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# EAT BREATHE EXCRETE REPEAT: Physiological Responses of the Mussel *Mytilus galloprovincialis* to Diclofenac and Ocean Acidification

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**Abstract:** Combined effects of the nonsteroidal anti-inflammatory drug diclofenac and lowered seawater pH were assessed on the physiological responses of the mussel *Mytilus galloprovincialis*. Bivalves were exposed for 1 week to natural pH (8.1) and two reduced pH values (pH −0.4 units and pH −0.7 units), as predicted under a climate change scenario. After the first week, exposure continued for additional 2 weeks, both in the absence and in the presence of environmentally relevant concentrations of diclofenac (0.05 and 0.5 µg/L). Clearance rate, respiration rate, and excretion rate were measured after 7 days of exposure to pH only and after 14 (T1) and 21 (T2) days of exposure to the various pH*diclofenac combinations. At all sampling times, pH significantly affected all the biological parameters considered, whereas diclofenac generally exhibited a significant influence only at T2. Overall, results demonstrated that the physiological performance of *M. galloprovincialis* was strongly influenced by the experimental conditions tested, in particular by the interaction between the two stressors after 21 days of exposure. Further studies are needed to assess the combined effects of climate changes and emerging contaminants on bivalve physiology during different life stages, especially reproduction.

**Keywords:** ocean acidification; diclofenac; bivalves; clearance rate; respiration rate; excretion rate

## 1. Introduction

Marine coastal ecosystems are characterized by high fluctuations of several environmental physical and chemical variables, which the ongoing global climate change (GCC), such as warming and ocean acidification (OA), may exacerbate. The combination of altered abiotic factors with environmental pollutants can pose major threats to marine biodiversity [1–5]. Along coastal areas, in particular, nearby harbors, marinas, and wastewater treatment plans, aquatic organisms may be exposed to a wide range of anthropogenic compounds, such as emerging contaminants that, because of their almost unknown environmental impacts, raise increasing concern [6–10].

Among emerging contaminants, pharmaceutical and personal care products (PPCPs) can be detected in various aquatic systems, such as seawater, surface waters, groundwaters, and effluents from wastewater treatment plants [11–17]. PPCPs are made to target specific metabolic and molecular pathways in humans and farmed animals, but they can exert detrimental effects on nontarget species when released into the environment.

Being used in both human and veterinary medicine, diclofenac (DFC), a nonsteroidal anti-inflammatory pharmaceutical (NSAID), is frequently detected in sewage and surface waters [18,19], and its concentrations in the marine environment range from a few nanogram per liter to several microgram per liter.

DFC inhibits the cyclooxygenase enzymes (COX-1 and COX-2), which function as a catalyst in the conversion of arachidonic acid to prostaglandins [20] in mammals, while in other taxa, DFC can trigger negative effects, such as gill damage and renal lesion in rainbow trout [21] and kidney damage in vultures [22]. DFC has been shown to induce oxidative stress, DNA damage, alteration in estrogenic activity and affects hemocytes functionality in marine mussels [23–27]. Despite this evidence, there is still a lack of knowledge on the possible negative effects of DFC on marine bivalves' physiological parameters, such as clearance, respiration, and excretion rates. In addition, bivalve physiological parameters might be strongly influenced by the alteration of abiotic factors such as pH and temperature, alterations that will be exacerbated by the ongoing climate change. In this regard, Liu and He [28] found that the exposure to predicted reduced levels of pH (7.7 and 7.4) significantly influenced the physiological responses of the scallop *Chlamys nobilis* and the mussel *Perna viridis* resulting in strongly reduced excretion and clearance rates. Alterations in physiological rates, such as clearance, respiration, and excretion, may help to understand detrimental effects on growth, survival, and other energy-dependent processes that were observed in several studies on mollusks. In juvenile mussels (*Mytilus chilensis*), 70-days exposure to high $pCO_2$ levels (750–1200 ppm) led to a significant decrease in energy available for growth (scope for growth) [29]. Matoo et al. [30] showed that long-term exposure to warming (+5 °C) and hypercapnia (~800 ppm $CO_2$) minimally affected the cellular redox status in the hard-shell clams *Mercenaria mercenaria* and eastern oysters *Crassostrea virginica*. However, the combined exposure to those stressors resulted in an elevated standard metabolic rate of clams revealing increased expenditure for basal maintenance.

The potential interaction between environmental emerging contaminants and OA is an issue that needs to be addressed. In marine organisms, the interactions between PPCPs and reduced pH have been studied experimentally mostly at biochemical and cellular levels [26,27,31,32]. At present, there is a gap of knowledge on how changes in environmental variables (temperature, salinity, dissolved oxygen, and pH) may influence animals' physiological performance. Consequently, in this study, we evaluated the effects of DFC under OA conditions on physiological responses (clearance rate, respiration rate, and excretion rate) of the mussel *Mytilus galloprovincialis*.

