


Article

Biological Responses of Oyster *Crassostrea gasar* Exposed to Different Concentrations of Biofloc

Léa Carolina de Oliveira Costa ¹, Andrezza Carvalho ², Mariana Holanda ³, Jorge Santos ², Lucélia Borges ², Bruna Guterres ² , Je Nam Junior ², Virginia Fonseca ², Larissa Muller ², Luis Romano ², Silvia Botelho ², Marcelo Pias ², Juliane Ventura ² and Luís H. Poersch ^{2,*} 

¹ Instituto Federal de Educação Ciência e Tecnologia do Pará, Rodovia BR 316, KM 61, Castanhal 68740970, PA, Brazil; lea.costa@ifpa.edu.br

² Marine Aquaculture Station, Institute of Oceanography, Federal University of Rio Grande—FURG, Rua do Hotel nº2, Cassino, Rio Grande 96210030, RS, Brazil; andrezzachagas@hotmail.com (A.C.); jorgerenato_82@hotmail.com (J.S.); lu.fito@gmail.com (L.B.); gutierrez.bruna@gmail.com (B.G.); jenamjunior@hotmail.com (J.N.J.); vikavetp@gmail.com (V.F.); larissamullern@gmail.com (L.M.); luisalbertoromano0@gmail.com (L.R.); silviac.botelho@gmail.com (S.B.); pias.marcelo@gmail.com (M.P.); juliane.lima@furg.br (J.V.)

³ Escuela de Ciencias del Mar, Pontificia Universidad Católica de Valparaíso, Valparaíso 2373223, Chile; mariana.holandalima@gmail.com

* Correspondence: lpoersch@gmail.com

Abstract: Oysters have the potential to be a part of more sustainable farming systems, such as multitrophic systems integrated into biofloc systems, due to their filtration activity, which enables them to act as organic consumers. However, the stress experienced by animals in a system with a high organic load can compromise their productive performance. The objective of this study was to evaluate the biological responses of *Crassostrea gasar* oysters when exposed to different concentrations of total suspended solids in biofloc systems. The oysters were exposed to four different concentrations of solids for 28 days. Hall effect sensors were installed on the outside of the shells to detect the movement of the oyster valves. Also, biochemical and histological analyses were conducted to assess the biological responses of the oysters to exposure to varying levels of solids. A difference in valve opening detected by the Hall sensors was observed from the second week of culture, indicating a relationship between shell closure and higher concentrations of suspended solids present in the system. In terms of biochemical analysis, a significant increase in lipid damage was observed in treatments with medium and high levels of total suspended solids compared with the control group. Conversely, no changes were observed in the gill structure of the oysters caused by the concentrations of suspended solids in the system when compared with the control. According to the analyses of gill activity and biochemistry, it is suggested that *C. gasar* should be cultured with total suspended solids at less than 200 mg/L. Oysters cultivated in a biofloc system keep their shells closed when subjected to high concentrations of total suspended solids; concentrations of total suspended solids below 200 mg/L do not induce oxidative stress, changes in behavior or histological alterations in *C. gasar* oysters cultivated in a biofloc system.

Keywords: nutrients; *Crassostrea gasar*; biofloc; density; biosensor; bioremediation

Key Contribution: Oyster cultivated in a biofloc system keep their shells closed when subjected to high concentrations of total suspended solids; Concentrations of total suspended solids below 200 mg/L do not induce oxidative stress, changes in behavior or histological alterations in oyster *C. gasar* cultivated in a biofloc system.



