *V. philippinarum* exposed to 4-nonylphenol (Matozzo & Marin, 2005). The same trend was observed in bivalves after exposure to heavy metals and fluoranthene (Pipe et al., 1999; Coles et al., 1994).

Also the reduction in environmental levels of pH demonstrated to influence in contrasting ways THC values in bivalves and other species as well. Significant reductions were reported in the echinoderm *Asteria rubens* after a week of exposure to pH 7.7 respect to pH 8.1 (Hernroth et al., 2011). On the contrary, Bibby et al. (2008) did not observe any significant variations of THC values in *Mytilus edulis* exposed to pH 6.5 for 32 days (when the control was pH 7.9).

Another biomarker used in this study was the Neutral Red uptake. Numerous studies have demonstrated that the endocytotic activity (phagocytosis, in particular) of bivalve haemocytes can be affected by both exposure to xenobiotics and changes in environmental parameters (Oliver & Fisher, 1999). NR uptake assay is faster than the phagocytosis assay, but equally responsive. NR dye has been extensively used to evaluate the effects of stressors on lysosomal membrane stability in bivalve haemocytes. Uptake of this cationic dye by viable haemocytes can occur by pinocytosis or passive diffusion across cell membranes, whereas non-viable cells do not take up the dye (Coles et al., 1995). Differences in the degree of dye uptake may reflect damage to cell membranes (including lysosomal membranes) and/or weakening of haemocyte pinocytotic capability (Matozzo & Marin, 2005). Our results demonstrated that exposure of *M. galloprovincialis* and *V. philippinarum* to reduced pH and diclofenac resulted in a different pattern of variation in haemocyte NR uptake between mussels and clams.

In particular, in mussels, a significant effect of pH was noticed at all haemolymph sampling times. An increase in NR uptake values was observed with diminishing pH at T0. Such increment was maintained only a pH 7.4 at T1, while at the same pH an opposite trend was recorded at T2. Similarly, in a study on mussels (*M. galloprovincialis*) collected in the Biscay Bay (Spain), Cajaraville et al. (1996) recorded a significant increase in NR uptake values in animals collected in a contaminated site compared to the reference site. On the contrary, results obtained by Matozzo et al. (2012b) showed a significant reduction in NR uptake levels in *C. gallina* kept at low pH (7.4), 22°C

and 34 psu. Similar results were observed in this study in *V. philippinarum,* where at T0 a significant effect of pH on NR uptake values was found, with reduced levels at pH 7.7 and 7.4 respect to pH 8.1. In the two-weeks exposure to different pH levels and diclofenac concentrations there was a strong effect due to the duration of the exposure, with a remarkable increase in NR uptake values recorded at T2 compared to those at T1 in animals kept at reduced pH. These findings highlighted that a prolonged time of exposure may cause different pH-driven responses in animals.

In conclusion, although it is not clear how pH and diclofenac affect haemocyte responses in bivalves, the results obtained in this study indicate that the experimental conditions tested can induce marked alterations in the immune parameters of *M. galloprovincialis* and *V. philippinarum*. Reduced pH, which are predicted to occur in the coming years, more than diclofenac affected the functional responses of haemocytes. Clams showed reduced pinocytotic activity that may result in decreased defence capability. This compromised state could lead up to increased susceptibility of animals to diseases. For this reason, further studies are needed to aim at determining the effects (mainly the combined effects) of GCCs and emerging contaminants on marine bivalves when their immune system is challenged with environmental pathogens.

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4.2. Research article II: Oxidative stress-related responses in the mussel *Mytilus galloprovincialis* and the clam *Venerupis philippinarum* under different combinations of pH values and diclofenac concentrations

4.2.1. Introduction

Changes in abiotic environmental factors, such as increases in temperature, surface water acidification and variations in precipitation regimes and in seawater salinity (IPCC, 2007) are the main aspects in the present and future scenarios of global climate changes (GCCs). GCCs are a consequence of increased impact of anthropogenic activities, in particular those related to the consumption of fossil fuels and deforestation. Indeed, the continuous increase in carbon dioxide (CO_2) emissions in the atmosphere is considered to be one of the most important factors causing GCCs.

The atmospheric CO_2 levels are currently around 380 ppm and could reach 800 ppm by 2100 (Feely *et al.*, 2004). When atmospheric CO_2 diffuses passively into ocean surface waters, it can lead not only to an increase in the partial pressure of CO_2 but also to changes in carbonate chemistry resulting in a reduction in ocean pH. Seawater pH values have already decreased by approximately 0.1 pH units with respect to the pre-industrial levels, and a further reduction by 0.3–0.5 pH units is predicted before the end of the 21st century (Caldeira and Wickett, 2005; Raven et al., 2005; IPCC, 2007). Research has shown that ocean acidification affects many marine organisms in a

variety of marine habitats from tropical to high-latitude ecosystems (Walter et al., 2002; Kroeker et al., 2013) by altering their physiological parameters (Liu and He 2012), antioxidant responses (Matozzo et al., 2013), immune parameters (Bibby et al., 2008; Matozzo et al., 2012) and also their growth rate and survival (Chaparro et al., 2009; Gazeau et al., 2010, Byrne et al., 2013). In particular, marine organisms that built calcareous structures such as corals, echinoderms, molluscs, crustaceans and coralline algae have been indicated as the most vulnerable to seawater acidification (Gattuso et al., 1998; Orr et al., 2005; Gazeau et al., 2007; Moy et al., 2009; Beaufort et al., 2011). Findings from recent studies provide some meaningful examples of this vulnerability. Matozzo et al. (2013) found that the exposure to different combinations of pH, temperature and salinity levels can affect the biochemical responses to oxidative stress in two marine bivalves, Mytilus galloprovincialis and Chamelea gallina.

But climate changes are not the only threat that aquatic organisms, those inhabiting coastal and estuarine habitats in particular, have to face. Indeed, they are continuously exposed to wide range of anthropogenic substances, such as heavy metals, pesticides and pharmaceutical products.

Pharmaceuticals and personal care products (PCCPs) are a class of emerging environmental contaminants that can be detected in various aquatic systems, such as seawater, surface waters, groundwater and effluents from wastewater treatment plants (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al.,

2003). They are usually found in significant quantities, in the ng/L-µg/L range, both in municipal effluents and in surface waters worldwide (Fent et al. 2006; Bound and Voulvoulis 2006; Kasprzyk-Hordern et al. 2008; Santos et al. 2010). Even though PCCPs are generally considered to be not persistent, their continuous release into aquatic ecosystems is a concerning issue. Indeed, PCCPs can be found in the environment either as unmetabolised substances or as metabolites (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003; Bringolf et al., 2010).

Among pharmaceuticals, diclofenac is one of the most common and widely used non-steroidal anti-inflammatory (NSAIDs) drugs. Diclofenac has been found in wastewater treatment plant effluents and coastal environments, in concentrations ranging between 1.23 and 4.47 µg/L (Weigel et al., 2004) and 0.022 and 0.298 µg/L (Rodrìguez-Navas et al., 2013), respectively.

Despite a large amount of analytical data, the knowledge about the possible ecotoxicological effects of NSAIDs on aquatic non-target species, mostly under conditions of chronic exposure to low environmental concentrations, is still very little. Studies should consider the high activity of these molecules, which can seriously affect the health status of the biocoenosis.

In human and veterinary medicine, NSAIDs are well-known cyclooxygenase (COX)-1 and -2 inhibitors, the latter being induced with the onset of pain and inflammation initiated by oxygen radicals, such as nitric oxide and hydrogen peroxide and cytokines (e.g. interleukin-1, 8 and tumor necrosis factor a) (Fent et al., 2006). However, the toxic effects induced by single substances on aquatic

vertebrates and invertebrates have been moderately documented. The exposure of the brown trout (*Salmo trutta*) to 0.5, 5 and 50 µg/L diclofenac for 7, 14 and 21 days significantly reduced haematocrit levels after 7 and 14 days of exposure. Furthermore, after 21 days, diclofenac exposure resulted in increased monocyte infiltration in the liver, telangiectasia in gills, and the occurrence of interstitial hyaline droplets, interstitial proteinaceous fluid and mild tubular necrosis in trunk kidney (Hoeger et al., 2005). The exposure to a mixture of three common NSAIDs, namely, diclofenac, ibuprofen and paracetamol, induced significant enhancements of DNA fragmentation in the zebra mussel *Dreissena polymorpha* (Parolini et al., 2011).

In a global change scenario, scientific community should start considering how shifts in environmental parameters, such as ocean acidification, may interact with chemical contamination. Indeed, it is generally poorly understood, at any level, how sensitivity to pollutants may be affected by changes predicted to occur in environmental conditions (temperature, pH or oxygen level) in the near future.

Marine bivalve molluscs are generally recognized as valuable bioindicator organisms in environmental pollution studies (Regoli, 1998; Torres et al., 2002; Lau and Wong, 2003; Krishnakumar et al., 2006; Verlecar et al., 2006), mostly due to their sedentary and filter feeding habits (Goldberg, 1975). Furthermore, bivalves are important components of coastal and estuarine communities, and they often have an important economical role, being fished and farmed.

For these reasons, in the present study potential oxidative stress and genotoxicity of the NSAID diclofenac under seawater acidification

were investigated for the first time in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis* and the Manila clam *Venerupis philippinarum*. The activities of anti-oxidant enzymes superoxide dismutase (SOD), catalase (CAT), and cyclooxygenase (COX) were evaluated as biomarkers of oxidative stress. Lipid peroxidation (LPO) and DNA strand-break formation were measured to highlight potential oxidative damage.

4.2.2. Materials and Methods

Animals

Specimens of *M. galloprovincialis* (4.0 ± 0.5 cm shell length) and *V. philippinarum* (3.5 ± 0.5 cm shell length) were collected respectively along the west coast of the Northern Adriatic Sea (near Cavallino Treporti, Italy) and in the Lagoon of Venice (near Chioggia, Italy) and immediately transferred to the laboratory. Bivalves were carefully checked for shell damage (damaged animals were not used for experiments), and epibionts (such as barnacles and algae) were removed from the mussels. In the laboratory, the bivalves were maintained for a few days in aerated seawater with salinity, temperature and pH values similar to those measured in the field when the animals were collected. Prior to starting exposure, bivalves were acclimatised to the experimental conditions by gradually increasing or decreasing the seawater temperature (2°C per day) to 22°C and by gradually decreasing pH values from 8.1 to 7.7 and 7.4. The experiments were performed outside of the periods of sexual maturity for mussels (summer) and clams (winter) to reduce the

potential for additional stress related to spawning during the experiments.

Experimental set-up for bivalve exposure

The experimental flow-through system (see Fig. 1, in Combined effects of seawater acidification and diclofenac on haemocyte parameters of two marine bivalve species, this thesis) used for both mussel and clam experiments was built inside the Hydrobiological Station 'Umberto D'Ancona' at Chioggia. It consisted of a main outside reservoir filled with natural seawater (about 1000 L of capacity). The seawater was then pumped into two tanks (about 300 L each) inside the laboratory and equilibrated to the controlled laboratory temperature (22°C) The seawater next moved on to three tanks of approximately 120 L capacity, where the pH was adjusted to experimental values by bubbling CO_2 using an automatic control system (ACQ110 Aquarium Controller Evolution by Aquatronica) connected with pH electrodes (ACQ310N-PH by Aquatronica). The seawater was finally pumped (25 ml min^{-1}, using ACQ450 Dosing pumps by Aquatronica) into the experimental tanks (A, B and C, approximately 50 L each) containing the bivalves.

During the first week of exposure, animals were subjected to three pH values (8.1, as control, 7.7 and 7.4). During the second and the third week, they were exposed to three concentrations of diclofenac (0, 0.05 and 0.5 µg/L) for each pH value. For each concentration, two replicates (with 70 bivalves each) were constituted (control without contaminant: C-A, C-B; diclofenac 0.05 µg/L: D1-A, D1-B; diclofenac 0.5 µg/L: D2-A, D2-B).

The experimental concentrations of diclofenac were maintained by distributing in continuous with peristaltic pumps stock solutions of the contaminant in distilled water (1 ml min^{-1}, using a MCP Process Pump, mod. ISM915A, provided with a MS/CA pumphead, mod. ISM724A, by ISMATEC) from two 15 L glass jars. Taking into account the two combined flows (25 ml min^{-1} for seawater and 1 ml min^{-1} for the contaminant solution, respectively), the concentrations of diclofenac inside the two glass jars were calculated to be 25 times higher than the concentrations needed inside the respective tanks with bivalves.

Of the experimental conditions tested, the control without diclofenac at 8.1 pH was considered as a reference.

During the experiments, animals were fed with microalgae (*Isochrysis galbana*). Twice a day, 250 ml of algae (3.5 x 10^6 cells ml^{-1}) were added in each 120 L tank, in order to keep algae concentration around 2000 cells/ml in the experimental tanks with animals. Bivalve mortality was checked daily.

Tissue collection and preparation

Tissues for biochemical analyses were collected at three time intervals throughout the experiments: after 7 days of exposure to differing pH values (T0), and after 7 and 14 days of exposure to differing pH/diclofenac combinations (T1 and T2, respectively). At each tissue sampling time, six pools (from 7 animals each) of gills and digestive gland were prepared for each experimental condition. The bivalve shells were opened, and the gills and digestive gland were excised, pooled and then placed in tubes on ice. Aliquots from each

pooled tissue were immediately frozen in liquid nitrogen and stored at -80°C until analysis. For the SOD, CAT and TBARS assays, the gills and digestive glands were thawed on ice and homogenised (1:4, w:v) in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM Dithiothreitol (DTT, Sigma) and 40 µg/ml Aprotinin (Sigma). They were sonicated for 2 min at 0°C with a Braun Labsonic U sonifier at 50% duty cycles and were then centrifuged at 12,000 g for 45 min at 4°C. Supernatants (S12) were collected for assays. For COX assay and DNA strand break assay, digestive glands and gills were homogenized in ice-cold homogenization buffer (25 mM Tris-HCl, 150 mM NaCl, 1 µg/ml Aprotinin, pH 7.5). For COX assay tissue aliquots were then centrifuged at 10,000 g for 30 min at 4°C and supernatant (S10) was collected, while for DNA strand breaks assay the homogenised tissues were used.

SOD activity assay

Total SOD activity was measured in the gills and digestive glands in triplicates with the xanthine oxidase/cytochrome C method in accordance with Crapo et al. (1978). Tissues were homogenised as described above, and the cytochrome C reduction by superoxide anion generated by xanthine oxidase/hypoxanthine reaction was detected spectrophotometrically at 550 nm at room temperature (20°C). Enzyme activity was expressed as U/mg of proteins with one unit of SOD being defined as the amount of sample producing 50% inhibition in the assay conditions. The reaction mixture contained 46.5 µM KH_2PO_4/K_2HPO_4 (pH 8.6), 0.1 mM EDTA, 195 µM hypoxanthine, 16 µM cytochrome c, and 2.5 µU xanthine oxidase. For

SOD assay, as well as for the other biochemical assays, tissue protein concentrations were quantified in accordance with Bradford (1976).

CAT activity assay

Gill and digestive gland CAT activity was measured in triplicate following the method described in Aebi (1984).

Decreases in absorbance of a 50 mM H_2O_2 solution (ε = -0.0436 mM^{-1} cm^{-1}) in 50 mM phosphate buffer (pH 7.8) and 10 µl of S12 were continuously recorded at 240 nm and at 10-s intervals for 1 min. The results were expressed in U/mg of proteins with one unit of CAT being defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H_2O_2/min.

COX activity assay

Cyclooxygenase activity assay is based on the oxidation rate of 2,7-dichlorofluorescein in the presence of horseradish peroxidase and arachidonic acid. 25 µl aliquots of digestive gland or gill S10 were mixed with 5 µl of 6 mM arachidonic acid, 20 µl of DCFH-DA/peroxidase 10x in 150 µl of 50 mM Tris–HCl, pH 8, containing 0.1% Tween 20. The reaction mixture was incubated at 30 °C for 0, 10, 20 and 30 min, and fluorescence was directly measured at 485 nm excitation/530 nm emission (Hitachi F-3010). The results were expressed as relative fluorescence units (RFU)/min/mg proteins.

Lipid peroxidation (LPO) assay

LPO was quantified in the gills and digestive gland S12 (200 µl) using the malondialdehyde (MDA) assay based on the method described in Buege and Aust (1978). Absorbance was read spectrophotometrically at 532 nm, and the results were expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. The concentration of TBARS was calculated as MDA equivalents using the molar extinction coefficient for MDA (ε= 1.56 x 105M^{-1} cm^{-1}).

DNA strand-break assay

The levels of DNA strand-breaks were determined by the alkaline DNA precipitation assay developed by Olive (1988) using fluorescent detection of DNA strands (Bester et al., 1994; Gagné and Blaise, 1995). The principle of the assay is based on the selective precipitation of protein-bound genomic DNA from the more soluble DNA strand fragments. Briefly, to 25 µL of gill and digestive gland homogenates were added 200 µL of alkaline lysis buffer composed of 2% SDS-10 mM EDTA (tetrasodium salt), 10 mM Tris-base and 40 mM NaOH (pH 12). After mixing at room temperature for 1 min, 200 µl of 0.12 M KCl was added and incubated at 60 °C for 10 min. The mixture was cooled at 4°C for 30 min and then centrifuged at 8,000 g for 5 min at 4 °C. A 50 µL aliquot of the supernatant was mixed with 150 µL of assay buffer containing 1µg/ml Hoechst working solution (Bisbenzimide H 33258) in a 96-wells microplate and incubated for 5 min on a plate shaker before reading. Fluorescence was measured at 360 nm and 460 nm for excitation and emission wavelengths, respectively. Standard solutions of salmon sperm DNA (D1626,

Sigma) were used for calibration. The results were expressed as µg of supernatant DNA/mg of proteins.

Statistical analysis

Results of antioxidant enzyme activity (CAT, SOD, COX) and oxidative damage assays (LPO and DNA strand-breaks) were analysed with a permutation-based nonparametric MANOVA (Pesarin, 2001). For each parameter, a nonparametric full factorial ANOVA was set with factors pH, Contaminant and Time (the last as a repeated measure). To take into account the dependence of measures within the same tank, a mixed-model approach was used (Finos and Basso, 2012, 2013).

4.2.3. Results

Results from the MANOVA analysis for mussels and clams are reported in Tab. 1.