## 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 Treporti, Italy; temperature of approximately 25 °C and pH of 8.2) in July, and transferred immediately to the laboratory. Only bivalves with no shell damage were chosen for the experiments, and epibionts (such as algae and barnacles) were gently removed from the mussels. In the laboratory, individuals were maintained for 7 days in aerated seawater at salinity, temperature, and pH values similar to those measured during bivalve collection.

### 2.1. Experimental Setup for Bivalve Exposure

Before the experiments, bivalves were acclimatized to the experimental conditions by gradually decreasing the seawater temperature (1 °C per day) to 22 °C, and by gradually reducing natural pH values of about −0.4 or −0.7 units (a reduction approximately of 0.1 units per day). The bivalves were fed daily with microalgae (*Isochrysis galbana*). The experiments were performed during the resting phase, when animals were not sexually active (June/July), to reduce any additional stress that might arise from spawning events during the experiments. The experimental flow-through system (Figure 1) used for mussel exposure was planned at the Hydrobiological Station "Umberto D'Ancona" in Chioggia. It consisted of a main outside reservoir filled with natural seawater (about 1000 L of capacity). The seawater (mean salinity and pH values throughout the experiments were 34 ± 0.4 and 8.07 ± 0.04, respectively) 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). In the end, the seawater was pumped (25 mL/min, using ACQ450 Dosing pumps by Aquatronica) into the experimental tanks (approximately 50 L each) containing 70 bivalves each. During the first week of exposure, animals were exposed to three pH values (6 experimental tanks per pH value): natural pH (named "pH N"), reduced pHs as projected under RCP8.5 scenario for the year 2100 (−0.4 pH units, named "pH −0.4"), and 2300 (−0.7 pH units, named "pH −0.7") [33]. During the second and the third week, they were exposed to two environmentally realistic concentrations of diclofenac (0.05 and 0.5 µg/L) [19], for each pH value. For each concentration (C: control without contaminant; D1: diclofenac 0.05 µg/L; D2: diclofenac 0.50 µg/L) two replicate tanks (A and B) were prepared (Figure 1). The experimental concentrations of DFC were maintained constant during the experiments by distributing in continuous the stock solutions of the contaminant with peristaltic pumps (1 mL/min, using an MCP Process Pump, mod. ISM915A, provided with an MS/CA pump head, mod. ISM724A, by ISMATEC) from two 15 L glass jars. Taking into account the two combined flows (25 mL/min for seawater and 1 mL/min for the contaminant solution, respectively), the concentrations of DFC in the two glass jars were calculated to be 25 times higher than the concentrations needed in the respective tanks with bivalves. Of the experimental conditions tested, the control without DFC at pH N was considered as a reference. During the experiments, animals were fed twice a day by adding 250 mL of microalgae ($3.5 \times 10^6$ cells/mL) (*Isochrysis galbana*) in each 120 L tank, thus keeping an algae concentration of about 2000 cells/mL in the experimental tanks with animals. Seawater carbonate chemistry parameters (i.e., $pH_T$, total alkalinity, dissolved inorganic carbon, $pCO_2$, and calcite and aragonite saturation state) were monitored two to three times a week. The methods in detail are reported in Munari et al. [26] and results are available in Table 1. Bivalve mortality was checked daily, and no dead mussel was found during the experiment.