Citation: Costa, L.C.d.O.; Carvalho, A.; Holanda, M.; Santos, J.; Borges, L.; Guterres, B.; Nam Junior, J.; Fonseca, V.; Muller, L.; Romano, L.; et al. Biological Responses of Oyster *Crassostrea gasar* Exposed to Different Concentrations of Biofloc. *Fishes* **2023**, *8*, 586. <https://doi.org/10.3390/fishes8120586>

Academic Editors: Wagner C. Valenti and Eduardo Luis Ballester

Received: 17 October 2023

Revised: 11 November 2023

Accepted: 14 November 2023

Published: 29 November 2023



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1. Introduction

The integration of filter-feeding species, such as the oyster *Crassostrea gasar*, into multi-trophic biofloc cultures provides mutual benefits: increased productivity, a reduced

organic load in the system, and a constant natural food source for the oysters. The inclusion of mollusks in integrated farming has gradually increased, with a view of using more sustainable and productive crops. According to the FAO [1], mollusks are the second most produced group, with the *Crassostrea* genus in first place, representing a group of economic interest with rising production. In addition to profitability, the use of more sustainable systems has been gaining ground in aquaculture, with Lima et al. [2] showing that the integration of the *Crassostrea* sp. oyster in the cultivation of white shrimp provided lower concentrations of settleable solids and better performance of the species compared with monoculture. The key to a successful integrated system lies in achieving the appropriate species composition as well as ensuring that waste from the feeding species can be utilized by species that consume organic material and that the selected species are tolerant to the conditions of the culture system.

When it comes to integrating species such as oysters that consume fine particulate organic material into biofloc system cultures, uncertainties arise regarding the physiological responses of these bivalves to high solid concentrations in this system. It is not definitively known whether the increased availability of solids might diminish the oysters' filtration potential or even induce stress that could lower their performance. This is because studies involving the optimal concentrations of total suspended solids in bivalve cultures are still limited [2]. The biological responses caused by environmental stress can affect various levels of organization, ranging from the cellular level to the entire population [3]. This occurs as an attempt to overcome challenges and aid the organism in returning to its normal physiological state [4]. However, these responses can lead to negative consequences, including reduced resilience, impacts on growth and reproduction, and heightened susceptibility to diseases.

However, defining the state of well-being of sessile bivalve mollusks is not a simple task. Indicators can be as conspicuous as the mortality of individuals or as subtle as alterations in the activities of biomolecules, such as enzymes. Enhancing the understanding of the physiological and behavioral reactions of mollusks is crucial for mitigating losses in aquaculture systems [5].

In this context, to comprehend the condition of animals in aquaculture environments, a range of analyses can be employed as indicators of environmental comfort [6]. Monitoring the oyster valve opening behavior through biosensors, oxidative stress biomarkers, and the histological examination of oyster gills are some of the methods utilized to observe animal behavior and health. The utilization of biosensors allows for the analysis of organism behavior in response to environmental changes, providing a swift, sensitive, and cost-effective approach [7–9]. The opening and closing movements of bivalve shells are closely linked to vital activities and environmental conditions [8], making the monitoring of this movement instrumental in understanding behavioral patterns influenced by environmental characteristics [10,11].

The development of biosensors based on the behavioral analysis of bivalve mollusks has been explored; these biosensors are sensitive tools for detecting pollutants and physicochemical conditions of interest, as well as their impacts on ecosystem services [12]. In terms of behavioral analysis, Le Moullac et al. [13] emphasized the measurement of valve activity (shell opening and closing) as a potentially valuable tool for biologically monitoring water quality and comprehending metabolic optimization strategies.

Likewise, Andrewartha and Elliott [14] introduced biosensors centered around sentinel animals, combined with the monitoring of environmental variables, as prospective key technologies for managing aquaculture farms. Generally, changes in behavior associated with bivalves' exposure to harmful algae [15–17] and other contaminants include a decrease in mean opening amplitude and an increase in transition frequency. To monitor the amplitude of shell opening in bivalve mollusks, valvometry techniques are employed. Among these techniques, Hall effect sensors in conjunction with magnets offer several advantages, such as durability, lightness, easy attachment, and reduced stress on the animals, which facilitates the measurement of their movements [16].