M. galloprovincialis

At T0, no significant effects of pH on SOD activity were observed in mussel gills. At T1 MANOVA did not highlight significant variation in SOD activity due to pH and its interaction with diclofenac, while a significant effect of diclofenac was found ($p<0.027$). In particular, when compared to control at pH 8.1, all the other conditions tested showed decreased SOD activity in gills. While in controls and in 0.05 µg/L diclofenac-exposed mussels a decreasing trend of SOD activity was observed with decreasing pH, an opposite trend of variation was

found in animals treated with the higher concentration of the contaminant. At T2, no significant effects were observed, even if at pH 8.1 and 7.7 animals exposed to 0.5 µg/ml of diclofenac showed a reduction of SOD activity in gills.

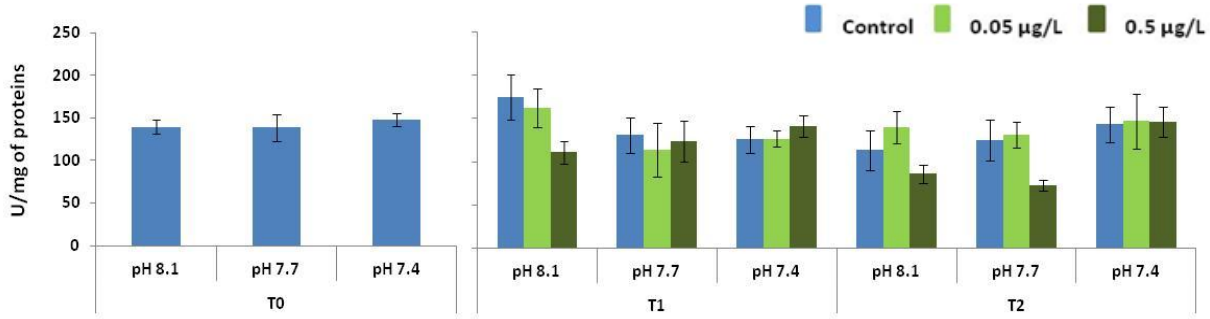

Fig. 1 - SOD activity in *M. galloprovincialis* gills. Values are means ± SE.

After one week of exposure to the different pH conditions (T0), no significant effects were observed in digestive gland SOD activity. At T1 and T2, no significant effects of pH, diclofenac or their interaction were recorded. Despite this, at T1 a reduction in SOD activity in animals exposed to diclofenac was observed at all pH levels tested, but it was not significant probably because of the high variability recorded among pools from the same treatment.

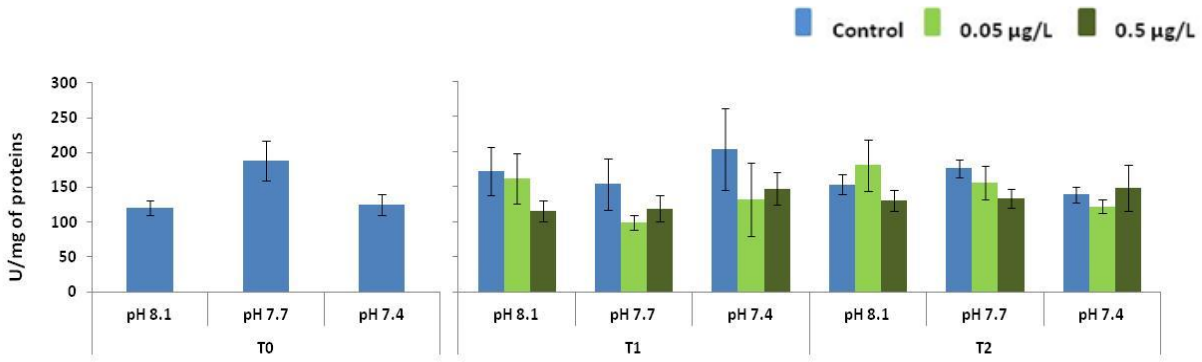

Fig. 2 - SOD activity in *M. galloprovincialis* digestive gland. Values are means ± SE.

After one week of exposure no significant effects were highlighted by MANOVA even if lower values were recorded at pH 7.7 and 7.4 compared to controls at pH 8.1. Both at T1 and T2, pH, diclofenac and their interaction did not significantly affect CAT activity in mussel gills. Reduced activity was observed in animals kept in presence of diclofenac at pH 8.1, both at T1 and T2, while in animals maintained at pH 7.7 and 7.4 there was not a clear trend of variation.

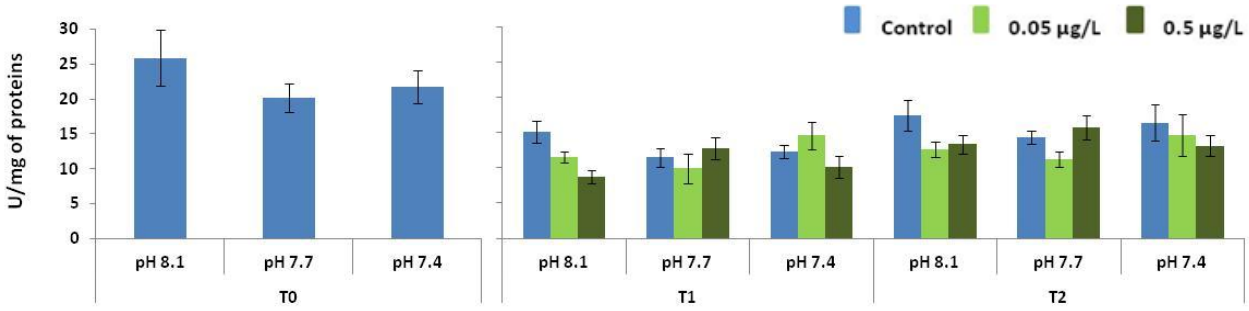

Fig. 3 - CAT activity in *M. galloprovincialis* gills. Values are means ± SE.

At T0, the exposure to different pH levels did not affect significantly the activity of CAT in mussel digestive gland. At T1, no significant effects were recorded.

Despite this, animals exposed to 0.5 µg/L of diclofenac showed higher CAT activity values in digestive glands both at pH 8.1 and 7.7 respect to those of controls and 0.05 µg/L-diclofenac exposed mussels. Conversely, at pH 7.4 no increases in CAT activity were found with growing diclofenac concentration. At T2, CAT activity was significantly influenced by pH ($p>0.007$), by diclofenac ($p>0.007$) and their interaction as well ($p>0.000$). An increased enzyme activity was observed in the digestive gland of mussels maintained at pH 8.1 with 0.05 µg diclofenac /L , at pH 7.7 without diclofenac and at pH 7.4 with

0.5 µg diclofenac/L, when comparing the results obtained with those from animals kept at pH 8.1 without diclofenac.

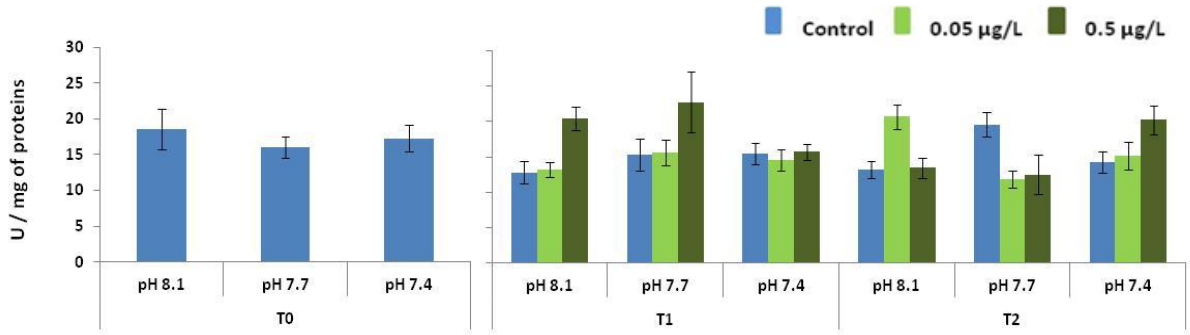

Fig. 4 - CAT activity in *M. galloprovincialis* digestive gland. Values are means ± SE.

COX activity in gills was significantly influenced by reduced pH ($p<0.000$) at T0. In particular, a reduction was observed in mussels kept at pH 7.7 respect to controls, while in gills of animals kept at pH 7.4 COX showed levels of activity almost two times higher than in controls at pH 8.1.

Both at T1 and T2, the activity of COX was influenced by pH (respectively $p<0.001$ and $p<0.006$) but not by diclofenac and its interaction with pH.

After two and three weeks of exposure, in mussel gills the same pattern of variation observed at T0 was recorded, with COX activity higher in animals kept at pH 7.4 respect to those at pH 8.1.

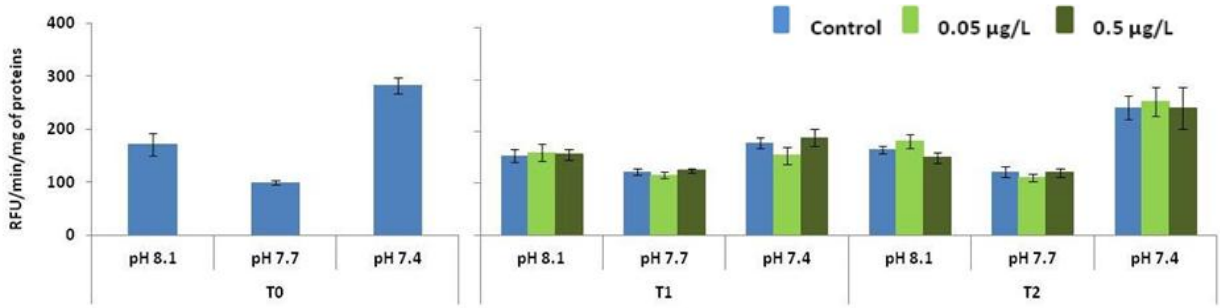

Fig. 5 - COX activity in *M. galloprovincialis* gills. Values are means ± SE.

At T0, COX activity in digestive gland was significantly influenced by pH reduction ($p<0.000$). Similarly to results obtained from mussel gills, COX activity dropped in animals exposed to pH 7.7 while at pH 7.4 it increased respect to controls at pH 8.1. At T1 and T2, COX activity was significantly influenced by pH ($p<0.006$ and $p<0.009$ respectively), but not by diclofenac and pH/diclofenac interaction. Although COX activity was lower in the digestive gland than in gills, the same variation pattern observed in gills was highlighted .

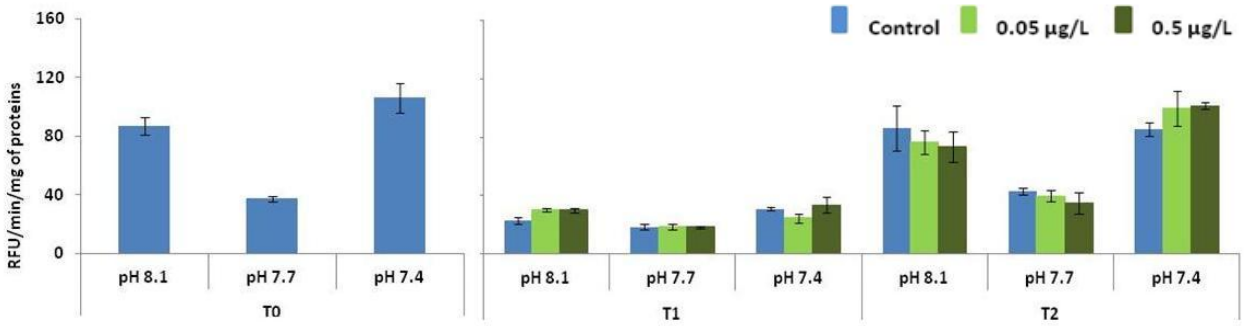

Fig. 6 - COX activity in *M. galloprovincialis* digestive gland. Values are means ± SE.

In mussel gills, MANOVA did not highlight any significant variation in lipid peroxidation during the entire experiment.

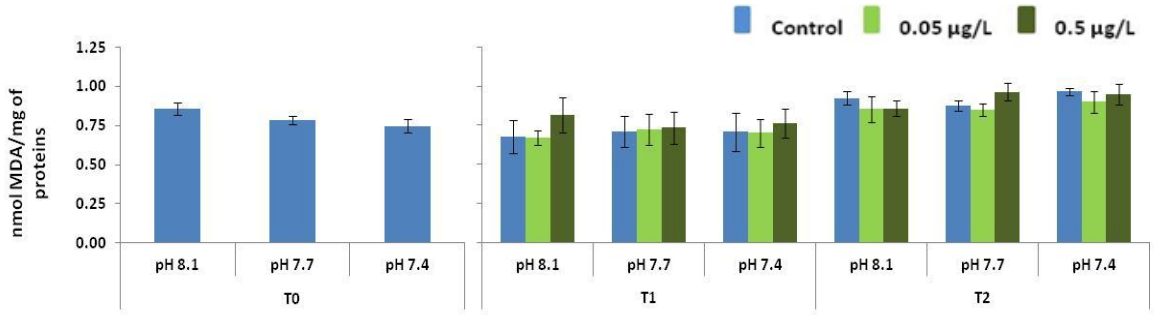

Fig. 7 - LPO in *M. galloprovincialis* gills. Values are means ± SE.

Like in gills , in digestive gland there were not significant evidences of an increase or a decrease in LPO levels, when comparing animals kept at reduced pH and controls at T0. Also after two (T1) and three weeks (T2) of exposure no significant effects due to the experimental conditions tested were observed.

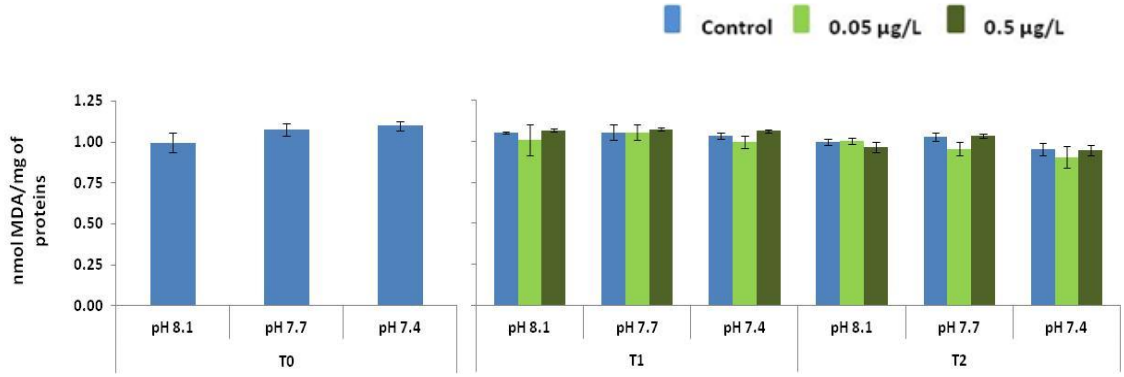

Fig. 8 - LPO in *M. galloprovincialis* digestive gland. Values are means ± SE.

At T0 mussel gills were negatively affected by reduced pH with a significant increase of DNA strand-break formation ($p<0.000$) in animals maintained at pH 7.7 and 7.4 compared to controls. At T1 and T2, reduced pH and diclofenac did not significantly affect the response measured in mussel gills. Only at T2, a significant effect of interaction between pH and diclofenac was observed ($p<0.006$). This effect was particularly evident in animals kept at pH 7.4 and 0.5 µg/L of diclofenac.

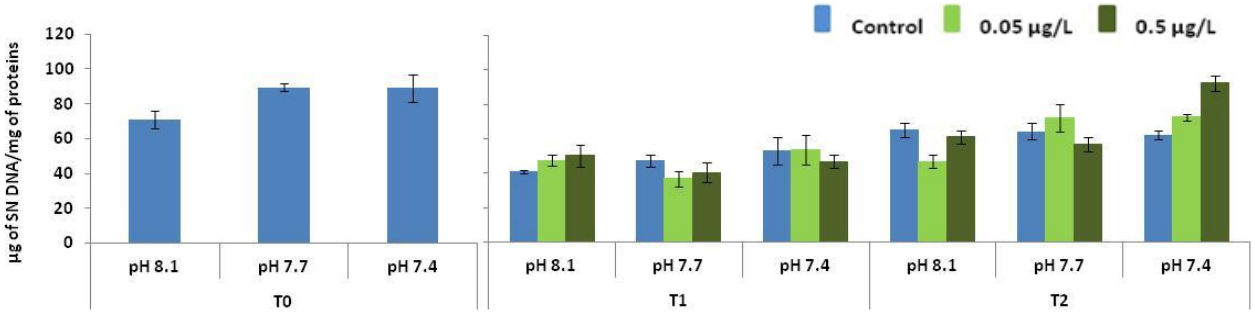

Fig. 9 – DNA strand-break in *M. galloprovincialis* gills. Values are means ± SE.

Contrary to what was observed in mussel gills at T0, in digestive gland a significant reduction in DNA damage was observed with decreasing pH ($p<0.000$). Indeed, DNA strand-break formation in controls at pH 8.1 was almost two times higher than in treatments at pH 7.7 and 7.4. The same trend was observed at T1 with higher values of DNA damage in animals kept at pH 8.1 without diclofenac compared to all the other conditions tested. However, no significant effects of pH, diclofenac or their interaction were recorded. Interestingly, at T2 an opposite pattern of variation was observed. DNA strand-break formation increased significantly because of reduced pH ($p<0.047$). No significant effects due to the presence of diclofenac, as well as to its interaction with pH, were observed, even if animals kept at pH 7.4 and 0.05 µg/L of the contaminant showed higher values of DNA strand-breaks.

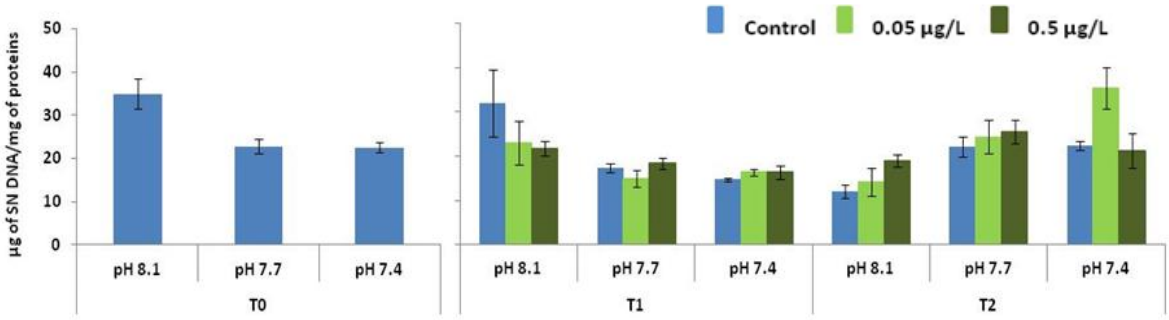

Fig. 10 – DNA strand-break in *M. galloprovincialis* digestive gland. Values are means ± SE.