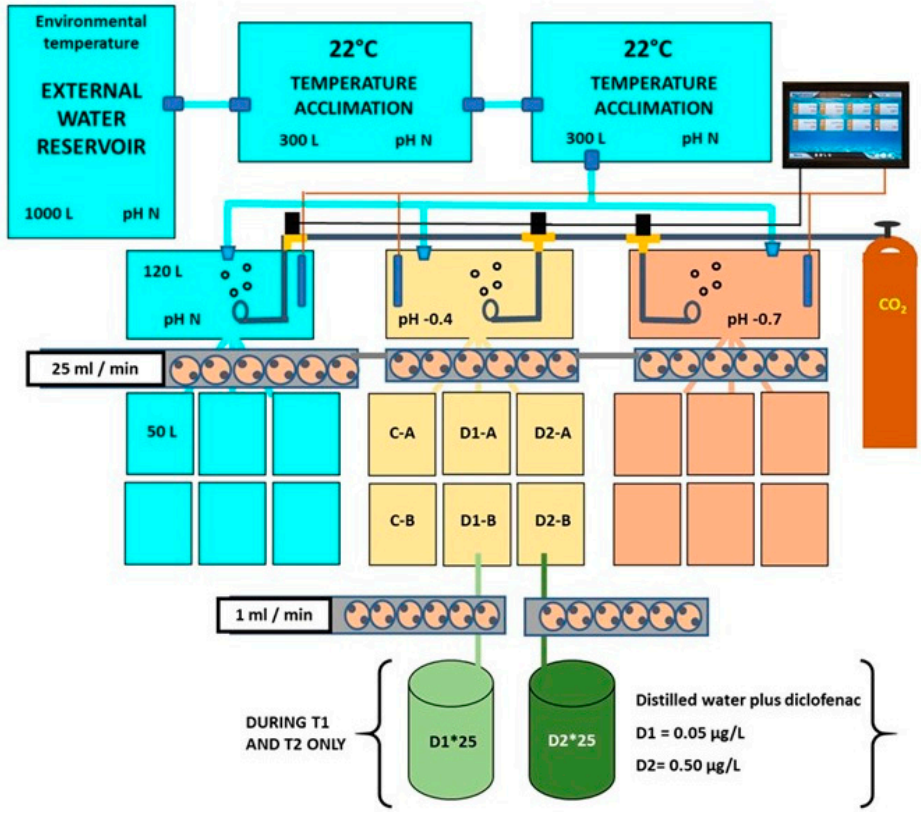


**Figure 1.** The experimental flow-through system.

**Table 1.** Seawater carbonate chemistry parameters (mean values ± SE) throughout the experiment. TA = total alkalinity; DIC = total dissolved inorganic carbon; $pCO_2$ = $CO_2$ partial pressure; $\Omega_{cal}$ = calcite saturation state; $\Omega_{arg}$ = aragonite saturation state.

| Sampling Time | Conditions (pH and Diclofenac) | | $pH_T$ | TA | DIC | $pCO_2$ | $\Omega_{cal}$ | $\Omega_{arg}$ |
|---|---|---|---|---|---|---|---|---|
| T0 | N pH | | 7.98 ± 0.01 | 2884.42 ± 52.45 | 2665.50 ± 50.58 | 631.47 ± 22.02 | 5.718 ± 0.11 | 3.76 ± 0.07 |
| | pH −0.4 | | 7.76 ± 0.01 | 2800.33 ± 12.39 | 2681.52 ± 8.95 | 1080.65 ± 20.59 | 3.62 ± 0.08 | 2.38 ± 0.05 |
| | pH −0.7 | | 7.34 ± 0.05 | 2894.53 ± 40.94 | 2923.77 ± 32.32 | 3248.09 ± 376.73 | 1.56 ± 0.17 | 1.02 ± 0.11 |
| T1-T2 | N pH | 0.00 µg/L | 8.14 ± 0.01 | 2848.91 ± 5.40 | 2539.97 ± 4.86 | 399.19 ± 8.67 | 7.71 ± 0.15 | 5.07 ± 0.10 |
| | | 0.05 µg/L | 8.14 ± 0.01 | 2840.80 ± 2.45 | 2529.65 ± 7.58 | 391.60 ± 10.54 | 7.76 ± 0.17 | 5.10 ± 0.11 |
| | | 0.50 µg/L | 8.14 ± 0.01 | 2841.81 ± 6.30 | 2531.97 ± 5.32 | 395.75 ± 5.85 | 7.73 ± 0.09 | 5.09 ± 0.06 |
| | pH −0.4 | 0.00 µg/L | 7.71 ± 0.03 | 2837.30 ± 7.66 | 2734.99 ± 17.47 | 1262.87 ± 113.96 | 3.34 ± 0.24 | 2.20 ± 0.16 |
| | | 0.05 µg/L | 7.73 ± 0.02 | 2825.60 ± 5.93 | 2713.87 ± 8.83 | 1167.10 ± 62.20 | 3.49 ± 0.14 | 2.30 ± 0.09 |
| | | 0.50 µg/L | 7.74 ± 0.02 | 2820.23 ± 6.24 | 2708.33 ± 10.45 | 1143.96 ± 47.08 | 3.49 ± 0.10 | 2.29 ± 0.07 |
| | pH −0.7 | 0.00 µg/L | 7.39 ± 0.02 | 2879.65 ± 11.71 | 2886.92 ± 15.69 | 2734.47 ± 106.67 | 1.72 ± 0.07 | 1.13 ± 0.04 |
| | | 0.05 µg/L | 7.42 ± 0.01 | 2883.54 ± 12.98 | 2882.98 ± 15.51 | 2566.18 ± 85.56 | 1.81 ± 0.06 | 1.19 ± 0.04 |
| | | 0.50 µg/L | 7.43 ± 0.01 | 2878.18 ± 9.64 | 2872.24 ± 12.93 | 2504.41 ± 81.93 | 1.87 ± 0.06 | 1.23 ± 0.04 |

### 2.2. Measurements of Physiological Parameters

Physiological measurements (clearance rate, respiration rate, and excretion rate) were performed according to the methods described by Widdows [34]. Fifteen mussels per experimental condition were used to determine physiological rates after 7 days (T0) of exposure to three pH levels and after 7 and 14 days (T1 and T2, respectively) of exposure to differing pH*DFC combinations.