In aquaculture, oxidative stress in cultured aquatic organisms can be induced by various conditional factors [18]. Molecular and biochemical biomarkers have been widely employed to demonstrate the biological responses of organisms to specific environmental conditions [19]. They are used to highlight alterations in biomolecules and other aspects of oxidative stress resulting from reactive oxygen species or shifts in redox balance in experimental animals [20]. Sies and Jones [21] conceptualized oxidative stress as an imbalance between oxidants and antioxidants in favor of oxidants. This imbalance can lead to changes in redox signaling and control as well as molecular damage. During stressful events, reactive oxygen species are generated [22,23]; however, aerobic organisms possess an antioxidant defense system that enables them to manage these species by either neutralizing/intercepting them or repairing the damage they cause [24,25].

Another way of assessing the condition of a cultured animal is through histological analysis, which allows the structure of tissues to be observed through microscopic examination of their components [26]. One of the advantages of this analysis is its ability to provide information on the state of health of the organism at the cellular level [27]. Many tissues can be used in these analyses, but the gills stand out as one of the most important organs for aquatic species.

This study aimed to evaluate the biological responses of *C. gasar* oysters when exposed to different concentrations of total suspended solids in biofloc culture systems on the basis of behavioral, biochemical, and histological analyses of the bivalves. The results obtained will demonstrate the best concentration of solids for the cultivation of *C. gasar* oysters in biofloc systems.

2. Materials and Methods

2.1. Oyster Origin

The oysters were obtained from a commercial farm in the state of Santa Catarina, Brazil. Upon arrival at the Marine Aquaculture Station (EMA-FURG), they underwent a four-week acclimatization period in the laboratory. During this phase, they were housed in 500 L water tanks filled with filtered seawater (salinity 27) and maintained with continuous aeration. Biofloc water from a marine shrimp farm with a total suspended solids (TSSs) concentration of 100 mg/L was introduced to feed the oysters. The water was completely renewed twice a week. Initially, the oysters' mean weight was 54.18 ± 13.45 g, and they measured 68.2 ± 4.44 mm in mean height, 50.4 ± 3.50 mm in mean length, and 24.1 ± 5.02 mm in mean width.

2.2. Experimental Design

Water recirculation systems were established comprising one 500 L water reservoir (considered the macrocosm) and three 100 L boxes where the oysters were distributed. The 500 L reservoir was equipped with a pump (Sarlo Better—1000 L/h, São Caetano do Sul, São Paulo, Brazil) that circulated water to the three 100 L boxes, and through gravity, the water returned to the 500 L reservoir. Throughout the experiment, constant water circulation was maintained in the boxes, and aeration was provided via blowers and micro-perforated hoses. Each set consisting of three 100 L boxes and one 500 L reservoir represented one treatment. A suspended screen was placed in each 100 L box to keep the oysters elevated from the bottom of the box (Figure 1). Four times a day, the water in the 100 L boxes was agitated to resuspend the solids that had settled on the box structures.

The oysters were subjected to varying concentrations of total suspended solids (TSSs) over a 28-day period in order to expose them to the concentrations of solids that can be found in the natural environment [28] and the higher concentrations found in biofloc shrimp farming [29]. The experiment comprised four treatments involving the exposure of oysters to three distinct TSS concentrations of water sourced from the cultivation of marine shrimps in a mature biofloc system (with the presence of nitrate). The treatments were as follows: control treatment—oysters were fed with microalgae; low treatment—maintained at a low TSS concentration, approximately 100 mg/L TSSs; medium treatment—maintained

at a nominal medium TSS concentration ranging from above 100 to 200 mg/L TSSs; and high treatment—maintained at a nominal high TSS concentration exceeding 200 mg/L TSSs. Each 100 L box represented one repetition of a treatment, resulting in a total of four treatments, each with three repetitions. Each experimental unit initially contained 30 oysters.



Figure 1. Experimental units (100 L) containing the oysters stored on the screens.