V. philippinarum

Throughout the experiment, no significant effects of the experimental conditions tested were highlighted by MANOVA in SOD activity measured in clam gills. The only evidence of variation was found at T0, when SOD activity increased, but not significantly, under reduced pH.

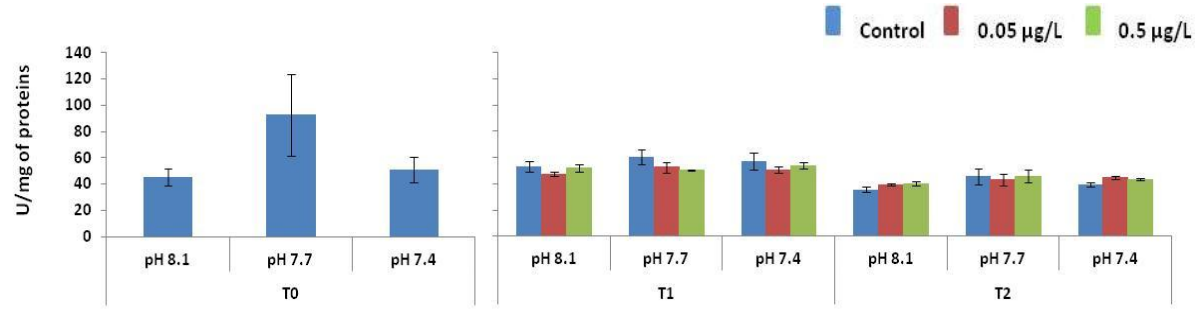

Fig. 11 – SOD activity in *V. philippinarum* gills. Values are means ± SE.

Like in mussels, also in clams digestive gland SOD activity was not significantly influenced by reduced pH at T0 and by reduced pH, diclofenac and their interaction at both T1 and T2. Despite this, some patterns of variation were observed. At T0 an increased activity was observed in animals kept at pH 7.4. Conversely at T1, decreased SOD

activity was recorded in animals exposed to diclofenac at all pH values tested compared to their equivalents without contaminant.

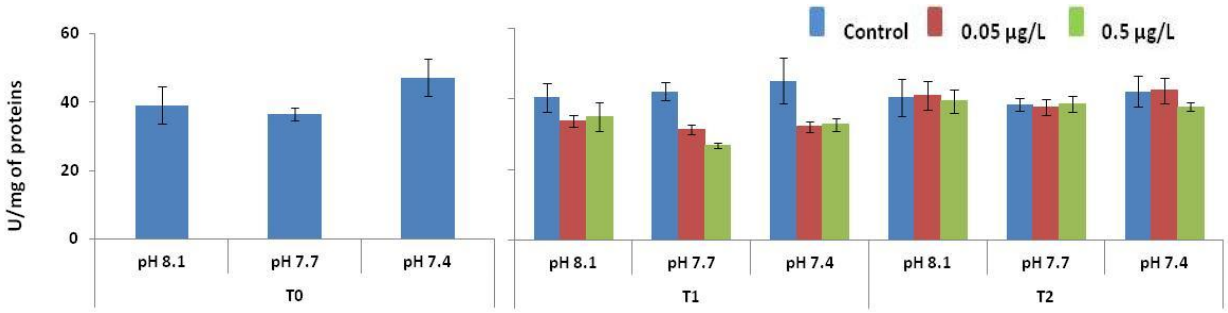

Fig. 12 - SOD activity in *V. philippinarum* digestive gland. Values are means ± SE.

In clams exposed to reduced pH levels for one week CAT activity showed to be higher in animals maintained at pH 7.4 (Fig. 13). However, the MANOVA did not highlight significant differences among treatments. When clams were exposed to different pH conditions combined with diclofenac, no significant effects due to reduced pH, the presence of diclofenac and their interaction were observed both at T1 and T2. However, as shown in Fig. 13, at T1 levels of activity in gills of animals exposed to diclofenac at all pH values tested were lower than those recorded in controls at pH 8.1. A decrease was also found in the absence of diclofenac at pH 7.4. Also at T2, decreasing levels of enzyme activity were observed with decreasing pH in animals kept without diclofenac, as well as in those exposed to the lower dose of the contaminant. Conversely, an opposite pattern of variation was shown at the higher diclofenac concentration, since CAT activity, lower than in controls at pH 8.1, progressively increased with decreasing pH.

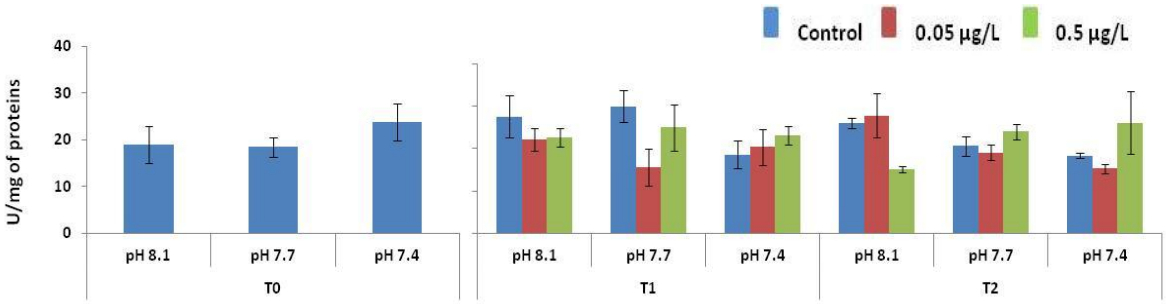

Fig. 13 - CAT activity in *V. philippinarum* gills. Values are means ± SE.

Similarly to what observed in clam gills, in digestive glands no significant variations in CAT activity with decreasing pH were recorded at T0. After two (T1) and three weeks (T2) of exposure no statistically significant variations induced by the experimental conditions were found. Nonetheless, at T2 under reduced pH values, the enzyme activity was lower in animals kept without diclofenac compared both to controls at pH 8.1 and to diclofenac-exposed clams at the same pH value.

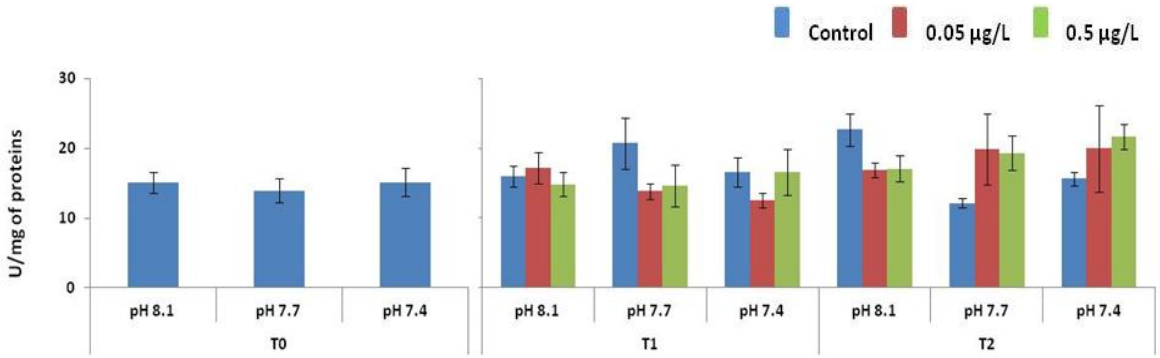

Fig. 14 - CAT activity in *V. philippinarum* digestive gland. Values are means ± SE.

Decreased pH significantly influenced COX activity in clam gills ($p<0.000$) resulting in lower values in clams kept at pH 7.7 and even more in those kept at pH 7.4. The same pattern of variation was maintained both after the second week (T1) and the third week (T2) of exposure when a significant negative effect of reduced pH

(p<0.000 and p<0.005 respectively) was found, while no effects due to the presence of contaminant or its interaction with pH were highlighted by the MANOVA.

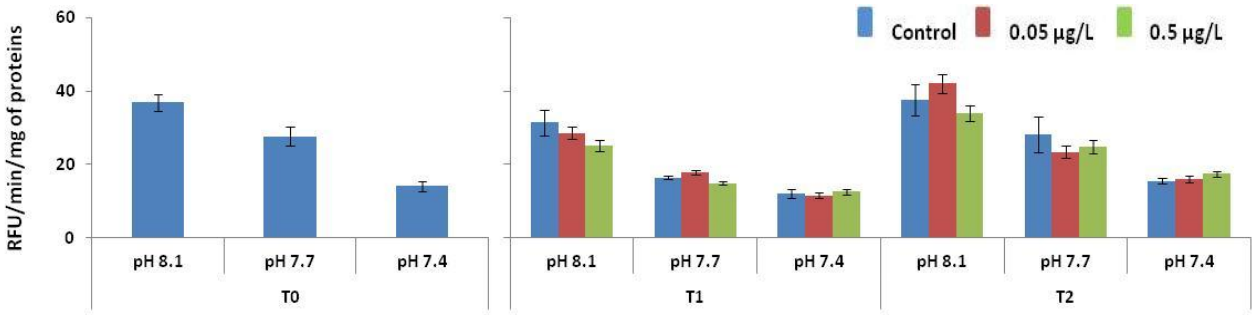

Fig. 15 - CAT activity in *V. philippinarum* gills. Values are means ± SE.

In clam digestive gland a reduction in COX activity due to decreased pH was observed at all the exposure times (p<0.000, 0.001 and 0.000, respectively). Again, no effects due to the exposure to diclofenac were observed.

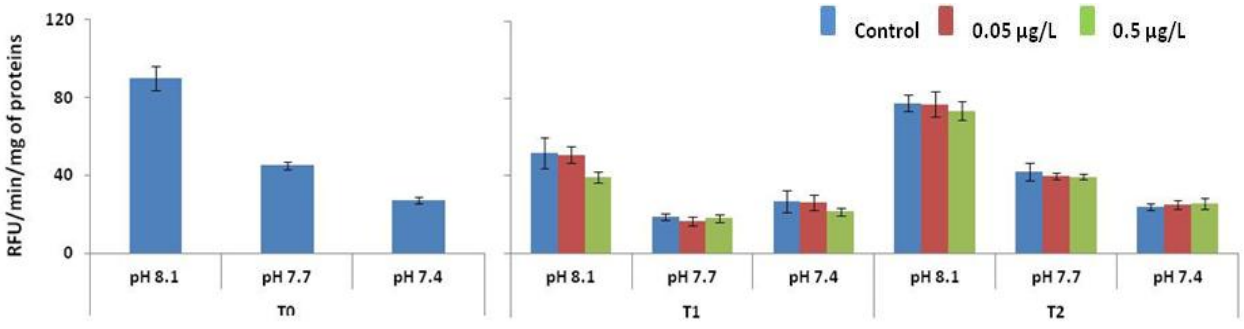

Fig. 16 - COX activity in *V. philippinarum* digestive gland. Values are means ± SE.

Results from TBARS test did not show any significant increase or decrease in LPO in gills of animals exposed for one week to lower pH

values. Also at T1 the experimental variables and their interaction did not significantly influence LPO, even if a LPO increase was observed in animals exposed to diclofenac at all pH tested. Interestingly, an opposite pattern was observed in the presence of diclofenac at T2, with slight decreases in LPO levels of animals kept at all pH values tested, except for the higher diclofenac concentration at pH 7.4. It can be noticed that at the higher diclofenac concentration LPO increased with decreasing pH. Indeed, at T2 MANOVA highlighted a significant effect of diclofenac ($p<0.005$) and pH/ diclofenac interaction ($p<0.019$).

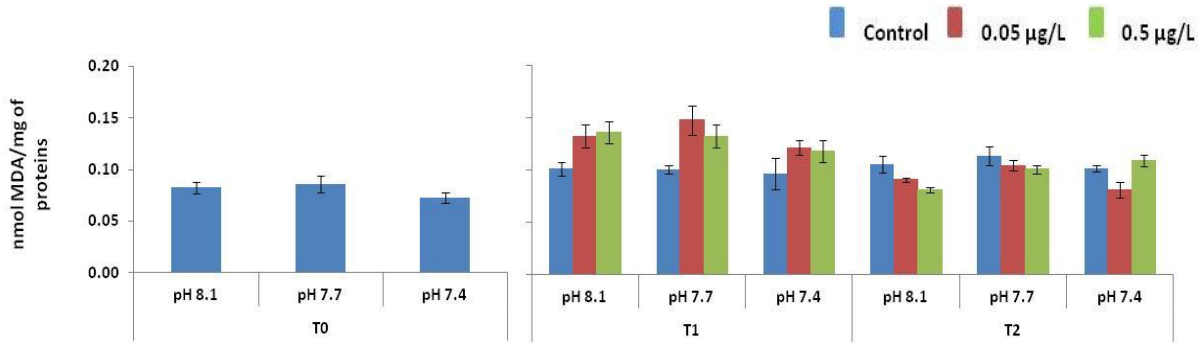

Fig. 17 - LPO in *V. philippinarum* gills. Values are means ± SE.

At T0, no significant effects were observed in clam digestive gland. At T1, only diclofenac significantly influenced LPO in digestive gland ($p<0.022$) with a reduction at all conditions tested compared to animals maintained at pH 8.1 without diclofenac. On the contrary at T2, MANOVA highlighted a significant influence of pH, ($p<0.027$) but neither diclofenac, nor their interaction significantly influenced LPO. Except for clams kept at pH 7.4 and 0.5 µg/L diclofenac, LPO

increased with decreasing pH both in the absence and in the presence of the contaminant.

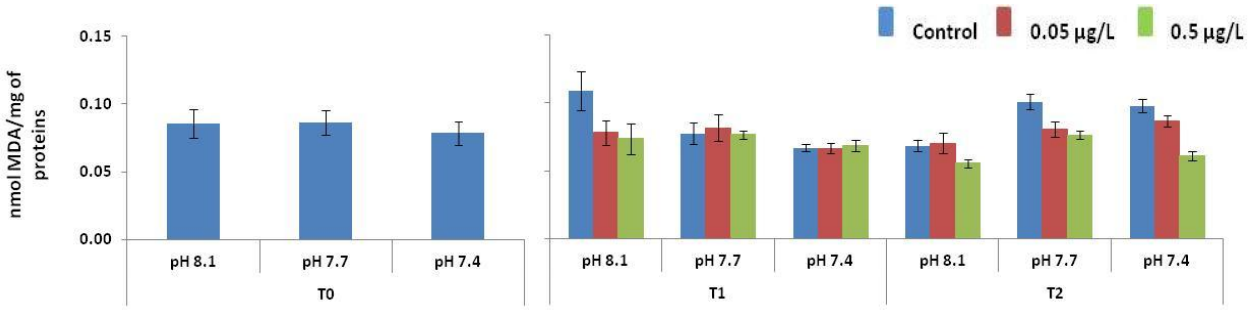

Fig. 18 - LPO in *V. philippinarum* digestive gland. Values are means ± SE.

Although a reduction in DNA strand-breaks was observed with decreasing pH, no significant effects of pH were revealed by MANOVA (Fig 19). Unlike results from T0, at T1 an opposite trend was observed, with the highest levels of DNA strand-breaks found in animals maintained at pH 7.4 in the presence of diclofenac. However, also at T1 no statistically significant effects due to the experimental conditions tested were highlighted. Conversely, at T2, MANOVA revealed a significant effect of pH ($p<0.012$) and its interaction with diclofenac ($p<0.014$). Indeed, when comparing results from animals kept at reduced pH with those from pH 8.1, a reduction in DNA strand-breaks formation was observed at pH 7.7 and 7.4 at all diclofenac concentrations tested, except for animals exposed to 0.05 µg/L at pH 7.7.

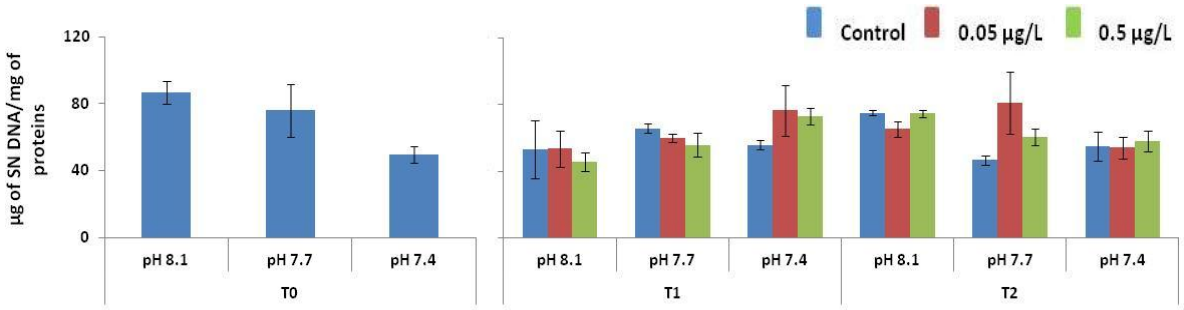

Fig. 19 – DNA strand-break in *V. philippinarum* gills. Values are means ± SE.

No significant effects due to reduced pH were found at T0, even if an increased DNA strand-break formation was observed with decreasing pH. The same trend was observed both at T1 and T2 with increased values in clams kept at reduced pH but with no significant effects induced by the experimental conditions.

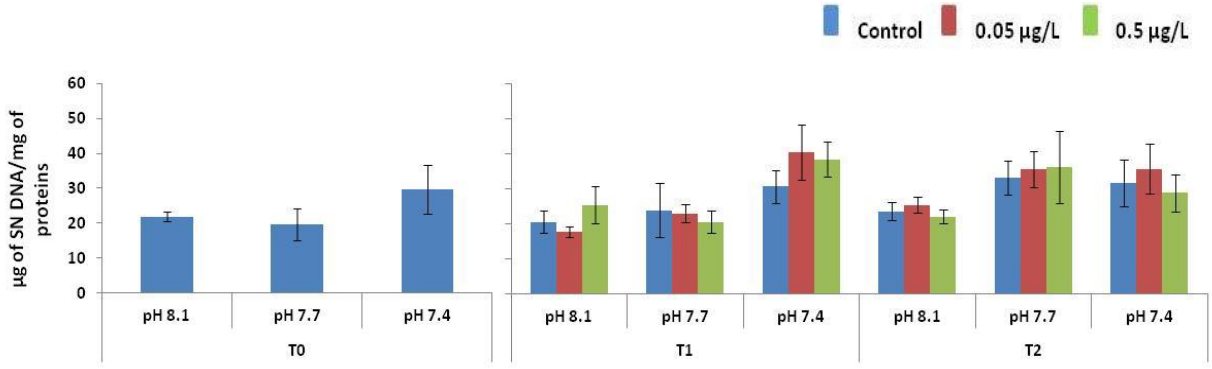

Fig. 20 – DNA strand-break in *V. philippinarum* digestive gland. Values are means ± SE.