All measurements were carried out at the same values of temperature, salinity, and pH used throughout the exposure. To avoid the presence of microorganisms, seawater was previously sterilized with a UV lamp (Scudo UVC 11 W) and filtered (Whatman GF/F glass microfiber filters). Filtered seawater (FSW) was then adjusted to the experimental values of pH by insufflating $CO_2$. The effect due to body size was removed from physiological measurements using the following allometric Equation (1)

$$Y = aX^b, \quad (1)$$

where "$Y$" is the physiological measurement, "$X$" is the dry weight of the mussel soft body tissue, "$a$" the intercept of the regression line indicating the value of the physiological parameter/unit of dry weight (1 g), and "$b$" the slope of the regression line. Coefficient "$b$" was equal to 0.4 for the clearance rate and to 0.65 for both respiration and excretion rates, as indicated by Widdows and Johnson [35] for *Mytilus edulis* and successively used by Widdows et al. [36] for *M. galloprovincialis*. After the three physiological trials, the dry weight of each organism was measured. The soft tissues were excised from the valves and dried until constant weight in an oven at 60 °C, for at least 48 h.

#### 2.2.1. Clearance Rate (CR)

The volume of seawater cleared of suspended particles per unit of time is defined as CR. Each mussel was maintained in a static condition in a beaker containing 2 L of FSW and 30,000 cell/mL of *I. galbana*, while one beaker without a mussel acted as a blank. Throughout the assay, seawater was gently aerated to avoid the settlement of microalgae. Every 30 min over 2 h, *I. galbana* concentration was determined on four 5 mL aliquots sampled from each beaker. From each aliquot, three 60 µL subsamples added with 60 µL of isotonic solution (Coulter ISOTON II Diluent) were used for the measurement with a Scepter 2.0 Handheld Automated Cell Counter, Millipore™.

The CR was calculated using Equation (2)

$$CR = (V/t) \times (\ln C1 - \ln C2), \quad (2)$$

where "V" is the volume of seawater (L), "t" is the time interval (h), and "C1" and "C2" are microalgae concentrations at the beginning and end of each time increment, respectively. For each mussel, the CR

value was defined based on a 1 h period (two consecutive time increments), during which the decline in cell concentration was greatest [37]. Results were expressed as liters/hour.

#### 2.2.2. Respiration Rate (RR)

Oxygen consumption rate or respiration rate (RR) of each mussel was determined using 700 mL closed plexiglass chambers (RC-400 Strathkelvin) kept at constant temperature (22 °C). Oxygen concentration within each chamber was maintained homogeneous using a magnetic stirrer moving a magnetic stir-bar beneath a perforated plate supporting the mussel. In each chamber, the decline in oxygen partial pressure ($pO_2$) was determined using a calibrated membrane oxygen electrode (Strathkelvin 951302) connected to a multichannel Strathkelvin oxygen meter (Model 928). Before the assay, 30 min were given to the mussels to let them open their shell and to restart pumping, then oxygen consumption was measured continuously for an hour. Oxygen uptake was not considered when $pO_2$ was below 100 mm Hg because *M. galloprovincialis* becomes an oxyconformer at a lower level of $pO_2$ [34]. A chamber filled with FSW only acted as a blank. RR was calculated with the following formula (3)

$$RR = [60\ (C0 - C1)\ (V)]/(t0 - t1) \quad (3)$$

where "C0" and "C1" are oxygen concentrations at the beginning and end of the assay, "V" the volume of the chamber, and "t0" and "t1" the start and finish times of the assay in minutes, respectively [34]. Results were expressed as micromole $O_2$/hour.

#### 2.2.3. Excretion Rate (ER)

ER was determined by measuring the [N-$NH_4^+$] in seawater from the RR chambers. At the end of the RR assay, a 50 mL aliquot was collected from each chamber and processed according to the spectrophotometric method described by Solorzano et al. [38].

ER was calculated using Equation (4) [34]

$$ER = (Ct - Cb) \times V/t \quad (4)$$

where "Ct" and "Cb" are, respectively, the [N-$NH_4^+$] in the chamber with and without a mussel respectively, both measured at the end of the RR assay, "V" is the chamber volume in liters and "t" the incubation period in hours. Results were expressed as µg-at N-$NH_4^+$/hour.