To feed the oysters in the control treatment, the diatom *Chaetoceros muelleri* was cultivated in f/2 media [30] with a salinity of 28, a temperature of 20 °C, and a light intensity of 40 $\mu\text{mol photons/m}^2/\text{s}^1$ in batch-type cultivation. Cell growth was monitored daily using a Neubauer chamber and an optical microscope. Daily counts of the remaining microalgae's density in the control experimental units were also conducted, and if necessary, aliquots of the microalgae culture were added to maintain a density of 16×10^4 cells/mL in the units. Every two weeks, three oysters were collected from each experimental unit for biochemical analysis. At the end of the experiment, two oysters were collected from each experimental unit for histological analysis. For biochemical and histological analysis, the oysters' adductor muscles were cut, the shells were opened, the visceral mass was removed from the shell, and only the gills were collected from the visceral mass for analysis. For biochemical analysis, the samples were stored at -80 °C, and for the histological analysis, the samples were stored in a 20% saline formalin solution.

2.3. Water Quality

Total suspended solids (TSSs) were measured twice a week using gravimetry, following a methodology adapted from AOAC [31] with adjustments made in accordance with treatment definitions. As needed, concentrated flake water was introduced to maintain the concentrations in the biofloc treatments. This matured floc, containing nitrate, originated from an ongoing marine shrimp culture and was stored in a 1000 L water tank with continuous aeration. The dissolved oxygen level was 8.6 ± 0.68 mg/L, the total alkalinity was 240 ± 20.8 mg CaCO_3/L , and the TSS concentration was 1460 ± 15.2 mg/L.

In the 100 L boxes where the oysters were stored, the following analyses were carried out twice a week. Settleable solids (SSs) were measured by an Imhoff Cone [32], and turbidity was measured by a portable turbidimeter (2100P, Hach®, Loveland, CO, USA). Temperature and oxygen were measured daily in the early morning and late afternoon using a multiparameter probe e (model Pro-20, YSI Inc., Yellow Springs, OH, USA). The pH reading was also taken daily in the morning by a bench pH meter (Seven2Go S7 Básico, Mettler Toledo, São Paulo, Brazil). Oyster mortality was observed daily.

Salinity, total alkalinity, ammonia, nitrite, nitrate, and phosphate were checked twice a week. Salinity was measured with an optical refractometer. Alkalinity was measured according to APHA [33], ammonia was measured according to UNESCO [34], and nitrite was measured according to Strickland and Parsons [35]. When total alkalinity values were below 150 mg CaCO_3/L , they were adjusted with calcium hydroxide according to Furtado

et al. [36]. Nitrate and phosphate were measured 1x/week according to the methodology of Aminot and Chaussepied [37].

2.4. Hall Sensor

Oyster valve activity was monitored using Hall effect sensors, following the methodology outlined by Guterres et al. [8]. The Hall effect sensor was affixed to the upper valve using epoxy resin (Araldite 5 min) [11], while neodymium magnets were attached to the lower valve using cyanoacrylate glue [10,15,16]. The choice of glue was informed by existing literature, along with the study conducted by Hartmann et al. [11], which evaluated the performance (mechanical strength and fixation time) of different substances in constructing biosensors based on bivalve behavioral analysis. The sensors were made waterproof using epoxy resin and linked to the electronic system via 3-way cables.

Positioned on opposing sides of the shell (see Figure 2), these sensors registered the oyster's opening by detecting their movement away from each other. The opening amplitudes for each individual were expressed as percentages, considering the maximum and minimum values recorded throughout the experiment [38]. The sensors were linked to an Arduino MEGA board and a computer to enable real-time recording of oyster openings throughout the experiment. The simultaneous connection of all sensors to the acquisition system was facilitated by employing two multiplexer boards, each with 16 channels. Within each experimental unit, 4 oysters were equipped with sensors, resulting in 12 sensor-equipped oysters per treatment and a total of 48 oysters in the experiment. Data were recorded using an SD memory card module.