Sampling time	Species	Factor	SOD in gills	SOD in dig. gland	CAT in gills	CAT in dig. gland	COX in gills	COX in dig. gland	LPO in gills	LPO in dig. gland	DNA strand-break in gills	DNA strand-break in dig. gland
T0	*M. galloprovincialis* *V. philippinarum*	pH	ns	ns	ns	ns	0.000	0.000	ns	ns	0.000	0.000
			ns	ns	ns	ns	0.000	0.000	ns	ns	ns	ns
T1	*M. galloprovincialis*	pH	ns	ns	ns	ns	0.001	0.006	ns	ns	ns	ns
		diclofenac	0.027	ns	ns	ns	ns	ns	ns	ns	ns	ns
		pH*diclofenac	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.029
	V. philippinarum	pH	ns	ns	ns	ns	0.000	0.001	ns	ns	ns	ns
		diclofenac	ns	ns	ns	ns	ns	ns	ns	0.022	ns	ns
		pH*diclofenac	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
T2	*M. galloprovincialis*	pH	ns	ns	ns	0.007	0.006	0.009	ns	ns	ns	0.047
		diclofenac	ns	ns	ns	0.007	ns	ns	ns	ns	ns	ns
		pH*diclofenac	ns	ns	ns	0.000	ns	ns	ns	ns	0.008	ns
	V. philippinarum	pH	ns	ns	ns	ns	0.005	0.000	0.027	ns	0.012	ns
		diclofenac	ns	ns	ns	ns	ns	ns	0.005	ns	ns	ns
		pH*diclofenac	ns	ns	ns	ns	ns	ns	0.019	ns	0.014	ns

Tab. 1 - MANOVA results for oxidative stress parameters in *M. galloprovincialis* and *V. philippinarum*.

Statistically significant effects are in red.

4.2.4. Discussion

In this study, the combined effects of decreased pH and diclofenac on oxidative stress-related parameters were evaluated for the first time in *M. galloprovincialis* and *V. philippinarum.*

Generally, oxidative stress occurs when antioxidant defences are overwhelmed by reactive oxygen species (ROS) produced in organism tissues. Antioxidant enzymes, such as SOD and CAT, are important enzymes that prevent ROS-mediated oxidative damage in bivalves (Soldatov et al., 2007). The exposure of molluscs to ROS-generating stressful conditions may result in either induction or inhibition of antioxidant enzymes. Eventually, a reduction in antioxidant defence results in a damage to membrane lipids, DNA and proteins.

The quantification of antioxidant enzyme activities and the evaluation of potential damage can be helpful to reveal oxidative stress induced by experimental conditions. However, comparisons of our results with data from the literature are difficult because, to our knowledge, there are no previous studies on the combined effects of reduced pH and diclofenac, or other NSAIDs, on bivalve antioxidant responses. Instead, there is a considerable amount of information about the effects of single environmental parameters on antioxidant defences in aquatic invertebrates (Abele et al., 1998; Khessiba et al., 2005; Verlecar et al., 2007; Tomanek et al., 2011; Zhang et al., 2012; Matozzo et al., 2013).

SOD catalyses the dismutation of superoxide anion to hydrogen peroxide in mitochondria and cytosol (Irato et al., 2007) and generally an increase of this enzyme indicates the ability to face a possible oxidative stress.

Altogether, in this study, experimental conditions tested did not affect SOD activity even if there were differences between the two species and between tissues from the same species. In particular, SOD activity was significantly influenced by diclofenac in mussel gills after one week of exposure to differing combinations of pH and contaminant. Generally in both the bivalve species, in particular at T1, a reduction in SOD activity was observed in animals exposed to diclofenac at all pH tested, both in gills and in the digestive gland with similar trend. This negative trend observed at T1, no longer persisted at T2 in clams, while in mussels inhibiting effect of diclofenac was maintained only under exposure to 0.5 µg/L diclofenac at pH 8.1 and 7.7. From these findings, it is possible to infer that the contaminant generally decreased SOD activity after one week of exposure, while after two weeks only clams seemed to be able to restore similar levels of activity independently of treatment. Similarly, Parolini et al. (2011b) exposed *D. polymorpha* to environmental concentrations of diclofenac (0.3, 1, and 2 nM) for 96 hours recording negligible effect on the activities of antioxidant and detoxifying enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx).

Other studies, conversely, recorded higher levels of SOD activity following NSAIDs exposure. As an instance, Gonzalez-Rey & Bebianno (2011) exposing *M. galloprovincialis* for 7 days to ibuprofen, recorded a significant increase of SOD activity.

In this study no significant effects on SOD due to reduced pH were observed, both in mussels and in clams, even if reduced activities were found in animals kept at low pH in mussel gills at T1. Data

available from literature demonstrated that shifts in environmental parameter can affect antioxidant defenses in aquatic organisms.

The exposure of the copepod *Centropages tenuiremis* to elevated pCO_2, for instance, caused a significant decrease in SOD activity on day 1 of exposure, with enzyme activity increasing on day 2 and decreasing on days 3 and 4 (Zhang et al., 2012).

Increased SOD activity results in high dismutation of O_2^- to H_2O_2, which should stimulate CAT activity. Along with glutathione peroxidase, CAT is the most important scavenger of H_2O_2 in cells. In this study, no significant variations in CAT activity were found in mussel gills, while significant effects due to pH, diclofenac and their interaction were observed in digestive gland after the third week of exposure, with different pattern of variation among the different levels of pH. In clam digestive gland instead, although no statistically significant effects were found , opposite trends of variation were observed at the end of the exposure in controls and diclofenac treated animals, the enzyme activity decreasing at reduced pH in the former ones, but increasing in the latter. This finding is consistent with the role of digestive gland in organic chemicals' biotrasformation processes. Overall, no evident relationship between SOD activity and CAT activity was observed in both species. In a study on *M. galloprovincialis* and *C. gallina* exposed to three different levels of pH (8.1, 7.7 and 7.4) combined with two temperatures (22 and 28°C) Matozzo et al. (2012) found that CAT was increased by low pH and high temperature in clam digestive gland. From data available in literature, also the exposure to NSAIDs can induce CAT activity, as observed in our study. Parolini et al. (2011a)

demonstrated that the exposure to 0,2 μg/L of ibuprofen for 4 days significantly induced CAT activity in soft tissues of *D. polymorpha*. Also in the amphipod *Hyalella azteca* a 72-hours exposure to diclofenac-contaminated sediment significantly increased CAT activity (Oviedo-Gómez et al., 2010).

In mammals, cyclooxygenase (COX) is the enzyme that catalyzes the committed step in the metabolism of arachidonic acid to prostaglandins (Vane and Botting, 1998). NSAIDs inhibit cyclooxygenase (COX)-1 and -2 resulting in decreased prostaglandin synthesis. Bivalves have been shown to possess prostaglandin synthase or COX activity, which is expressed during gamete maturation and spawning (Osada and Nomura, 1990). Furthermore, it is known that COX expression is increased by reactive oxygen species (Dokmeci, 2004).

In this study, COX activity was investigated both in gills and digestive gland of mussels and clams. In mussels, COX was significantly influenced by pH at all times of tissue collection with the lowest levels found in animals kept at pH 7.7 and the highest in those kept at 7.4. Interestingly, a similar pattern of variation was observed both in gills and digestive gland . Instead, diclofenac did not significantly affect COX activity at T1 and T2. As in mussels, also in clams pH, but not diclofenac, significantly influenced the enzyme activity throughout the experiment, with the same pattern of variation in the two tissues. Unlike mussels, however, the lowest COX activity in clams was observed at pH 7.4. To our knowledge, these are the first results on the effects of seawater acidification on COX activity. As mentioned above, it is known that shifts in environmental

parameters (temperature, salinity, and pH) can alter enzymatic activity. In this study reduced pH may have altered COX functionality or its expression as well. Further studies are needed to understand the role of environmental parameters' shifts in modulating this. Unlike other studies, in our experiments COX was not influenced by diclofenac, at the concentrations considered, at least. In the freshwater mussel *Elliptio complanata*, exposed to domestic wastewater aeration lagoons on the eastern shores of the Richelieu River (Quebec, Canada), COX activity was significantly increased probably because of the presence of several pharmaceutical products (Gagnè et al., 2007). However, the concentrations of pharmaceuticals, including diclofenac, in riverine waters receiving effluents from wastewater treatment plants are known to be also ten times higher than those in surface waters of estuarine and coastal environments (Rodrìguez-Navas et al., 2013). Recently, Farcy et al. (2011) did not highlight increases in COX activity in *E. complanata* after a two-week exposure to tertiary-treated municipal effluents in which considerable amounts of NSAIDs were measured.

LPO is a well-established mechanism of cellular injury, and its measurement is used as an indicator of oxidative damage in cells and tissues. In this study, both in mussel gills and digestive gland no significant increases in LPO levels were recorded, and for the entire experiment values were rather stable at all conditions tested. Instead in clam significant variations were observed, both in gills and in digestive gland with different trends. In gills, increased LPO values were observed at T1 in animals exposed to diclofenac, whereas a decrease occurred at T2, except for the higher concentration at the

lowest pH. Conversely, in clam digestive gland, at T1 lowered LPO levels with respect to controls at pH 8.1 were recorded in all the other experimental conditions tested, while at T2 significant LPO increases were observed in animals exposed to reduced pH values.

In clams, but not in mussels, a relationship between LPO levels and CAT and COX activities was observed. Indeed, at T1 in clam gills a reduction in these enzyme activities corresponded to a higher lipid peroxidation, thus suggesting a reduced defense against oxidative stress, that occurred in the tissue, the gills, directly exposed to the environment. In digestive gland, a similar relationship was not evidenced.

Also DNA strand-break formation was considered in this study as an index of oxidative damage. Mussel gills showed to be significantly affected by the experimental conditions since the first week of exposure, when DNA strand-break formation increased in mussels exposed to low pH. At the end of the experiment, the interaction between low pH and diclofenac resulted in the highest levels of DNA strand-breaks. Significant variations in DNA damage were also observed in mussel digestive gland, both at T0 and T2. Unlike in gills, however, a decreased genotoxic effect was found at low pH after the first week of exposure. Interestingly, in mussels maintained at low pH both the increase in DNA strand-break formation observed in gills and the decrease observed in digestive gland corresponded to an increase in COX activity. These results suggested that under reduced pH mussels tried to cope with increased oxidative stress by increasing COX activity, even though in the gills, which directly experience

changes in seawater, this response was not able to prevent genotoxic effects.

Unlike mussels, clams did not show significant variations in genotoxicity after one week of exposure to low pH. In both tissues, at T1 increased DNA strand-break formation was observed with increasing pH at all diclofenac concentrations. The same pattern was maintained in digestive gland at T2, whereas a reverse behavior occurred in gills which showed significant reduction of DNA damage. Interestingly, at T1, DNA strand-breaks increased with decreasing pH, whereas COX activity reduced, thus confirming the possible relationship between the two responses above suggested for mussels. Nonetheless, at the end of the experiment other mechanisms of DNA protection and repair may have led to decreased DNA damage in gills of clams kept at reduced pH, since COX activity remained low.

Overall, both species considered in this study demonstrated to be influenced by reduced pH more than by exposure to diclofenac, even though interactions between the two variables were often highlighted. Among the various biomarkers measured, SOD and CAT activities generally did not show significant changes under the differing experimental conditions tested, in both mussels and clams. These antioxidant enzymes are involved in an organism's response to increased oxidative stress. In this regard, Tomanek et al. (2011) suggested that elevated CO_2 levels may cause oxidative stress by increasing the production of ROS either indirectly by lowering intra- and extracellular pH, which may enhance the Fenton reaction, or directly by interacting with other ROS to form more free radicals.

Matozzo et al. (2013) reported in *M. galloprovincialis* exposed to various combinations of pH, temperature and salinity a high capability of modulating biochemical responses to cope with environmental stress. We cannot exclude that other enzymatic and non enzymatic ROS scavengers, not measured in this study, may have played a relevant role in antioxidant defence.

Although measurements of ROS levels were not performed, their increase under low pH can be inferred by increased oxidative damage resulting in higher LPO for clams and higher DNA strand-break formation for mussels.

It is important to point out the opposite response in COX activity of the two studied species. While it increased in mussels exposed to low pH suggesting enhanced antioxidant response, a decrease was found in clams. COX inhibition results in decreased biosynthesis of prostaglandins, which are involved in many physiological processes, such as reproduction, water transport and osmoregulation (Ruggeri and Thoroughgood, 1985; Osada and Nomura, 1990). As in scallops prostaglandins are known to be implicated in gonad maturation and spawning (Osada and Nomura, 1990), reduction in COX activity observed in clams is cause for concern and deserves in-depth investigation in future studies.

4.2.5. References

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4.3. Research article III: Exposure to decreased pH and caffeine affect haemocyte functionality in the mussel *Mytilus galloprovincialis*

4.3.1. Introduction

Organic wastewater contaminants, such as caffeine, present in fluvial, estuarine and coastal environments, have produced increasing concern on their potential ecotoxicological effects (Maul et al., 2006). Several studies have reported the presence of caffeine in surface and ground waters across the world (Weigel et al. 2002; Buerge et al. 2003; Metcalfe et al. 2003; Thomas & Foster 2005). Caffeine (3,7-dihydro-1,3,7-trimethyl-1h-purine-2,6-dione) has been indicated as the most commonly consumed stimulant by humans (Lawrence et al. 2005). It is used in medicine as a cardiac, cerebral, and respiratory stimulant, and it also functions as a diuretic (Buerge et al. 2003). Furthermore, it is a key ingredient of coffee, chocolate, tea and soft and energy drinks. Data from literature estimate that about 460,000 kg of caffeine are consumed daily by human population worldwide (Buerge et al. 2003) and a portion of this amount takes its way to aquatic environments. Caffeine was found in U.S. streams at maximal levels of 6.0 μg/L (median 0.1μg/L) (Kolpin et al., 2002). Ferguson et al. (2013) reported concentrations of caffeine from 18 to 1250 ng/L in near-shore habitats of southern Lake Michigan, while Siegener & Chen (2002) found concentrations ranging from 140 to 1600 ng/L in Boston Harbour seawater. Despite the great

consumption of caffeine and its environmental presence are well reported in several studies, the effects that caffeine may have on aquatic organisms are not nearly known, especially those on marine organisms living in estuarine and coastal environments mostly affected by human presence .

In addition to emerging contaminants, marine organisms have to face with another issue that is presently giving a great concern, global climate changes (GCCs).

Climate change is a threat to marine biota because increased atmospheric CO_2 due to human activity, is causing ocean warming, acidification, hypercapnia and decreased carbonate saturation (IPCC, 2007). The capability of the oceans to take up CO_2 may influence seawater carbonate chemistry, with a consequent decrease in pH values, concentration of carbonate ions and the related calcium carbonate ($CaCO_3$) saturation state of seawater (Orr et al., 2005). Several reports have implied that the regular and constant release of CO_2 into the atmosphere has already caused a reduction in ocean pH values of approximately 0.1 pH units with respect to the preindustrial levels and those reductions from 0.3 to 0.5 pH units are predicted to occur before the end of the 21st century (Caldeira & Wickett, 2005; IPCC, 2007). Under unrestricted CO_2 emissions, the maximum predicted reduction in ocean surface pH is 0.77 units (Caldeira and Wickett, 2003, 2005; Raven et al., 2005).

It is known that shifts of environmental parameters, such as temperature, salinity and pH can affect marine organisms by altering many biological pathways.

Generally, when an organism is subject to stressful conditions it can cope with stress modifying its physiological, biochemical and behavioral responses. At immunological level, responses comprise a complex network of specific and non-specific humoral and cell-mediated components. In bivalves, haemocytes are involved in many crucial physiological functions, including nutrient transport and digestion, tissue and shell formation, maintenance of homeostasis, and immune response (Cheng et al., 1996, 2000; Chu et al., 2000).

Haemocyte-mediated immune parameters are suggested to be particularly sensitive to variations in environmental factors (Chu, 2000). In *V. philippinarum* kept for 7 days at various temperature/salinity combinations (5, 15, 30°C and 18, 38 and 38 psu) the number of circulating haemocytes (total haemocyte count, THC) significantly decreased in animals exposed to 5 and 30°C at both the lowest (18 psu) and the highest (38 psu) salinity (Munari et al., 2011).

More recently, Matozzo et al. (2012b) showed that in *Mytilus galloprovincialis* and *Chamelea gallina* the combined exposure to different levels of temperature, salinity and reduced pH can significantly alter haemocyte functionality. In another study, high temperatures affected some important functional responses of haemocytes in the clam *C. gallina* (Monari et al., 2007). In that study clams were exposed for 7 days at 20, 25 and 30 °C and THC, phagocytosis, lysozyme activity (in both haemocyte lysate and cell-free haemolymph), activity and expression of the antioxidant enzyme superoxide dismutase (SOD) (in both haemocyte lysate and cell-free haemolymph) were measured. Results showed that the highest

temperature increased significantly THC in *C. gallina*, while total SOD activity significantly decreased in haemocytes with increasing temperature.

However, GCCs not only will influence aquatic organisms, they will also play a role in the fate and behaviour of chemical toxicants, by modifying their environmental distribution and bioavailability (Noyes et al., 2009).

As an instance, ocean acidification is predicted to affect the speciation of metals (Millero et al., 2009). Moreover, variations in environmental parameters could alter marine organisms' susceptibility to pollutants.

With the aim of reducing the knowledge gap on these issues, in this study the combined effects of low pH, as predicted by climate change scenarios, and caffeine, on haemocyte parameters, such as total haemocyte count (THC), Neutral Red (NR) uptake and haemocyte proliferation, were investigated for the first time in the Mediterranean mussel *M. galloprovincialis*.

4.3.2. Materials and Methods

Animals

Specimens of *M. galloprovincialis* (4.0 ± 0.5 cm shell length) were collected along the west coast of the Northern Adriatic Sea (near Chioggia, Italy) and immediately transferred to the laboratory. Bivalves were carefully checked for shell damage (damaged animals were not used for experiments), and epibionts (such as barnacles and algae) were removed from the mussels. Prior to starting exposure,

bivalves were acclimatised to the experimental conditions by gradually increasing or decreasing the seawater temperature (2°C per day) to 22°C and by gradually decreasing pH values from 8.1 to 7.7 and 7.4.

Experimental set-up for mussel exposure

The experimental flow-through system used in this study is previously described in detail (Combined effects of seawater acidification and diclofenac on haemocyte parameters of two marine bivalve species, this thesis).

During the first week of exposure, mussels were subjected to three pH values (8.1, as control, 7.7 and 7.4). During the second and the third week, they were exposed to three concentrations of caffeine (0, 0.05 and 0.5 µg/L) for each pH value. For each concentration, two replicates (with 70 bivalves each) were constituted (control without contaminant: C-A, C-B; caffeine 0.05 µg/L: D1-A, D1-B; caffeine 0.5 µg/L: D2-A, D2-B).