#### 2.2.4. Statistical Analyses

Data were checked for normal distribution (Shapiro–Wilk test) and homogeneity of variances (Bartlett's test). As ANOVA assumptions were not always fulfilled, a nonparametric approach was used for statistical analysis of the results. The whole data set obtained at each sampling time was statistically analyzed using a Permutational Multivariate Analysis of Variance (PERMANOVA, with 9999 permutations), considering pH and DFC as fixed factors. The same approach was then used to analyze the effects of pH, DFC and their interaction on each single biomarker. PERMANOVA was also used to perform statistical pairwise comparisons. The null hypotheses tested were: at T0, no significant differences exist among pH levels (N, −0.4, and −0.7); at T1 and T2, (i) for each pH tested, no significant differences exist among DFC concentrations (0.00, 0.05, and 0.50 µg/L) and (ii) for each DFC concentration tested, no significant differences exist among pH levels (N, −0.4, and −0.7). Statistical significance was set at $p \leq 0.05$. If the number of unique values from permutations was too low, *p*-values was calculated using the Monte Carlo procedure. The software package PRIMER 6 PERMANOVA Plus (PRIMER-E Ltd., Plymouth, UK) was used for the statistical analyses.

## 3. Results

Results of the PERMANOVA analysis are reported in Table 2. Significant effects of pH on the overall physiological responses of mussels were detected during the whole experiment, whereas a significant effect of DFC and its interaction with pH was observed only at T2. For each physiological rate, significant effects of the experimental factors (i.e., pH at T0; pH, DFC, and pH*DFC at T1 and T2) were also found (Table 2).

**Table 2.** Permutational Multivariate Analysis of Variance (PERMANOVA) results. Pseudo-F values (indicated as F) and Monte Carlo *p*-values for all physiological parameters and for each single parameter measured in *Mytilus galloprovincialis* throughout the experiment performed at three pH levels (pH N, pH −0.4, and pH −0.7), in the absence (T0: 7-days exposure) or in the presence of 0.05 and 0.50 µg/L diclofenac (T1: from day 7 to 14; T2: from day 14 to 21) are listed. Significant results are in bold. Abbreviation: CR = clearance rate, RR = respiration rate, ER = excretion rate.

| Sampling Time | Factors | All Variables in | CR | RR | ER |
|---|---|---|---|---|---|
| T0 | pH | $F_{(2,44)}$ = 4.591<br>$p_{(MC)}$ = **0.011** | $F_{(2,44)}$ = 6.266<br>$p_{(MC)}$ = **0.005** | $F_{(2,44)}$ = 5.301<br>$p_{(MC)}$ = **0.006** | $F_{(2,44)}$ = 8.321<br>$p_{(MC)}$ = **0.004** |
| T1 | pH | $F_{(2,44)}$ = 38.919<br>$p_{(MC)}$ < **0.001** | $F_{(2,44)}$ = 2.832<br>$p_{(MC)}$ = 0.073 | $F_{(2,44)}$ = 43.027<br>$p_{(MC)}$ < **0.001** | $F_{(2,44)}$ = 5.773<br>$p_{(MC)}$ = **0.007** |
| | Diclofenac | $F_{(2,44)}$ = 2.070<br>$p_{(MC)}$ = 0.127 | $F_{(2,44)}$ = 1.651<br>$p_{(MC)}$ = 0.199 | $F_{(2,44)}$ = 1.780<br>$p_{(MC)}$ = 0.183 | $F_{(2,44)}$ = 5.854<br>$p_{(MC)}$ = **0.007** |
| | pH*diclofenac | $F_{(4,44)}$ = 2.185<br>$p_{(MC)}$ = 0.073 | $F_{(4,44)}$ = 1.514<br>$p_{(MC)}$ = 0.212 | $F_{(4,44)}$ = 2.248<br>$p_{(MC)}$ = 0.082 | $F_{(4,44)}$ = 1.732<br>$p_{(MC)}$ = 0.162 |
| T2 | pH | $F_{(2,44)}$ = 13.544<br>$p_{(MC)}$ < **0.001** | $F_{(2,44)}$ = 2.577<br>$p_{(MC)}$ = 0.086 | $F_{(2,44)}$ = 14.453<br>$p_{(MC)}$ < **0.001** | $F_{(2,44)}$ = 10.961<br>$p_{(MC)}$ < **0.001** |
| | Diclofenac | $F_{(2,44)}$ = 5.903<br>$p_{(MC)}$ = **0.004** | $F_{(2,44)}$ = 1.650<br>$p_{(MC)}$ = 0.206 | $F_{(2,44)}$ = 6.747<br>$p_{(MC)}$ = **0.003** | $F_{(2,44)}$ = 1.002<br>$p_{(MC)}$ = 0.996 |
| | pH*diclofenac | $F_{(4,44)}$ = 6.947<br>$p_{(MC)}$ < **0.001** | $F_{(4,44)}$ = 3.748<br>$p_{(MC)}$ = **0.012** | $F_{(4,44)}$ = 7.438<br>$p_{(MC)}$ < **0.001** | $F_{(4,44)}$ = 3.939<br>$p_{(MC)}$ = **0.010** |

Significant differences between treatments are shown in Figure 2, at T1 and T2, only when a significant effect of the pH*DFC interaction was found. Furthermore, statistically significant differences between groups of animals exposed to different experimental pHs or different DFC concentrations are reported in Table 3.