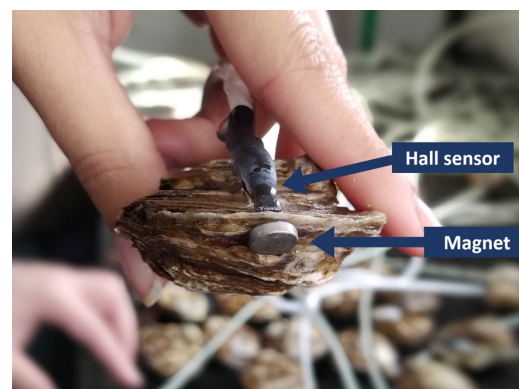


Figure 2. Positioning of the Hall sensor and magnet on the right and left valves, respectively, of *C. gasar* oysters.

The acquisition system captured two readings per second (2 Hz) in bytes. These bytes were then converted into percentages of open state (%) on the basis of the individual oyster's lowest (0%) and highest (100%) observed values. To analyze the extensive volume of readings gathered during the experiment effectively, the average openings were computed every 1 min. Consequently, the behavioral data collected over the course of 24 h were represented by 1440 observations.

To facilitate data organization, this work designated oyster opening levels as follows: closed for 0% opening, slightly open for openings ranging from 0.1% to 25%, moderately open for 25.1% to 50%, open for 50.1% to 75%, and fully open for 75.1% to 100% openings.

2.5. Biochemical Analyses

After freezing in an ultrafreezer, the gills of the *C. gasar* oysters were homogenized according to Bebianno et al. [39] in a chilled buffer solution (TRIS-HCl 50 mM, EDTA 1 mM, sucrose 0.5 M, DTT 1 mM, KCl 0.15 M, PMSF 0.1 M), adjusted to pH 7.6, at a ratio of 1:5 (*w/v*). Subsequently, the tissue was centrifuged for 30 min at 4 °C and 10,000 × *g*, and the resulting supernatant was collected and stored at −80 °C for the analysis of enzyme

activity, including glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) activity, along with total antioxidant capacity against peroxy radicals (ACAP) and peroxidized lipids (TBARS). For the assessment of reduced glutathione (GSH) and protein-associated sulfhydryl groups (P-SH), the same solution was employed, excluding DTT, and the same protocol as the other analyses was followed.

To determine the total protein in the tissues (gills), the Bradford method [40] was adopted using a Sensiprot commercial kit and a microplate reader (BioTek LX 800, Winooski, Vermont, USA). For the analysis of GST enzyme activity, the methodology of Habig et al. [41] was adopted, in which the increase in the CDNB-GSH product formed from the consumption of GSH in the presence of the extracts of the tissue samples and 0.1 M potassium phosphate buffer solution at pH 7.0 was monitored. The analysis was performed in a microplate reader at a temperature of 25 °C with a wavelength of 340 nm. The units used were nmol of CDNB per mg of protein per minute.

For catalase (CAT), the methodology described by Beutler [42] was adopted, in which the consumption of hydrogen peroxide (H₂O₂) in the presence of the samples was recorded in a spectrophotometer at 240 nm, pH 8.0, and a temperature of 25 °C. The units adopted were μmol of H₂O₂ per min and per mg of protein, which are expressed as CAT units.

The analysis of reduced glutathione (GSH) levels was performed using the protocol described by Sedlak and Lindsay [43]. Samples were adjusted to a concentration of 2 mg of protein per mL, followed by the addition of 50% (*w/v*) trichloroacetic acid (TCA) and centrifugation at 20,000 × *g* for 10 min at 4 °C to precipitate the proteins. The resulting supernatant was collected for GSH analysis and mixed with 0.4 M Tris-base buffer at pH 8.9 along with DTNB. Following a 15 min incubation, the mixture was read at 405 nm for subsequent analysis. The units used for measurement were μmol of GSH per mg of protein. The pellet was collected, resuspended in homogenization buffer, and then treated with 0.2 M Tris-base at pH 8.2 and DTNB. After another 15 min incubation, the mixture was read at 405 nm to assess P-SH levels, with the unit of measurement of μmol of P-SH groups per mg of protein.