During the experiments, the experimental concentrations of caffeine were maintained by distributing in continuous with peristaltic pumps stock solutions of the contaminant. Mussels were fed with microalgae (*Isochrysis galbana*) twice a day, a concentration of about 2000 cells/ml being attained in the experimental tanks. Bivalve mortality was checked daily. At the end of the first (T0), the second (T1) and the third (T2) week of exposure, samples of haemolymph were collected to measure haemocyte parameters.

Haemolymph collection

For each experimental condition, six pools of haemolymph (3 pools per tank, from 7 mussels each) were prepared. Haemolymph (approximately 300 ml per animal) was collected from the anterior adductor muscle with a 1-ml plastic syringe and stored in ice. An equal volume of 0.38% sodium citrate (Sigma) in 0.45 mm-filtered sea water (FSW) with a pH of 7.5 was added to haemolymph samples to prevent clotting. One hundred microlitres of pooled haemolymph were used to determine the THC, 500 µl for the NR uptake assay and 400 µl for haemocyte proliferation assay.

THC, haemocyte volume and diameter determination

A Scepter^{TM} Handheld Automated Cell Counter (Millipore) was used to determine THC and haemocyte volume and diameter. Twenty µl of pooled haemolymph were added to 2 ml of isotonic solution (COULTER ISOTON II Diluent). THC results were expressed as the number of haemocytes ($x10^6$) ml haemolymph^{-1}. The haemocyte diameter and volume were expressed in µm, and in picolitres (pl), respectively.

Neutral Red uptake (NRU) assay

NR uptake assay was performed according to the procedure reported in previous studies (Cajaraville et al., 1996; Matozzo et al., 2002). Pooled haemolymph was centrifuged at 780 g for 10 min. Haemocytes (at a final concentration of 10^6 cells ml^1) were resuspended in an equal volume of 8 mg l^{-1} NR dye (Merck) solution in FSW, and incubated at room temperature for 30 min. They were

next centrifuged at 780 g for 10 min, re-suspended in distilled water, sonicated at 4°C for 30 s with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12000 g for 15 min at 4°C. The supernatant, corresponding to haemocyte lysate (HL), was collected for the NRU assay. Absorbance at 550 nm was recorded on a Beckman 730 spectrophotometer. The results were expressed as optical density per ml haemolymph (OD ml haemolymph^{-1}).

Haemocyte proliferation

Hemocyte proliferation was evaluated using a colorimetric method with a commercial kit (Cell proliferation Kit II, Roche). The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye in metabolically active (viable) cells. Briefly, the XTT labelling reagent and electron-coupling reagent were thawed at 37°C and mixed immediately before use to obtain the XTT labelling mixture. A total of 200 µl of the mixture was added to 400 µl of pooled hemolymph and incubated for 4 h in a dark humidified chamber. The absorbance at 450 nm was subsequently recorded using a Beckman 730 spectrophotometer. The data were normalized to THC values that were recorded in clams from each experimental condition and expressed as optical density (OD) values at 450 nm.

Statistical analysis

Haemocyte parameters (i.e. THC, haemocyte volume and diameter, NRU, haemocyte proliferation) were analysed with a permutation-based nonparametric MANOVA (Pesarin F., 2001). For each parameter, a nonparametric full factorial ANOVA was set with factors

pH, Contaminant and Time (the last as a repeated measure). To take into account the dependence of measures within the same tank, a mixed-model approach was used (Finos L. and Basso D., 2012, 2013).

4.3.3. Results

For each immunomarker measured in this study, statistical results from the MANOVA analysis are reported in Table X.

After one week of exposure to reduced pH (T0), an increase in THC values was observed (Fig. 1), but MANOVA did not reveal any significant effect of pH. At T1, a similar trend was observed with increased THC levels in animals exposed at pH 7.4 compared to those exposed at pH 8.1 at all caffeine concentrations tested. Increased THC was also found at the dose of 0.5 µg/L at pH 7.7. Despite the observed changes, no significant effects of reduced pH, caffeine or their interaction were shown by the statistical analysis. Conversely at T2, THC decreased in animals exposed to caffeine at all pH values tested respect to controls at pH 8.1. A marked decrease was also observed in animals kept at pH 7.4 in the absence of caffeine. Again, no significant effects were highlighted by MANOVA.

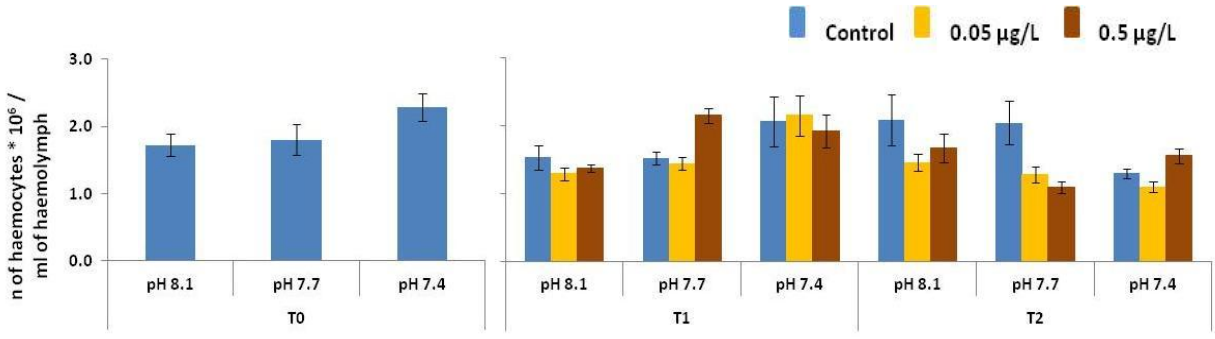

Fig. 3 – THC in *V. philippinarum*. Results are means ± SE.

Also haemocyte volume increased (Fig. 2), but not significantly, after one week of exposure to low pH. At T1, haemocyte volume was increased by the presence of caffeine mostly at the highest dose at all pH tested. However, no statistically significant variations due to the experimental conditions were highlighted. MANOVA revealed a significant effect of caffeine at T2 ($p<0.029$). Indeed, a strong increase of haemocyte volume was shown in animals kept at the highest dose of 0.5 µg/L caffeine at all pH tested, as well as in animals exposed to 0.05 µg/L at pH 8.1.

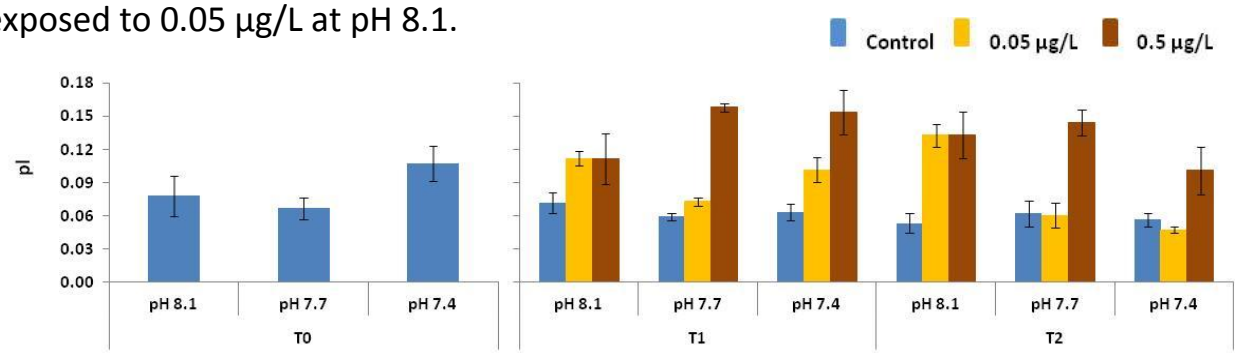

Fig. 4 – Haemocyte volume *V. philippinarum*. Results are means ± SE.

At T0 haemocyte diameter slightly increased (Fig. 3), but not significantly, with reducing pH. Similarly to what observed for haemocyte volume, the exposure to caffeine, especially to 0.5 µg/L, increased haemocyte diameter, but only at T2 a significant variation was highlighted by MANOVA ($p<0.033$).

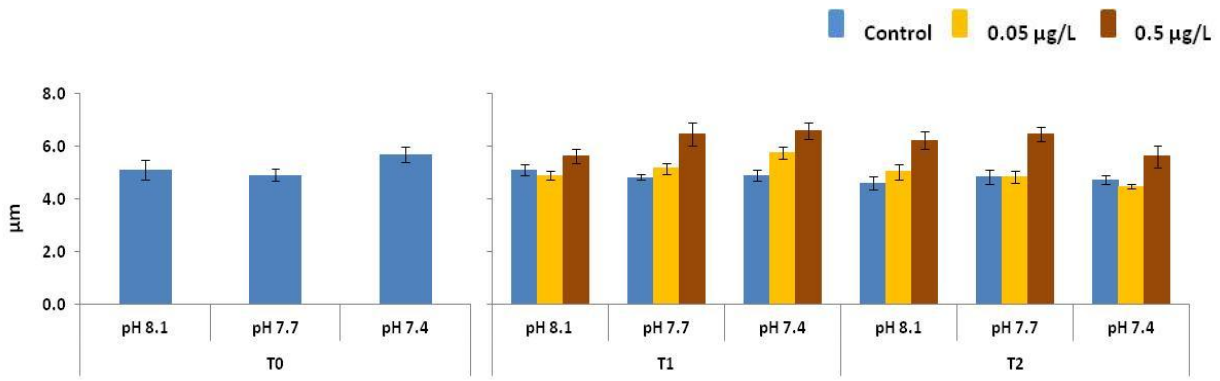

Fig. 5 - Haemocyte diameter in *V. philippinarum*. Results are means ± SE.

NRU was significantly increased by reduced pH ($p<0.000$) after one week of exposure (Fig. 4). Compared to T0, NRU at T1 was two-fold higher at almost all conditions tested. A decrease in animals kept at pH 7.7 and 7.4 was observed, but no significant effects of pH, caffeine or their interaction were recorded. At T2, NRU levels decreased again with lower levels in animal maintained at pH 8.1. Also at T2 no significant variations due to the experimental conditions were highlighted by MANOVA.

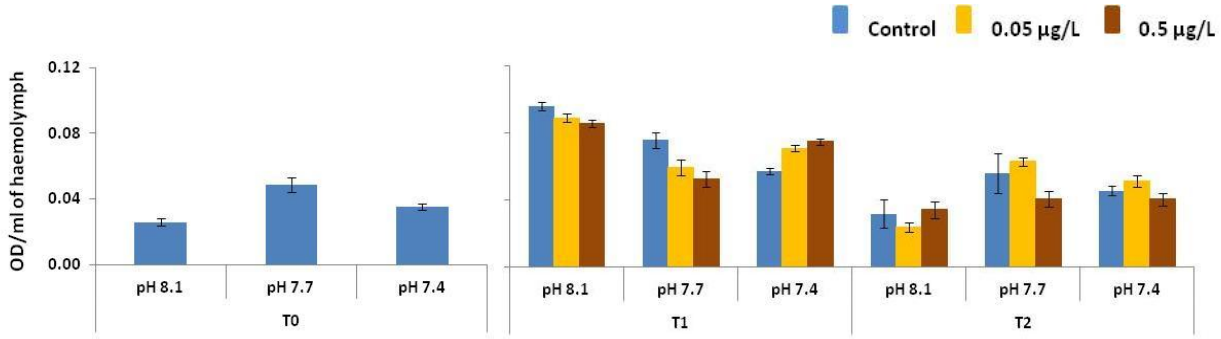

Fig. 6 – NR up-take in *V. philippinarum*. Results are means ± SE.

The exposure of mussels to decreased pH values induced an increase in haemocyte proliferation after one week of exposure (Fig. 5). However, MANOVA did not reveal any significant variation. At T1 and T2, proliferation values decreased in animals kept at pH 7.7 and 7.4

respect to those at pH 8.1 at all caffeine concentrations tested, but a significant effect of pH was observed at T1 only ($p<0.007$).

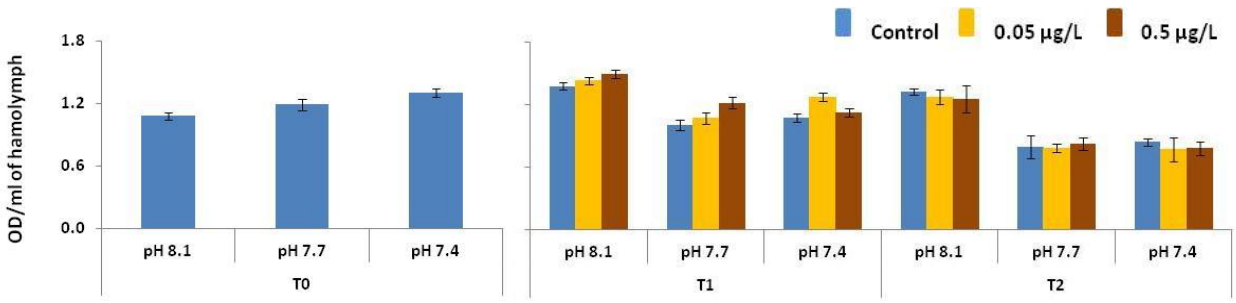

Fig. 7 – Haemocyte proliferation in *V. philippinarum*. Results are means ± SE.

Sampling time	Factor	THC	Haemocyte volume	Haemocyte diameter	NR up-take	Haemocyte proliferation
T0	pH	ns	ns	ns	0.000	ns
T1	pH	ns	ns	ns	ns	0.007
	diclofenac	ns	ns	ns	ns	ns
	pH*diclofenac	ns	ns	ns	ns	ns
T2	pH	ns	ns	ns	ns	ns
	diclofenac	ns	0.029	0.033	ns	ns
	pH*diclofenac	ns	ns	ns	ns	ns

Tab. 1 - MANOVA results for haemocyte parameters in *M. galloprovincialis* and *V. philippinarum*.

Statistically significant effects are in red.

4.3.4. Discussion

Alterations in haemocyte morpho-functional parameters have been reported for bivalves after exposure, both in vitro and in vivo, to organic and inorganic contaminants, such as heavy metals (Matozzo et al. 2001, Sauvé et al. 2002), organotins (Matozzo et al. 2002), polycyclic aromatic hydrocarbons (PAHs) (Guerra-Rivas et al. 2002). Only recently, increasing attention has been addressed to the evaluation of potential immunotoxic effects of emerging

contaminants, such as pharmaceuticals and personal care products (PPCPs) (Parolini et al., 2009; Matozzo et al., 2012a, 2012c; Luna-Acosta et al., 2012) and nanoparticles (Canesi et al., 2008).

In this study, the effects of caffeine, commonly used as a stimulant in a variety of products, were investigated under different seawater pH values for the first time in *M. galloprovincialis* haemocytes.

Literature about caffeine toxicity in non-target organisms is very limited. Recently, the sub-lethal cellular toxicity of environmentally relevant concentrations of caffeine (del Rey et al., 2011) was investigated in the mussel *Mytilus californianus*. In that study, Hsp70 concentrations in the gill and mantle tissues of mussels exposed to 0.05, 0.2, and 0.5 μg/L of caffeine for 10, 20, and 30 days were compared to basal levels in control mussels. Exposure to caffeine at 0.05 μg/L induced a moderate up-regulation of Hsp70 in the gill lamellae of *M. californianus* after 20 days of exposure while in the highest caffeine concentration (0.5 μg/l), a similar increase in Hsp70 expression was not observed throughout the experiment.

Not only the exposure to contaminants can alter haemocyte parameters. It is well documented that shifts in environmental parameters can affect haemocyte functionality as highlighted in several studies, which reported effects of temperature (Monari et al., 2007), salinity (Munari et al., 2011) ,and pH alone (Bibby et al., 2008) or in combination with different temperature and salinity values (Matozzo et al., 2012b).

When evaluating potential effects of environmental stressors, variations in haemocyte functionality represent crucial endpoints as they are expression of altered physiological performance and, most

of all, they may result in a reduced immunosurveillance, which increases susceptibility to diseases and lowers survival capability.

THC is one of the most commonly used bivalve immunomarkers to evaluate negative effects of stressors (including pollutants) to animals (Oliver and Fisher, 1999). Generally, increased THC may be due to either proliferation or movement of cells from tissues into haemolymph, whereas decreased THC are caused by cell lysis or increased cell movement from haemolymph to tissues (Pipe and Coles 1995; Parry and Pipe 2004). In this study, MANOVA did not show any significant variations in THC values among treatments at all haemolymph sampling times. However, after the first week of exposure an increase in THC was observed at reduced pH. It is important to note that circulating haemocytes from bivalves, such as demonstrated in mussels and clams, can divide in the haemolymph (Matozzo et al., 2008; Renvrantz et al., 2013) and that contaminants , including pharmaceuticals , can stimulate the mitotic activity of bivalve haemocytes (Matozzo et al., 2012a; Mayrand et al., 2005). Therefore, higher THC levels at low pH may be related to the increased haemocyte proliferation (although not significant) recorded at T0 as a response to decreased pH. At T1 an increase in THC levels was also observed mostly in mussels exposed to pH 7.4. At this time, however, increases in THC cannot be related to similar variations in haemocyte proliferation that was significantly lower under reduced pH, showing similar values in the absence and in the presence of caffeine. At the end of the experiment, a reverse pattern of THC variation respect to T1 was observed, higher values being occurred in the absence of caffeine at pH 8.1 and 7.7, and lower ones

in all conditions tested at pH 7.4. Like this study, in another study (Bibby et al., 2008) a 32-day exposure to different pH levels (7.8, 7.7, 7.5 and 6.7) did not cause any significant effects on total and differential cell counts in *Mytilus edulis*.—Interestingly, both haemocyte volume and diameter showed to be particularly affected by the experimental conditions tested, in particular under exposure to caffeine. Indeed, both parameters were significantly increased by caffeine at T2 with the highest values at the higher caffeine concentration (0.5 μg/L) at all pH tested.

The cationic probe NR is commonly used to evaluate the effects of stressors on lysosomal membrane stability in bivalve haemocytes (Hauton et al., 1998; Matozzo et al., 2001; Canesi et al., 2007; Binelli et al., 2009; Aguirre-Martínez et al., 2013). The uptake of NR by haemocytes occurs either by pinocytosis or by passive diffusion across cell membranes (Coles et al.,1995). Consequently, alterations in dye uptake reflect damage to cell membranes (including lysosomal membranes) and/or weakening of haemocyte pinocytotic capabilities.