**Table 3.** Statistical comparisons between experimental groups. Significant results are in bold.

| | | *pH N vs. pH −0.4* | *pH N vs. pH −0.7* | *pH −0.4 vs. pH −0.7* |
|---|---|---|---|---|
| T1 | Respiration rate | 0.258 | **<0.001** | **<0.001** |
| | Excretion rate | **0.008** | 0.860 | **0.002** |
| | | *0.00 µg/L vs. 0.05 µg/L* | *0.00 µg/L vs. 0.50 µg/L* | *0.05 µg/L vs. 0.50 µg/L* |
| | Excretion rate | 0.630 | **0.004** | **0.018** |
| | | *pH N vs. pH −0.4* | *pH N vs. pH −0.7* | *pH −0.4 vs, pH −0.7* |
| T2 | Respiration rate | **0.049** | **0.008** | **<0.001** |
| | Excretion rate | **<0.001** | **<0.001** | 0.482 |
| | | *0.00 µg/L vs. 0.05 µg/L* | *0.00 µg/L vs. 0.50 µg/L* | *0.05 µg/L vs. 0.50 µg/L* |
| | Respiration rate | **0.002** | **0.048** | 0.101 |

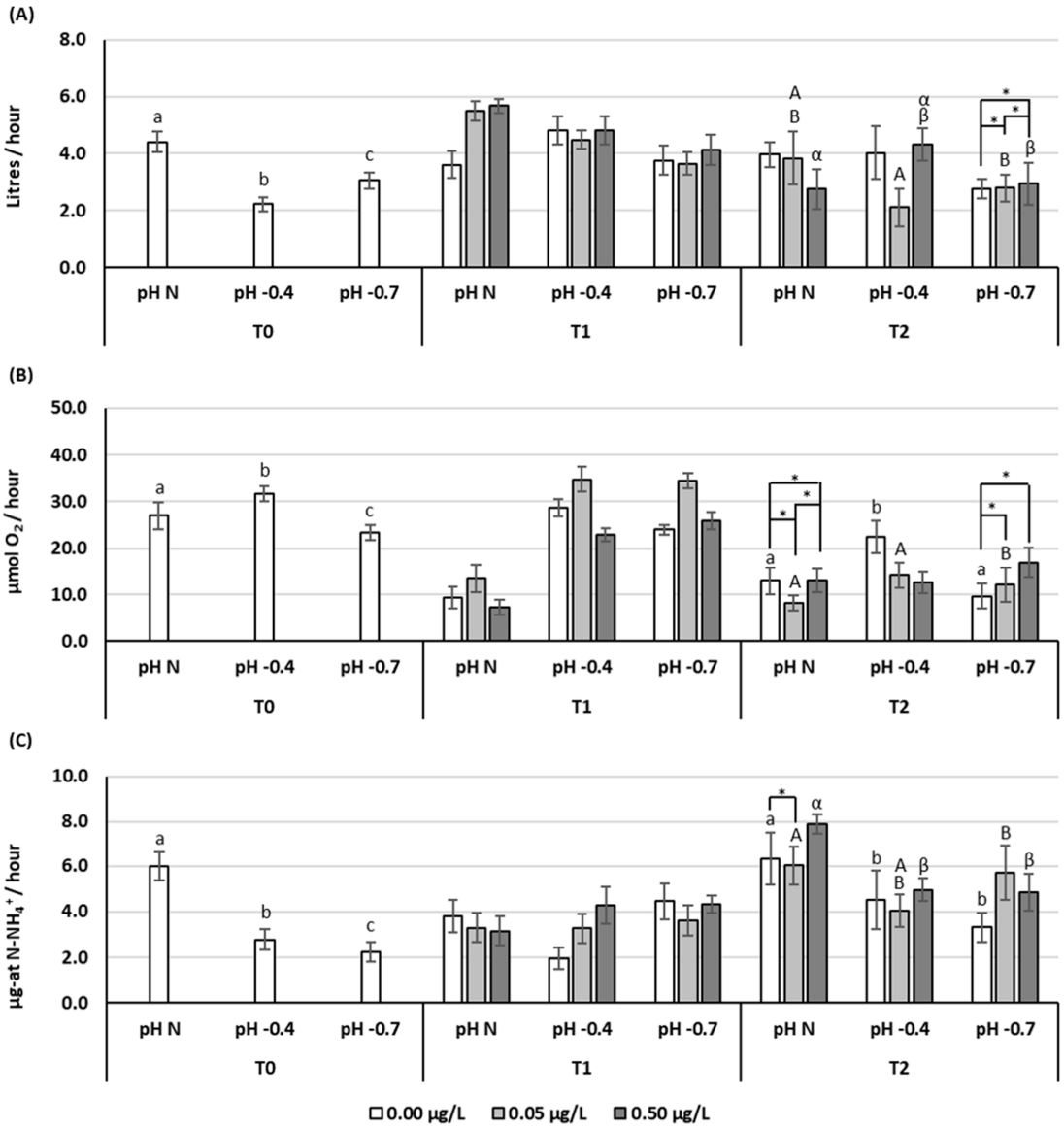


**Figure 2.** Clearance rate (**A**), respiration rate (**B**), and excretion rate (**C**) in *M. galloprovincialis*. Values are the means ± SE (n = 15). White columns refer to a diclofenac concentration of 0.00 µg/L, light grey to 0.05 µg/L diclofenac, and dark grey to 0.50 µg/L diclofenac. At T0, significant ($p < 0.05$) differences among pH N, pH −0.4, and pH −0.7 are presented with lower-case letters (a–c). At T1 and T2, significant differences ($p < 0.05$) among pH N, pH −0.4, and pH −0.7 are presented with lower-case letters (a, b, and c) at the diclofenac concentration of 0.00 mg/L, with capital letters (A, B, and C) at the diclofenac concentration 0.05 mg/L, and with Greek letters (α and β) at the diclofenac concentration of 0.50 mg/L. At each pH value, asterisks represent significant ($p < 0.05$) variations among different diclofenac concentrations.

### 3.1. Clearance Rate (CR)

After 1 week of exposure to the three pH values, CR showed to be affected by pH with a significant reduction in mussels kept at pH −0.4 and −0.7 compared to pH N (Table 2 and Figure 2A). At T1 no significant effects of pH, DFC, or pH*DFC interaction were found. Nevertheless, at T1 CR in mussels kept at pH N without DFC was lower than that measured in all the other conditions tested. At T2, a significant pH*DFC interaction was found. No clear pattern of variation with increasing DFC concentration was observed at pH N and −0.4, whereas CR significantly increased with increasing DFC concentration at pH −0.7 (Figure 2A).

### 3.2. Respiration Rate (RR)

RR was significantly influenced by pH at T0, with a reduction in oxygen consumption in mussels kept at pH −0.7 (Table 2, Figure 2B). At T1 as well, a significant effect of pH on $O_2$ consumption was revealed by PERMANOVA. Bivalves kept at pH −0.4 and −0.7 showed higher RR than those kept at pH N at all DFC concentrations. At T1, no significant effects of DFC were shown, even though increased $O_2$ consumption was recorded in individuals exposed to 0.05 µg/L, at all pH levels. At T2, significant effects of pH and pH*DFC interaction were highlighted. Two opposite responses of mussels maintained at pH −0.4 and those at pH −0.7 were found. In particular, at pH −0.7, there was a significant increase in $O_2$ consumption with the increasing concentration of DFC (Figure 2B).

### 3.3. Excretion Rate (ER)

After 1 week of exposure (T0), ER significantly decreased in mussels kept at low pH values, with respect to controls (Table 2 and Figure 2C). At T1, significant effects of pH and DFC were observed with an opposite trend of response between organisms kept at pH N and those exposed to a reduction in 0.4 units of pH. Despite this, no significant interaction among the two factors was recorded. On the contrary, at T2, ER was significantly affected by pH*DFC interaction. A decrease in ER was measured at all DFC concentrations tested when comparing pH N with pH −0.4 and −0.7. At all pH levels, slightly increased excretion values were observed in bivalves exposed to the highest concentration of DFC compared to their controls (Figure 2C).

## 4. Discussion

Under stressful conditions, mussels are subject to changes in their physiological performance, owing to decreased energy assumption through filtration and/or increased metabolic expenditure during respiration and excretion [39]. Reduced CR, as a measure of the bivalves' filtering activity, cannot always guarantee the proper amount of energy that is required to sustain all metabolic processes, especially those with a high energetic cost, such as growth and reproduction [40,41].

In this study, CR significantly reduced after 1 week of exposure to OA. This result is consistent with those of Liu and He [28], that reported a decrease in the clearance rate of two bivalve species, the noble scallop *Chlamys nobilis*, and the green-lipped mussel *Perna viridis*, when exposed to pH 7.7 and 7.4, if compared to control pH (8.1). The effect of OA on feeding may explain the detrimental effects on physiological processes, such as growth rate, survival and calcification, observed in different species of mollusks [42–44].