In this study, we demonstrated that the maintenance at reduced pH significantly induced NRU by haemocytes after one week of exposure, suggesting an increase in pinocytotic activity, probably related to the increased THC levels and the greater haemocyte volume and diameter observed at T0 in animals kept at reduced pH. In the study of Matozzo et al. (2012b), the exposure of *C. gallina* to reduced pH, high temperature (28°C) and low salinity (28 psu) induced haemocytes to become more active, increasing NRU. Also Bibby et al. (2008) found increased levels of phagocytosis in *M. edulis*

exposed to reduced pH values. In a recent study (Aguirre-Martìnez et al., 2013), a 35-day exposure of *V. philippinarum* to environmental concentrations of caffeine (0.1, 5, 15, 50 µg/L) caused a significant decrease in Neutral Red retention time. However, this effect occurred at 15 µg caffeine/L, a concentration 30-fold higher than the highest used in the present study. In our study, although a slight dose-dependent reduction in NRU by haemocytes was observed in animals kept at pH 8.1 and 7.7 at T1, no statistically significant effects due to caffeine or its interaction with pH were highlighted both at T1 and T2.

These results suggest that environmental caffeine concentrations and ocean acidification may impact the physiological condition and functionality of the haemocytes in mussels by altering their size and pinocytotic activity. Further investigations, however, are needed to address the toxicity of this contaminant on sentinel species. In particular, since effects on cell membrane functionality were highlighted also in other studies under acidified-conditions (Bibby et al., 2008; Matozzo et al., 2012b), further studies should investigate the combined effects of GCCs and emerging contaminants on marine bivalves when their immune system is challenged with environmental pathogens.

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4.4. Research article IV: Can the combination of decreased pH values and diclofenac exposure influence physiological performance in the mussel Mytilus galloprovincialis?

4.4.1. Introduction

The oceans have taken up to 50% of the anthropogenic CO_2 released to the atmosphere by the combustion of fossil fuels, contributing to partial mitigation of global warming (Sabine et al., 2004). However, the dissolution of anthropogenic CO_2 has dramatically altered the inorganic carbon chemistry of seawater by reducing the carbonate ion saturation and the pH (Feely et al., 2004; Orr et al., 2005). Furthermore, simulations based on ocean circulation models predict an increase in atmospheric CO_2 levels up to 2000 ppm by the year 2300 and a corresponding decrease in surface ocean pH by 0.77 units (Caldeira & Wickett, 2003).

Ocean acidification is predicted to have a significant effect on marine organisms, particularly calcifying animals such as molluscs and corals (Orr et al., 2005). Molluscs play an important role in marine food webs. Furthermore, they can provide a habitat for a number of marine organisms (Nagarajan et al., 2006) and they are important carbonate producers (Gutiérrez et al., 2003).

Several studies investigated which effects ocean acidification could have on marine species like bivalves, by measuring variations in biological responses at different levels, from molecular to organism. Michaelidis et al. (2005) demonstrated that in the mussel *Mytilus*

galloprovincialis haemolymph pH and metabolic rate were depressed during 90 days exposure to acidified water (pH 7.3). Matozzo et al. (2012; 2013) showed that the exposure to different pH values combined with different levels of temperature and salinity significantly affected both haemocyte parameters and the activity of antioxidant enzymes in two bivalves, *M. galloprovincialis* and *Chamelea gallina*. Liu & He (2012) found that the exposure to low seawater pH (7.7 and 7.4) significantly affected metabolism of the scallop *Chlamys nobilis* and the mussel *Perna viridis* with a strong reduction of excretion rates and clearance rate. The alteration of metabolic rates such as clearance, respiration and excretion may explain the effects on parameters such as growth rate, survival and other energy dependent processes as observed in other studies on molluscs. In juvenile mussels (*Mytilus chilensis*), 70 days of exposure to elevated pCO_2 levels (750–1200 ppm) resulted in a significant reduction of energy available for growth (scope for growth) (Navarro et al. 2013). Matoo et al. (2013) demonstrated that long-term exposure of the hard shell clams *Mercenaria mercenaria* and eastern oysters *Crassostrea virginica* to moderate warming (+5 °C) and hypercapnia (~800 ppm CO_2) minimally affects the cellular redox status, however, the combined exposure to elevated temperature and hypercapnia led to elevated standard metabolic rate in clams indicating elevated costs of basal maintenance.

Not only ocean acidification, or climate changes more in general, can affect the metabolism of marine organisms. Mostly in coastal environments, they are constantly exposed to a wide range of anthropogenic substances whose behaviour and fate after being

released are still unclear. Among these emerging contaminants, pharmaceutical products are cause for increasing concern since they are produced to have a biological effect and thus they can act even on non-target organisms when released into the environment.

Among pharmaceuticals, the non-steroidal anti-inflammatory drug (NSAID) diclofenac is widely applied for both veterinary and human use. This compound has been frequently detected in effluents from sewage treatment plants (STP) and surface waters worldwide. For example, median concentrations of 0.02 and 0.42 mg/L of diclofenac were detected, respectively, in streams and STP effluents of the United Kingdom (Ashton et al., 2004), while in coastal environments concentration between 0.022 and 0.298 µg/L were found (Rodrìguez-Navas et al., 2013).

Lethal toxic effects of diclofenac, that have led vulture species near to extinction in south Asia and south Africa, were reported (Naidoo et al.,2009; Oaks et al., 2004), and since then potential consequences of diclofenac release into ecosystems have received worldwide interest. Indeed, there is increasing evidence that this compound can affect non-target organisms in many ways.

Parolini et al. (2011) and Parolini & Binelli (2012) found that the exposure to diclofenac, as well as to a mixture of three non-steroidal anti-inflammatory drugs (diclofenac, ibuprofen and paracetamol), was able to induce significant increments in DNA fragmentation in haemocytes of the freshwater mussel *Dreissena polimorpha*, which preluded fixed genetic damage, as highlighted by the increase in both apoptotic and micronucleated cells. Lee et al. (2011) reported that the exposure to diclofenac caused a marked decrease in reproduction

rate, in two cladoceran species (in *Daphnia magna*-at 25mg/L, and in *Moina macrocopa* at 50 mg/L). In the same study, three-month exposure of the medaka fish *Oryzias latipes* to 0.001–10 mg/L of diclofenac resulted in a significant dose-dependent decrease in hatching success and delay in hatch.

An issue that still needs to be addressed is the potential interaction between environmental contaminants, like pharmaceutical products, and seawater acidification. It is known that ocean acidification affects the speciation of metals (Millero et al., 2009), possibly altering their toxicity, and it would also modify the nutrient cycles (Hutchins et al., 2009). But the occurrence of contaminants/reduced pH interactions has been little studied experimentally. Although some studies has been focused on interactions of differing environmental stressors with regard to invertebrate development and other life history traits (Byrne, 2011, 2012), generally, it is poorly understood how shifts in environmental parameters (temperature, pH or oxygen level), as predicted by climate changes scenarios, may influence animals' tolerance to pollutants.

In this study, potential effects of diclofenac under acidified conditions on physiological parameters (clearance rate, respiration rate and excretion rate) were evaluated in the mussel *Mytilus galloprovincialis.*

4.4.2. Materials and Methods

Specimens of *M. galloprovincialis* (4.0 ± 0.5 cm shell length) were collected along the west coast of the Northern Adriatic Sea (near

Cavallino Trepporti, Italy) in August 2011 and immediately transferred to the laboratory. Bivalves were carefully checked for shell damage (damaged animals were not used for experiments), and epibionts (such as barnacles and algae) were removed from the mussels. In the laboratory, the bivalves were maintained for a few days in aerated seawater with salinity, temperature and pH values similar to those measured in the field when the animals were collected. Prior to starting exposure, bivalves were acclimatized to the experimental conditions by gradually increasing or decreasing the seawater temperature (2°C per day) to 22°C and by gradually decreasing pH values from 8.1 to 7.7 and 7.4. The experiments were performed outside of the periods of sexual maturity to reduce the potential for additional stress related to spawning during the experiments.

Experimental setup for bivalve exposure

The experimental flow-through system (see Fig. 3, Combined effects of seawater acidification and diclofenac on haemocyte parameters of two marine bivalve species, this thesis) used for both mussel and clam experiments was built inside the Hydrobiological Station 'Umberto D'Ancona' at Chioggia. It consisted of a main outside reservoir filled with natural seawater (about 1000 L of capacity). The seawater was then pumped into two tanks (about 300 L each) inside the laboratory and equilibrated to the controlled laboratory temperature (22°C) The seawater next moved on to three tanks of approximately 120 L capacity, where the pH was adjusted to experimental values by bubbling CO_2 using an automatic control

system (ACQ110 Aquarium Controller Evolution by Aquatronica) connected with pH electrodes (ACQ310N-PH by Aquatronica). The seawater was finally pumped (25 ml min^{-1}, using ACQ450 Dosing pumps by Aquatronica) into the experimental tanks (A, B and C, approximately 50 L each) containing the bivalves.

During the first week of exposure, animals were subjected to three pH values (8.1, as control, 7.7 and 7.4). During the second and the third week, they were exposed to three concentrations of diclofenac (0, 0.05 and 0.5 µg/L) for each pH value. For each concentration, two replicates (with 70 bivalves each) were constituted (control without contaminant: C-A, C-B; diclofenac 0.05 µg/L: D1-A, D1-B; diclofenac 0.5 µg/L: D2-A, D2-B).

The experimental concentrations of diclofenac were maintained by distributing in continuous with peristaltic pumps stock solutions of the contaminant in distilled water (1 ml min^{-1}, using a MCP Process Pump, mod. ISM915A, provided with a MS/CA pumphead, mod. ISM724A, by ISMATEC) from two 15 L glass jars. Taking into account the two combined flows (25 ml min^{-1} for seawater and 1 ml min^{-1} for the contaminant solution, respectively), the concentrations of diclofenac inside the two glass jars were calculated to be 25 times higher than the concentrations needed inside the respective tanks with bivalves.

Of the experimental conditions tested, the control without diclofenac at 8.1 pH was considered as a reference.

During the experiments, animals were fed with microalgae (*Isochrysis galbana*). Twice a day, 250 ml of algae (3.5 x 10^6 cells ml^{-1}) were added in each 120 L tank, in order to keep algae concentration

around 2000 cells/ml in the experimental tanks with animals. Bivalve mortality was checked daily.

Measurements of physiological parameters

Measurements of the physiological parameters (clearance rate, respiration rate, excretion rate) were carried out following the procedures described by Widdows (1985). The physiological rates were determined in 12-15 mussels per experimental condition after 7 days of exposure to differing pH values (T0),and after 7 and 14 days of exposure to differing pH/diclofenac combinations (T1 and T2, respectively).

All measurements were performed at the same temperature, salinity and pH conditions used during the experiment. Seawater was previously treated with UV light (lamp model Scudo UVC 11 W) and filtered (Whatman GF/F glass microfibre filters) and then equilibrated to the experimental pH values by bubbling CO_2.

To remove the effect of body size from physiological measurements, the allometric equation $Y=aX^b$ was used, where Y is the physiological parameter, X body size, expressed as dry weight of mussel soft tissue, *a* the intercept of the regression line, representing the value of the physiological measurement/unit of dry weight (1 g), and *b* the slope of the regression line. The values of the coefficient '*b*' were 0.4 for the clearance rate, and 0.65 for the respiration and excretion rates, as reported for *Mytilus edulis* by Widdows & Johnson (1988) and subsequently applied in *M. galloprovincialis* by Widdows et al. (1997).

Clearance rate (CR)

CR, defined as volume of water cleared of suspended particles per unit of time, was measured using a static approach. Each mussel was maintained in a beaker containing 2 litres of filtered seawater and 30000 cell /ml of *I. galbana*. Seawater was kept gently aerated during the experiment and one beaker without a mussel acted as a blank. Microalgae concentration was measured on four 20-ml aliquots collected from each beaker every 30 min over a period of 2 hours by a Scepter 2.0 Handheld Automated Cell Counter, Millipore^{TM}. Each measurement was performed in triplicate on 60-µl subsamples added with 60 µl of isotonic solution (Coulter Isoton II Diluent).

The CR was calculated using the following equation:

$$CR=(V/t)\times(\ln C1 - \ln C2),$$

where V is volume of seawater (L), t the time interval (h), and C1 and C2 microalgae concentrations at the beginning and end of each time increment, respectively. For each clam, the CR value was defined on the basis of an 1 h period (two consecutive time increments), during which the decline in cell concentration was greatest (Widdows, 1993). Results were espressed as litres/hour.

Respiration rate (RR)

The rate of oxygen consumption or respiration rate (RR) of individual mussels was measured in closed plexiglas chambers (modified Quickfit flasks, volume 700 ml), which were held in a temperature controlled water bath placed on a magnetic stirrer. Air-saturated filtered seawater was added to each chamber and stirred by means of a magnetic stirrer bar beneath a perforated glass plate supporting

a mussel. The rate of decline in oxygen partial pressure ($p0_2$) in each chamber was measured by a calibrated membrane oxygen electrode (Strathkelvin 1302) connected to a multi-channel Strathkelvin oxygen meter (Model 928). Before measurements, a 30 min period was provided for allowing mussels to open their valves and to resume pumping, then mussel oxygen uptake was monitored continuously over the next hour. Oxygen consumption was not measured below a partial pressure of about 100 mm Hg because the rate of 0_2 uptake by *M. galloprovincialis* and other oxyconforming species becomes dependent on external $p0_2$ at lower oxygen tensions. A chamber without an animal was used as a blank. The RR was the calculated following the equation (Widdows, 1985):

$$RR=[60\ (C0-C1)\ (V\ )]\ /\ (t0-t1),$$

where C0 and C1 are oxygen concentrations at the beginning and end of the experiments, V the volume of the respiration chamber, and t0 and t1 the start and finish times (min) of the measurement period. Results were expressed as µmol O_2/hour.

Excretion rate (ER)

ER was calculated measuring the concentration of $N\text{-}NH_3$ in the water from the RR chambers. In particular, at the end of the measurement period for RR, an aliquot (50 ml) was collected from each chamber and analyzed spectrophotometrically following the method of Solorzano et al. (1969).

ER was determined using the formula (Widdows, 1985):

$$ER\ (\mu atm/h) = (C_t\text{-}C_b) \times [(14/(1000/V)] \times 1/t$$

where C_t and C_b are, respectively, the $N\text{-}NH_3$ concentration in the chamber containing the mussel and that in the blank, both measured at the end of the RR measurement period, V is the chamber volume in litres and t the incubation period expressed in hours. Results were expressed as

Dry weight

After CR and RR measurements, the dry weight of each mussel was determine. Soft body was removed from the shell and dry in an oven at 60°C for 48 hours, constant weight being attained.

Statistical analyses

Results of physiological parameters (CR, RR, ER) were analysed with a permutation-based nonparametric MANOVA (Pesarin, 2001). For each parameter, a nonparametric full factorial ANOVA was set with factors pH, Contaminant and Time (the last as a repeated measure). To take into account the dependence of measures within the same tank, a mixed-model approach was used (Finos and Basso, 2012, 2013).

4.4.3. Results

After one week of exposure to different pH levels clearance rate was significantly influenced by pH ($p<0.012$) with a reduction in animals kept at pH 7.7 and 7.4 respect to controls at pH 8.1 (Fig. 1). At T1 and T2 no significant effects due to pH or diclofenac or pH/diclofenac interaction were recorded. Despite this, at T1 clearance rate in

controls kept at pH 8.1 was lower than that measured in all the other conditions tested . Conversely, no clear pattern of variation was observed at T2.

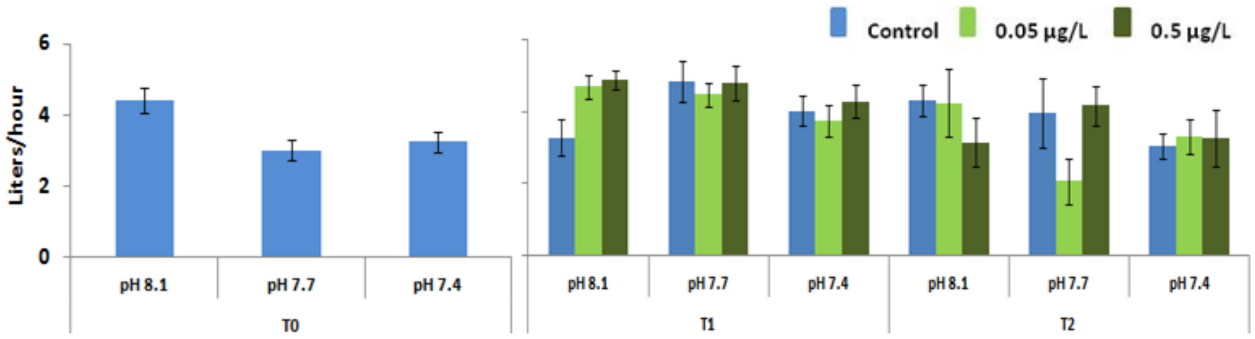

Fig. 8 – CR in *M. galloprovincialis*. Results are means ± SE

At T0 respiration rate was significantly affected by pH with a reduction of oxygen consumption in animals maintained at pH 7.4 (Fig.X). A significant effect of pH on respiration was also highlighted by MANOVA at T1. Animals kept at pH 7.7 and 7.4 showed higher oxygen consumption than those kept pH 8.1 at all concentration of diclofenac tested. No significant effects due to diclofenac were found at T1, even if an increase was observed in animals exposed to 0.05 µg/L, at all pH levels. At the end of the experiment (T2) no significant effects due to pH, diclofenac and their interaction were recorded. Interestingly, two opposite trends between results from animals kept at pH 7.7 and those kept at pH 7.4 were observed. At pH 7.7 exposure to diclofenac induced a decrease of respiration rate while at pH 7.4 an increase, but they were not statistically significant probably because of the high variability observed (Fig. 2).

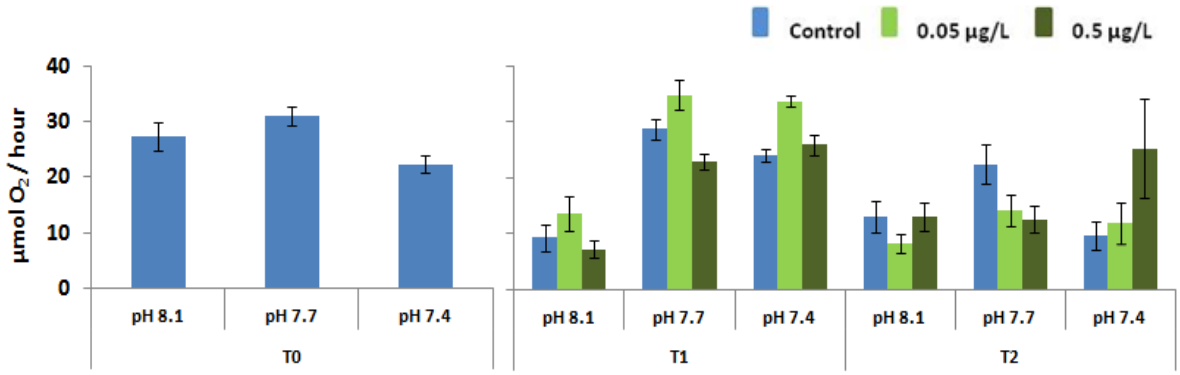

Fig. 9 – RR in *M. galloprovincialis*. Results are means ± SE.