However, not all species respond at the same way to stressors during experimental studies. A 3-months laboratory exposure to various $pCO_2$ levels (290, 380, 750 and 1140 µatm) did not lead to significant alterations in CR, RR, condition index, or cellular turnover (RNA: DNA) in the king scallop, *Pecten maximus*, under unrestricted food supply [45]. Similarly, clearance, ingestion and respiration rates of juvenile mussels (*M. galloprovincialis*) were not significantly affected when animals were exposed to a reduction in seawater pH of about 0.3 and 0.6 for 78 days [46]. As hypothesized by the authors, the high alkalinity levels in coastal waters of Ria Formosa (SW Portugal) were probably the reason for the lack of effects. The diverging pattern of responses to OA found in literature could lead to the conclusion that there might be a different capability among species and even populations to adapt [47,48].

Although the exposure to several organic and inorganic environmental contaminants is known to cause CR reductions in bivalves [35], in the present study, no significant effects of DFC or pH*DFC interaction were observed, even though at T1, an increasing trend due to the presence of DFC was observed in animals kept at pH N at both concentrations tested. We hypothesize that the apparent lack of effects was probably due to the environmentally relevant DFC concentrations tested. Conversely, in another study, the Baltic Sea blue mussels, *Mytilus trossulus*, was exposed to DFC, ibuprofen and propranolol in concentrations ranging from 1 to 10,000 µg/L [39]. The compounds were used both

individually and as a mixture. Bivalves exposed to high drug concentrations showed significantly lower scope for growth, leading to the hypothesis that organisms had less energy available for normal metabolism, growth, and reproduction.

Measuring RR and ER correspond to quantify metabolic expenditure, the energy used and lost through respiration and the elimination of nitrogen compounds. Variations in abiotic factors, such as temperature, dissolved oxygen, or seawater pH, can alter RR in marine organisms. Highest $pCO_2$ values in seawater may acidify tissues and body fluids, influencing the oxygen transport [49]. In particular, *M. galloprovincialis* is unable to regulate the acid–base composition of the extracellular fluid under reduced pH conditions [50].

In this study, OA significantly reduced RR at T0. Similar findings were reported in other studies on various bivalve species, such as *M. galloprovincialis* [50], *Crassostrea virginica* [51], *Crassostrea gigas* [52], *Ruditapes decussatus* [53], and *C. nobilis* [28]. In bivalves, oxygen consumption often increases under exposure to environmental contaminants [34]. Interestingly, in the present study, RR values measured at T1 significantly increased with decreasing pH, and at all pH tested, the highest value of oxygen consumption was measured at the lowest concentration of DFC. When compared to T1, an overall decrease in RR was recorded in organisms exposed to pH −0.4 and −0.7 at T2, mostly in the presence of DFC.

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 bivalve species, $NH_4^+$ is the main excreted component. Like for the other physiological rates measured in this study, nitrogen excretion can be influenced by changing environmental variables as well [54]. As an example, in three bivalve species, namely, *Pinctada fucata*, *C. nobilis*, and *P. viridis*, ER was significantly lower at pH 7.4 than at pH 8.1 [28]. Conversely, no data are available in literature on the possible effects of DFC on ER in aquatic invertebrates, to our knowledge at least.

In this study, 1-week exposure to OA caused a significant ER reduction, with respect to controls. At T2, the same trend of variation was found in mussels kept in the absence of DFC at all pH values tested. At this time, the effects of both DFC and pH*DFC interaction were better revealed at pH −0.7, with higher ER at both DFC concentrations.

*M. galloprovincialis*, commonly distributed and farmed in estuarine and coastal areas, are easily exposed to wide environmental changes and to many anthropogenic compounds [55].

In agreement with Michaelidis et al. [50], the decrease in ER recorded in this study at low pH values suggests that a reduced amino acid catabolism might represent an adaptation strategy to overcome stressful environmental variations and survive. However, this kind of plasticity seems to be affected by the presence of a contaminant, i.e., metabolic cost increases again, as confirmed by increased RR values, while the energy intake through filtration does not increase. The interaction of reduced pH and DFC, even at low concentrations, might lead to decreased energy availability with harmful outcomes for main life processes, such as reproduction. This could have consequently negative effects on mussel natural populations, as well as on the whole ecosystem since mussels are common keystone species on open coasts and in estuaries.

Overall, our results suggest that environmental concentrations of DFC and seawater acidification may impact the physiological performance of mussels. Further investigations, however, are needed to investigate the combined effects of global climate changes and emerging contaminants on different life stages of marine bivalves, to better understand if they can adapt to stressful conditions through generations.

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