At T0 a significant reduction ($p < 0.006$) in excretion rate was recorded in condition of low pH respect to controls at pH 8.1 (Fig. 3). At T1 no significant effects of pH, diclofenac or their interaction were observed. Conversely, at T2 the interaction between pH and contaminant significantly influenced excretion rate ($p<0.002$). A decrease in excretion rate was observed at all diclofenac concentrations tested when comparing pH 8.1 with pH 7.7 and 7.4. At all pH levels tested, mussels kept at the highest concentration of diclofenac showed slightly increased excretion values with respect to their controls (fig.3)

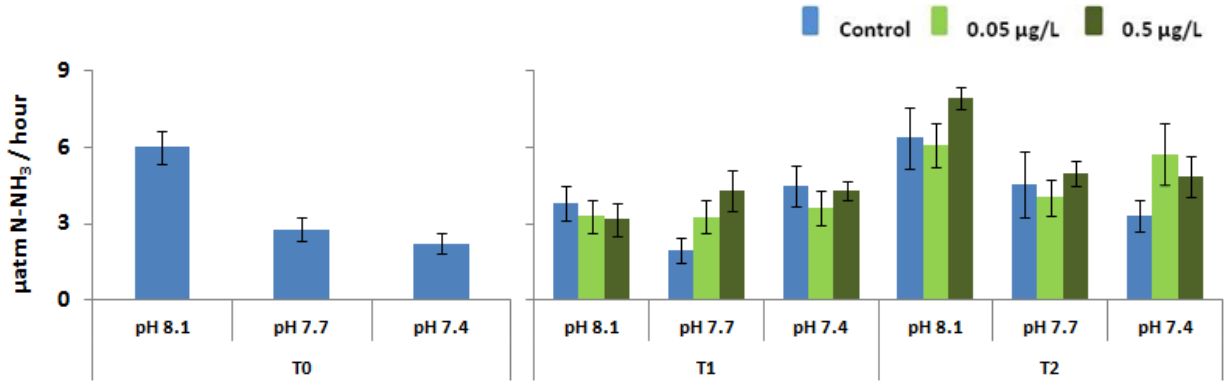

Fig. 10 – ER in *M. galloprovincialis*. Results are means ± SE.

4.4.4. Discussion

Under stressful conditions, mussels are subject to alterations in physiological rates resulting in a decreased acquisition of energy through filtration and/or an increase of metabolic consumption during respiration and excretion (Ericson et al., 2010). It is well-known that a reduced clearance rate, as a measure of the bivalves' filtering activity, often cannot guarantee the right amount of energy required to sustain all metabolic processes, growth and reproduction (Beesley et al., 2008; Thomsen & Melzner, 2010).

In the present study, a significant reduction in clearance rate was observed after one week of exposure to low pH. Conversely, in a previous study on the combined effects of pH (8.1, 7.7and 7.4), temperature (22 and 28°C) and salinity (28, 34 and 40 psu) on physiological parameters of *M. galloprovincialis*, a significant increase in clearance rate was highlighted at pH 7.7 and 7.4, 22°C and 34 psu (conditions similar to those used in this study) compared to control at pH 8.1 (Chinellato et al., 2010). In this study, however, although not significant, slightly increases in clearance rate were observed after two weeks of exposure to reduced pH values (T1). In their study, Liu & He (2012) evaluated the effects of different pH levels (pH 8.1, 7.7 and 7.4) on clearance, respiration, and excretion rates in three bivalve species, the pearl *oyster Pinctada fucata*, the noble scallop *Chlamys nobilis*, and the green-lipped mussel *Perna viridis*.

They found that clearance rate was higher at pH 7.7 for *P. fucata* and at pH 8.1 for *C. nobilis* and *P. viridis*. Sanders et al. (2013), found that 3 months laboratory exposure to four pCO_2 treatments (290, 380,

750 and 1140 µatm) did not induce any significant variations in clearance rate, respiration rate, condition index or cellular turnover (RNA: DNA) in the king scallop, *Pecten maximus*, when food was unrestricted. Also Fernandez-Reiriz et al. (2012) did not find significant effects on clearance, ingestion and respiration rates in juvenile of the mussels *M. galloprovincialis* exposed to a reduction of seawater about of -0.3 and -0.6 pH for 78 days. This lack of effects was due to the high alkalinity levels in coastal waters of Ria Formosa (SW Portugal).

It is known that the exposure to environmental contaminants, both organic and inorganic, can cause reductions in bivalve clearance rate (Widdows & Johnson, 1998). However in this study, no significant effects of diclofenac or its interaction with pH were observed even if at T1 a positive trend due to the presence of the contaminant, at both the concentration tested, was observed in animals kept at pH 8.1. This lack in toxicity is probably due the environmental-range concentrations used. Indeed, conversely to this study, Ericson et al. (2010) exposed the Baltic Sea blue mussels, *Mytilus trossulus*, to diclofenac, ibuprofen and propranolol in concentrations ranging from 1 to 10,000 µg/L. The pharmaceuticals were added both separately and in combination. Mussels exposed to high concentrations of pharmaceuticals showed significantly lower scope for growth, which indicates that the organisms had a smaller part of their energy available for normal metabolism.

To measure respiration and excretion rate is the same as to quantify metabolic cost, the energy used and lost through respiration and nitrogen compound elimination. Alterations of respiration rate can

be caused by abiotic factors such as variations in temperature, dissolved oxygen, or seawater pH. Those organisms which use gills to breath are more vulnerable than terrestrial ones to the increase in CO_2 concentration and to the decrease in pH values. Highest pCO_2 values in the water may acidify tissues and body liquids, influencing the oxygen transport (Raven et al., 2005). In the present study a significant reduction of respiration rate was observed at T0 due to decreasing pH. Similar results were obtained in other bivalve species: *M. galloprovincialis* (Michaelidis et al., 2005), *Crassostrea virginica* (Wilson & Burnett, 2000), *Crassostrea gigas* (Lanning et al., 2010), *Venerupis decussatus* (Fernandez-Reidriz et al., 2011) and *Clamys nobilis* (Liu & He, 2012). Conversely, Chinellato et al. (2010) did not observe significant variation in *M. galloprovincialis* exposed to pH 7.7 and 7.4 (22°C, 34 psu) for 7 days compared to control (pH 8.1).

Similar results were obtained by Chinellato (2012) in *Mimachlamys varia* (at 18°C) and *Callista chione* (at 16°C) and by Liu &He (2012) in *Pinctada fucata* and *P. viridis*.

In bivalves, oxygen consumption increases in presence of many environmental contaminants (Widdows et al., 1995). Interestingly in this study, respiration rate values measured at T1 showed a significant increase with decreasing pH. At all pH tested, the highest value of oxygen consumption was recorded at the lowest concentration of diclofenac. When compared to T1, at T2 an overall decrease was found in the respiration rates of mussels kept at pH 7.7 and 7.4, mostly in diclofenac exposed animals.

In the energy balance, excretion represents a part of the energy loss through release of nitrogen compounds coming from protein and

nucleic acid catabolism. In many bivalves species, NH_4^+ is the main excreted component. Like for the rest of physiological parameters considered in this study, also nitrogen excretion can be affected by environmental parameters (Han et al., 2008; Vedpathak et al., 2008). As an instance, Liu & He (2011) found that the excretion rate was significantly lower at pH 7.4 than pH 8.1 for the three species of bivalve used (*P. fucata, C. nobilis* and *P. viridi*s). On the contrary, there are no data available on the possible effect of diclofenac on excretion rate in aquatic invertebrates.

In the present study, an one-week exposure to low pH caused a significant reduction of the excretion rate respect to control. The same pattern of variation was observed at T2 in controls. At this time, the effect of diclofenac and its interaction with pH was better highlighted at pH 7.4 where the excretion rate increased at both the concentrations of the contaminant.

M. galloprovincialis, which is widely distributed and commonly farmed in the North Adriatic Sea and in the Lagoon of Venice, are exposed to rapid variations of a wide range of environmental parameters and to many natural and anthropogenic stressors (Bussell et al., 2008).

In agreement with Michaelides et al. (2005), the decrease of excretion rate observed in this study at reduced pH suggests that a lower amino acid catabolism may be an adaptation strategy to face stressful environmental condition and survive.

However, in the presence of a contaminant, this compensatory ability seems to be lost: metabolic cost increases again, as confirmed also by the increase in respiration rate, while the acquisition of energy

through filtering activity does not increase. The interaction of reduced pH and diclofenac, even at low concentrations, may cause an overall reduction of energy availability with harmful outcomes for the maintenance of the main life processes including reproduction with consequently negative effects on mussel population and on the entire ecosystem since mussels are common keystone species on open coasts and in estuaries.

4.4.5. References

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4.5. Research article V: Can seawater acidification combined with diclofenac exposure affect larval development in the clam *Venerupis philippinarum*?

4.5.1. Introduction

CO_2 levels in the atmosphere have increased from the pre-industrial 280 ppm to the current 380 ppm (Caldeira & Wickett, 2003), the main cause of this phenomeon being human activities like fossil fuel consumption and deforestation.

As a result of increased anthropogenic emission of CO_2, sea water pH will decrease by an estimated value of 0.2 to 0.4 units by the end of this century (Caldeira & Wickett, 2003, 2005; Royla Society, 2005; Cao *et al.*, 2007) in a process commonly known as ocean acidification. These changes are predicted to occur faster than any other which has been faced for hundred millions of years (Sabine *et al.*, 2004), and the knowledge about how marine organisms could cope with them is still limited.

Ecologically relevant CO_2 perturbation studies have demonstrated adverse effects of ocean acidification for a wide range of marine organisms but in particular calcifying organisms have proved to be the most vulnerable to low pH levels (Hofmann et al., 2010; Kroeker et al., 2010).

More than adult organisms, early life stages may be particularly impacted by ocean acidification (Kurihara, 2008), because of their higher sensitivity to changing environmental conditions.

Indeed, recent studies have observed negative effects of ocean acidification on early developmental processes including fertilization, larval growth, larval duration, and settlement in bivalves (Range et al., 2013), gastropods (Crim et al., 2011) and echinoderms (Dupont et al. 2008).

Early life stages of marine organisms can be affected not only by environmental stressors like pH, temperature or salinity but also by the presence of anthropogenic substances.

In particular, apprehension is growing internationally owing to the widespread detection of pharmaceuticals in sewage water treatment plant (STP) effluents, surface waters, sediment and biota (Enick and Moore, 2007; Bound and Voulvoulis, 2006; Han et al., 2006; Rodrìguez-Navas et al., 2013). Because of high bioavailability and biological activity of pharmaceuticals (Fent et al., 2006; Jjemba, 2006), there is a risk of bioaccumulation and detrimental effects in non-target organisms following chronic exposure in the environment (Bengtsson et al., 2005; Daughton and Ternes, 1999).

The knowledge about the effects of such emerging contaminants is very little as until recently the attention has been mostly focused on the role of heavy metals, antifouling substances, pesticides, and the so-called legacy pollutants in general (Hutchinson et al., 2013), in altering normal biological processes in aquatic organisms (Fathallah et al., 2010; Wang et al., 2010; Inoue et al., 2005).

In a recent study, Méndez et al. (2013) found that a 18 days exposure to different environmental fluoxetine concentrations in sediments favoured the occurrence of males with abnormal genital spines in the

marine-estuarine polychaete *Capitella teleta* suggesting that fluoxetine can have important reproductive implications.

One of the most common pharmaceutical products used both in human and veterinary medicine is the non-steroidal anti-inflammatory drug (NSAID) diclofenac. Diclofenac is considered to be fairly persistent (Bendz et al., 2005), bioaccumulative (Schwaiger et al., 2004), rather water soluble, with low volatility (Breton and Boxall, 2003) and low tendency for adsorption to organic matter (Johnson et al., 2007; Fent et al., 2006).

Some studies have shown negative effects of diclofenac to aquatic organisms at environmentally realistic concentrations (Pomati et al., 2004; Schwaiger et al., 2004; Gagnè et al., 2005; Parolini & Binelli, 2012). However, they focused on adult stages while nothing is known about the effects of diclofenac on early life stages, of marine bivalves in particular. To properly assess the ecotoxicological effects of a pollutant, interactions with other environmental parameters have to be taken into consideration, as they may influence not only the chemical's behavior and fate but also the organisms' susceptibility to that chemical.

Changes in environmental parameters, as predicted by climate change scenarios, may alter the susceptibility of organisms to anthropogenic compounds (González-Ortegón et al., 2013). Presently, the possible effects of emerging contaminants and seawater acidification on early life stages have never been addressed before. Here we have investigated for the first time the combined effects of low pH and diclofenac on the early stages of the clam *Venerupis philippinarum*.

4.5.2. Materials and methods

Animals and experimental setup

The experimental flow-through system used for rearing clam larvae was built inside a hatchery facility (Veneto Agricoltura) located in Pellestrina Island (Lagoon of Venice) (Fig. 1).

It consisted of twelve experimental tanks (80 L each) individually equipped with two 20 µm filters, a pH-meter and a gassy CO_2 dispenser. The seawater, directly taken from the lagoon, was pumped into each tank (300 ml/min) after being mechanically filtered and sterilized with UV light and maintained at 25°C of temperature and 32 psu of salinity for the entire period of larvae rearing.

Fig. 11 - Experimental flow-through plant.

Microalgae (*Isochrysis galbana*) were distributed in continuous in all the experimental tanks, in order to have a final concentration of about 5 x 10^6 cell/ml.Sexually mature clams were collected in the Lagoon of Venice in April 2013 and reared under controlled conditions (22 °C, 32 psu) until ready to spawn. Spawning was

obtained by thermal induction, following standard hatchery protocols.

Embryos were obtained by pooling gametes of 15 males and 15 females. Just after the fertilization, embryos were distributed in the experimental tanks at a density of 10/mL. Two different levels of pH (8.1 and 7.7) combined with two concentrations of diclofenac (0 and 0.5 µg/L) were tested. Three replicate tanks for each experimental condition were set up. The experimental concentrations of diclofenac were maintained by distributing continuously a stock solution of the contaminant (1 ml/min) from a 15 L glass jar by means of peristaltic pumps. Of the experimental conditions tested, the control without diclofenac at pH 8.1 was considered as a reference. Larval mortality and growth were daily checked and recorded. The experiment was stopped 96h after fertilization. At the end of the experiment, pools of larvae (9 per experimental condition, 130,000 larvae each) were constituted to evaluate oxidative stress (CAT enzyme activity) and lipid peroxidation.

Mortality and growth

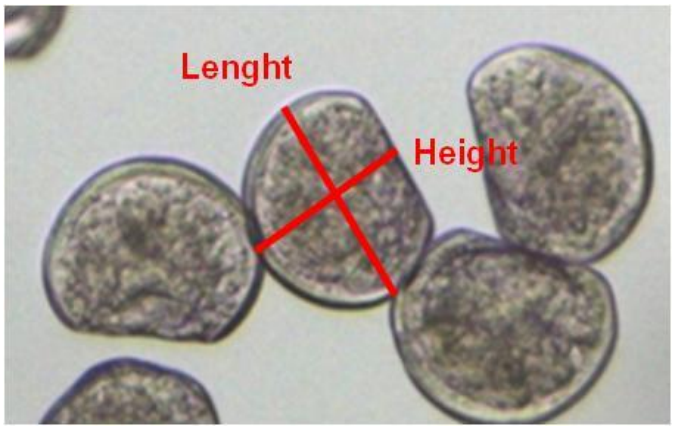

Fig. 12 – Shell length and shell height measured in V. philippinarum larvae.

Every 24 hours, larvae from each rearing tank were opportunely sieved and resuspended in 50 ml of filtered seawater.

From each suspension, four 1-ml aliquots were used

to count and photograph the larvae under a compound inverted microscope (Microscope: Leica DM750, camera: Leica DFC295, program: Leica LAS V3.8). Mortality was then calculated in relation to the initial number of embryos in each replicate tank. To assess larval growth, photographs were analyzed with IMAQ^{TM} Vision (National Instrument) and shell length and shell height (Fig.2) were measured in 200 individuals per replicate tank. Shell length/shell height ratio was also calculated.

Preparation of samples for biochemical analyses

Larvae pools were immediately frozen in liquid nitrogen and stored at - 80 °C until analysis. Samples were thawed on ice and centrifuged at 1,000 g for 2 min to remove seawater. Then larvae were added with 500 µl of 0.1 M Tris–HCl buffer (pH 7.5) containing 0.15 MKCl, 0.5 Msucrose, 1 mM EDTA, 1 mM Dithiothreitol (DTT, Sigma) and 40 µg/ml Aprotinin (Sigma). They were sonicated for 2 min at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles and were then centrifuged at 12,000 g for 30 min at 4 °C. Supernatant (SN) was collected for CAT and LPO assays.

Catalase activity assay

CAT activity was measured in triplicate following the method described in Aebi (1984).

Decreases in absorbance of a 50 mM H_2O_2 solution (ξ = -0.0436 mM^{-1} cm^{-1}) in 50 mM phosphate buffer (pH 7.8) and 10 µl of SN sample were continuously recorded at 240 nm and at 10-s intervals for 1 min. The results were expressed in U/mg of proteins with one unit of CAT being defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H_2O_2/min. For CAT assay, as well as for lipid peroxidation assay, tissue protein concentrations were quantified in accordance with Bradford (1976).

Lipid peroxidation (LPO) assay

LPO was quantified in sonicated larvae SN (200 µl) using the malondialdehyde (MDA) assay based on the method described in Buege and Aust (1978). Absorbance was read spectrophotometrically at 532 nm, and the results were expressed as nmoles of thiobarbituric reactive substances (TBARS)/mg protein. The concentration of TBARS was calculated as MDA equivalents using the molar extinction coefficient for MDA (ε = 1.56 x $10^5 M^{-1} cm^{-1}$).

Statistical analysis

Results from investigated parameters (i.e. shell length, shell height, their ratio, mortality, catalase activity and lipid peroxidation) were analysed with a permutation-based nonparametric MANOVA (Pesarin, 2001). For each parameter, a nonparametric full factorial ANOVA was set with factors pH, Contaminant and Time (the last as a repeated measure). To take into account the dependence of

measures within the same tank, a mixed-model approach was used (Finos and Basso, 2012, 2013).

2.5.3 Results

Larvae survival was significantly affected both by pH ($p<0.000$) and diclofenac ($p<0.038$) but not by their interaction.

96 hours after fertilization, about 50% of mortality was observed in larvae exposed to pH 7.7 and diclofenac, while in controls kept at pH 8.1 mortality was lower than 10% (Fig. 3).

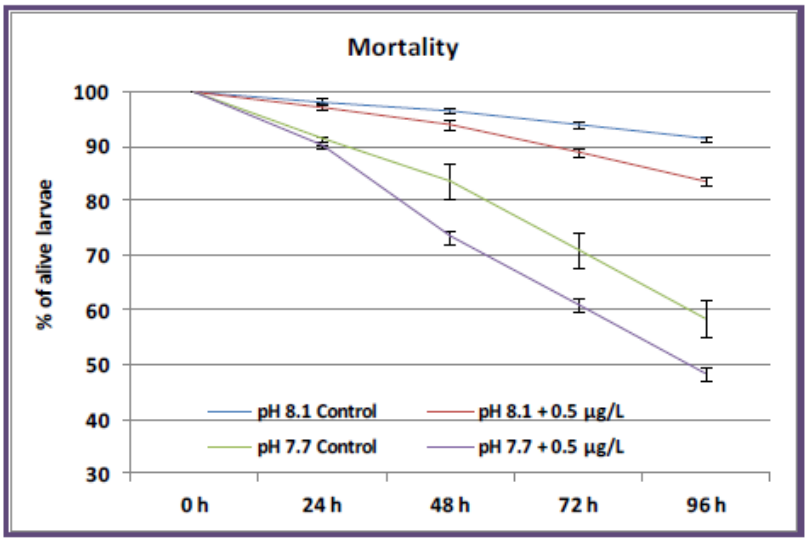

Fig. 13 – Larval mortality among different treatments. Results are means ± SE.

pH significantly influenced all shell parameters considered.

Results showed significant differences in length ($p<0.000$), height ($p<0.000$) and their ratio ($p<0.034$) during the 96-hs experimental period, with smaller shells observed in larvae kept at pH 7.7 respect to control, just after the first 24 hours post fertilization (Fig. 4A, B, C).

Significant effects due to the presence of diclofenac were highlighted by MANOVA only on shell length ($p<0.004$), while a significant interaction with pH was observed for both shell length and height but not for their ratio. Furthermore, since the very first day of exposure , deformed shells and prolapsed tissue were observed in larvae kept at low pH both in the absence and in the presence of diclofenac.

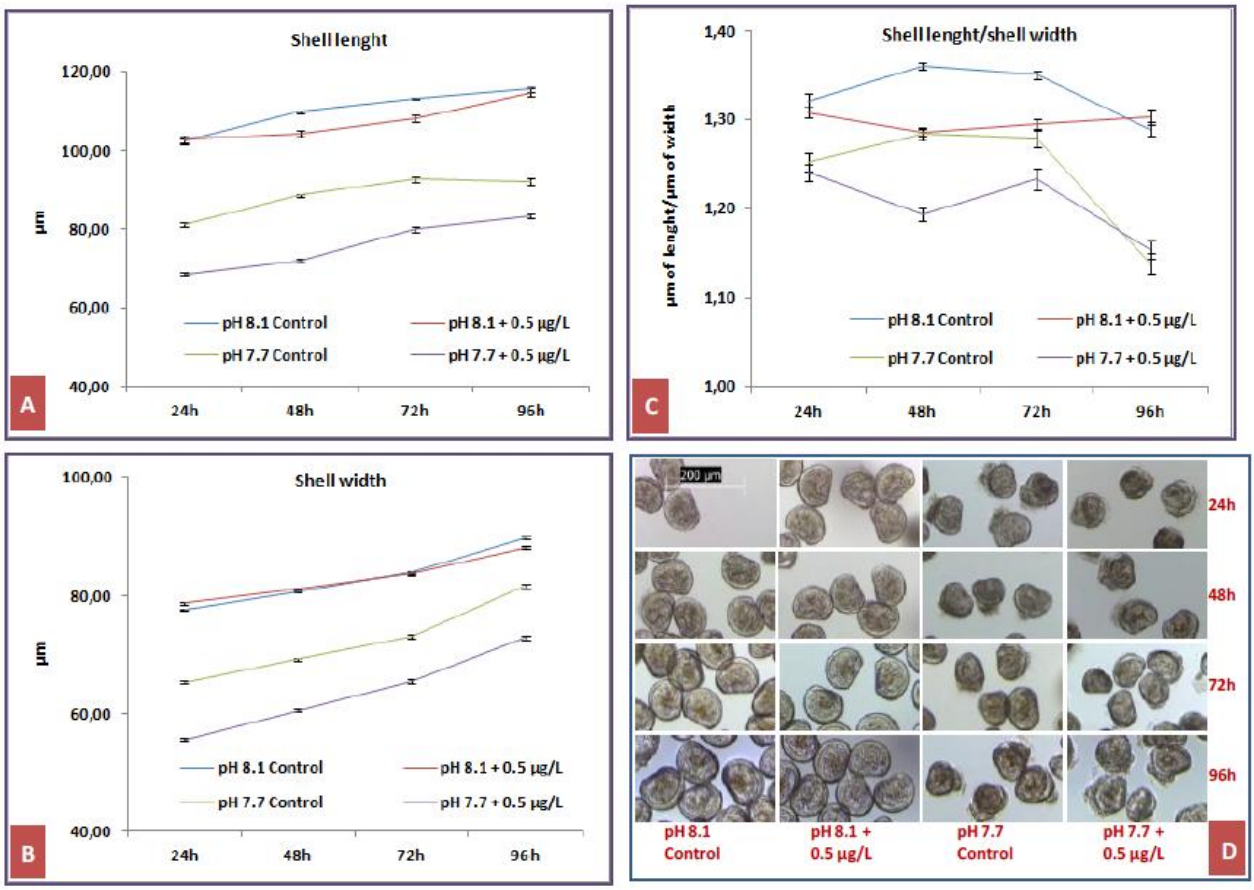

Fig. 14 – Shell length (A), shell height (B) and shell length/shell height variations during 96 hours post-fertilization. Results are means ± SE. Larvae development in the different treatments are showed in picture D.

CAT activity was significantly increased by low pH ($p<0.004$) with levels of activity three times higher in larvae reared at pH 7.7 compared to those maintained at pH 8.1, both in the absence and in the presence of diclofenac. Diclofenac and its interaction with pH did not significantly influence CAT activity, even if at both pH levels, an increasing trend was observed in the response measured in treated larvae with respect to controls (Fig. 5A).

MANOVA showed that differences in LPO levels among treatments were not significantly influenced neither by pH nor by diclofenac nor by their interaction, even though a slight increase in lipid

peroxidation was observed in larvae maintained in the presence of diclofenac, mainly at pH 7.7 (Fig. 5B).

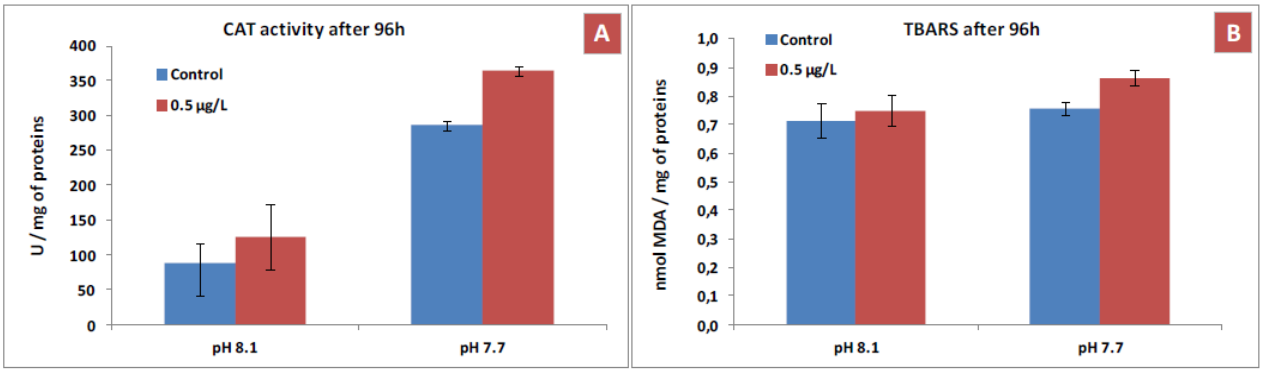

Fig. 15 – CAT activity (A) and LPO in V. philippinarum larvae. Results are means ± SE.

Factor	Mortality	Shell lenght	Shell height	Shell lenght/ shell height	CAT	LPO
pH	0.000	0.000	0.000	0.003	0.004	ns
diclofenac	0.038	0.004	ns	ns	ns	ns
pH*diclofenac	ns	0.001	0.000	ns	ns	ns

Tab. 1 - MANOVA results for larvae parameters in *V. philippinarum*.

Statistically significant effects are in red.

2.5.4. Discussion

Ecological consequences of climate changes have been predicted as widespread and dramatic (Fabry et al., 2008; Doney et al., 2009), with the possibility of loss in biodiversity and even extinctions (Veron et al., 2009). Furthermore, many areas around the world are contaminated by anthropogenic substances that may affect marine life through various biological pathways. In this study, the combined

effects of low pH, as predicted by climate change scenarios, and the non-steroidal anti-inflammatory drug diclofenac on the early life stages of the clam *V. philippinarum* were investigated for the first time.

Results showed that low pH affected *V. philippinarum* larvae more than diclofenac by reducing larvae survival and shell growth. Although shell shape, as represented by the length/ height ratio, did not change under low pH, morphological evidence of alteration in larval soft body was found. Similar alterations were observed in early stages of *Mytilus trossulus* exposed to copper concentrations exceeding 10 µg/L (Fitzpatrick et al., 2008) and in *Crossostrea gigas* veliger larvae exposed to low values of pH (ΔpH = -0.4 and ΔpH = -0.7) (Barros et al., 2013). Just 24 hours after fertilization, development of *V. philippinarum* larvae under control and reduced pH conditions diverged morphologically. Also CAT activity in larvae was significantly increased by pH only, suggesting strengthened anti-oxidant response to potentially increased oxidative stress. In another study, increased enzymatic activities (superoxide dismutase, CAT and glutathione peroxidase) were detected in D-shaped larvae and pediveligers of the bivalve *Meretrix meretrix* under Cd exposure (25 µg/L) (Wang et al., 2010). Conversely, in this study, no increase was found in LPO following exposure to reduced pH and /or diclofenac.

Acidified seawater affects early stages' survival and normal development in other species of bivalves and also in other taxa. For example, the survival of larvae of the bay scallop *Argopecten irradians* kept at pH 7.3 was significantly lower than that of larvae in ambient conditions after 1 day post-fertilization (White et al., 2013).

Likewise, the shell length in larvae from the high CO_2 treatment was significantly smaller than in larvae from the ambient conditions throughout the experiment and it was reduced by 11.5% within 7 days.

In their study, Barros et al. (2013) found high sensitivity of *C. gigas* veliger larvae to low values of pH (ΔpH = -0.4 and ΔpH = -0.7), as reflected by a decrease in survival and growth rates, as well as by an increased frequency of prodissoconch abnormalities. Moreover, results also showed that sperm motility, fertilization rate, and hatching success, were negatively influenced by acidification.

In the northern abalone *Haliotis kamtschatkana* (Crim et al., 2011), larval survival decreased by *ca.* 40% in elevated CO_2 treatments (800 and 1800 ppm) relative to the 400 ppm control. In the Antarctic sea urchin *Sterechinus neumayeri* (Byrne et al., 2013), near future warming-acidification treatments (24 °C/pH 7.6 and 7.8) induced a 5–25% decrease in normal development. An increase of CO_2 (about 1030 µatm) in seawater caused increased embryonic abnormalities and egg loss in the two-spotted goby *Gobiusculus flavescens* (Forsgren et al., 2013).

In this study, diclofenac affected larval survival and shell length, while no evidences of induced oxidative stress were found. There are no data available from literature about the possible effects of this pharmaceutical on early stages of marine invertebrates. Studies on chronic toxicity of diclofenac in fish reported cellular damage after 20 days of exposure to 0.5–1 µg/L, renal lesions after exposure to 5 µg/L (Hoeger et al., 2005; Schwaiger et al., 2004; Triebskorn et al., 2004)

and delayed hatching on embryos at 1 and 2 mg/L (Hallar et al., 2004).

V. philippinarum is a well exploited species in the Lagoon of Venice. After its introduction for aquaculture purposes in the ’80, *V. philippinarum* has been spreading widely in the Lagoon, replacing the native species *Venerupis decussatus.* Presently, *V. philippinarum* represents one of the main economical resources in the Lagoon area, being both fished and cultured. Therefore, population loss, as potentially driven by seawater acidification, could have both ecological and economical consequences. In this context, the measurements of morphometric parameters in embryos and larvae of calcyfing organisms, such as bivalve veligers and echinoderm plutei, provide a suite of informative endpoints to understand the effects of seawater acidification and thus potential risk to natural populations (Dupont et al., 2008; O’Donnell et al., 2010; Sheppard Brennand et al., 2010).

However, it has to be taken into account, that pH levels in nature will change over coming decades gradually, while in this study larvae were obtained from adults kept at pH 8.1 during gametogenesis and were subject to acidification only after fertilization. Like other marine invertebrates, more information is needed about clam susceptibility to reduced pH across the entire life cycle. Indeed, depending on the stage investigated (gametes, fertilization, embryos, larvae) responses to projected near-future (2100) acidification may be species-specific and some stages could be more resilient than others (Byrne, 2012). In this regard, also potential effects of diclofenac on clam gametogenesis should be considered in future

studies, since the target molecule of this pharmaceutical is the prostaglandin-endoperoxide synthase (PTGS), also called cycloxygenase (COX). A reduced activity of PTGS may occur under exposure to diclofenac resulting in a decreased production of prostaglandins that are involved in the gonad maturation in bivalves (Gagnè et al., 2005).

Overall, interference in reproductive processes due to environmental contaminants and synergistic detrimental effects due to seawater acidification in this clam species, as well as in other bivalves, need to be fully investigated in future studies as they are pivotal aspects in assessing and predicting ecological health in marine environments.

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5.1 CONCLUSION

In adults of both *M. galloprovincialis* and *V. philippinarum*, the evaluation of haemocyte and oxidative stress parameters allowed to highlight different patterns of response to the combination of seawater acidification and the emerging contaminant diclofenac. Exposure to seawater acidification-diclofenac and acidification–caffeine also affected physiological responses and haemocyte parameters, respectively, in *M. galloprovincialis*.

The results obtained showed that cellular, biochemical and physiological parameters measured in both mussels and clams were influenced more by pH than by the contaminants (diclofenac and caffeine). In both species, immune parameters were the most responsive to differing experimental conditions. In bivalves, circulating haemocytes are involved in important functions, such as immune defence (Cheng, 1981; Hine, 1999; Donaghy, 2009) and shell deposition (Mount, 2004). Increases in the THC values are generally considered as a consequence of proliferation or movement of cells from tissues into haemolymph, whereas decreases are likely due to cell lysis or increased movement of cells from haemolymph to tissues (Pipe et al.,1995). In our experiments on *V. philippinarum*, THC and haemocyte proliferation significantly increased with decreasing pH values at T0. Interestingly, the value of NR uptake decreased significantly at pH 7.4, suggesting that clams were trying to compensate a weak pinocytotic capability, due to damage to cell membranes, by increasing the number of circulating haemocytes to

maintain immunesurveillance. Despite COX is involved in inflammatory processes and it should be inhibited by NSAIDs, no significant effects due to diclofenac were revealed in both bivalve species, whereas pH significantly influenced this enzymatic activity. However, an opposite pattern of variation was observed in tissues of clams and mussels kept at low pH, decreasing in clams but not in mussels.

In *M. galloprovincialis*, differing modulation patterns were found in physiological rates measured at T0, T1 and T2. Reduction in filtration and nitrogen excretion and increase in oxygen consumption highlighted stressful condition under higher seawater CO_2 levels.

This study demonstrates that elevated levels of CO_2 and diclofenac negatively impact larval stages of *V. philippinarum*. Like in adults, also in larvae the effect of low pH (7.7) was more detrimental than that of diclofenac, being attained the worst conditions in larvae kept under the combination of the two stressors.

In particular, both reduced pH and diclofenac dramatically reduced larval survival and 50% mortality was observed in reduced pH/diclofenac treated larvae after only 96 hours post-fertilization. pH, more than diclofenac, compromised a normal growth in larvae but a significant interaction between low pH and contaminant was found. Just 24 hours after fertilization, development of *V. philippinarum* larvae under control and reduced pH conditions diverged morphologically. Oxidative stress parameters showed to be more sensible to pH variations *per se*. The increased CAT activity found and the absence of significant variation in LPO values suggested strengthened anti-oxidant response to potentially

increased oxidative stress. These results are the first evidence of negative impact of decreased pH on early-stages of the clam *V. philippinarum*. Moreover, those obtained are the first findings on toxicological effects of NSAIDs in bivalve early life stages.

Recently many studies provided important new data on how planktonic life stages and early juveniles may cope with changing environmental parameters. Seawater acidification, as a sole stressor, is exhaustively studied on different life stages of several invertebrate species (Dupont et al. 2010; Hofmann et al. 2010; Byrne 2012; Parker et al. 2013). However, there is an increasing need to consider the effects of acidification and other concurrent stressors, both biotic and abiotic, by enhancing multi-factorial studies on the whole life history of marine invertebrates. Indeed, depending on the stage investigated (gametes, fertilization, embryos, larvae, adults) some species (Byrne, 2012) demonstrated to be quite resilient to projected near-future (2100) warming and/or acidification. In tidally influenced habitats, in particular, this may reflect genetic adaptation and phenotypic plastic responses.

Thus, as suggested by Byrne (2012) in order to answer the question 'Can marine calcifying organisms cope with future ocean acidification?' some crucial issues need to be properly addressed: 1) Which effects seawater acidification may have on gamete maturation in adults? 2) Can fertilization occur in a high pCO2/acidic marine environment? 3) Can embryos survive during the whole larval stage until settlement? 4) Will calcifying larvae be able to build their skeleton? 5) Can larvae develop to the juvenile stage?, and

furthermore ‘Can acidified conditions alter susceptibility of bivalves, at different life-stages, to emerging contaminants?’

Gamete maturation, gamete fertilization and developmental failure may result in recruitment failure with negative flow on effects for marine populations and ecosystems.

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