Lipid peroxidation levels (TBARS) were determined using the methodology outlined by Oakes and Van Der Kraak [44]. This method involves the reaction of lipid peroxidation byproducts with thiobarbituric acid (TBA) under conditions of high temperature and acidity, leading to the generation of a chromogen that can be detected through fluorimetry (excitation at 520 nm and emission at 580 nm). The units of measurement used were nmol of malondialdehyde (MDA) per mg of protein.

The assessment of total antioxidant capacity against peroxy radicals (ACAP) was conducted in accordance with the methodology established by Amado et al. [45]. This involved quantifying the reactive oxygen species (ROS) in the samples, both treated and untreated, with a peroxy radical generator. The samples were adjusted to a concentration of 2 mg of protein per mL and exposed to peroxy radicals, which reacted with a fluorescent substrate (H₂DCF-DA). Using fluorimetry, with excitation at 485 nm and emission at 520 nm, readings were taken on a microplate reader at 5 min intervals over a 35 min period. The results were expressed as an area relative to the difference between the area of ROS with ABAP (2,2-azobis (2-methylpropionamidine) dichloride) and the area of ROS without ABAP. In terms of results interpretation, a larger relative area indicated a lower antioxidant capacity.

The SOD (superoxide dismutase) activity in the oyster gills was evaluated following the methods described by McCord and Fridovich [46] and Bainy et al. [47]. The reduction of cytochrome c absorbance at a wavelength of 550 nm was measured in a potassium phosphate buffer at pH 7.8. The specific activity of the SOD enzyme is expressed as SOD activity per mg of total protein.

2.6. Histological Analysis

At the end of the experiment, 6 oyster samples were collected from each treatment, resulting in a total of 24 oysters. After opening the oysters, the visceral mass was extracted

and preserved in 20% formalin saline. Subsequently, only the gills were removed from the visceral mass for analysis. The samples underwent a dehydration process using increasing ethanol concentrations in sequence, followed by clarification in xylene, and were subsequently embedded in Paraplast[®] using an automatic tissue processor (PT 05, LUPETEC, São Carlos, Brazil).

Sections with a thickness of 5 μm were prepared using a microtome (MRP03, LUPETEC, Brazil), stained with hematoxylin and eosin (H&E), and examined using an optical microscope (ZEISS Primo Star) at magnifications of 4 \times and 40 \times . The analysis of the gills was qualitative, involving the observation of structures and the identification of any damage. For histochemical assessment, specialized staining with periodic acid-Schiff (PAS) dye was employed, whereby PAS-positive structures were stained in magenta/red [26].

2.7. Statistical Analysis

The statistical analysis of water quality parameters, including pH, turbidity, alkalinity, salinity, ammonia, nitrite, nitrate, and phosphate, was conducted using one-way ANOVA. Assumptions of normality and homoscedasticity were verified using the Shapiro–Wilk and Levene tests, respectively [48]. If these assumptions were not met, the data were subjected to logarithmic transformation. For oxygen, temperature, suspended solids (SS), and total suspended solids (TSS) parameters, the non-parametric Kruskal–Wallis test was employed.

Regarding the statistical analysis of oyster valve activity monitored by the Hall sensor, two separate analyses were carried out. Initially, descriptive statistics were performed, and subsequently, a non-parametric Kruskal–Wallis analysis was conducted to assess the mean opening values within each treatment. The second analysis involved creating a frequency table for the data, categorizing them according to their opening level within each treatment and per week of the experiment. The non-parametric Kruskal–Wallis analysis was then performed to compare the values of each opening level across the weeks.

For the statistical analysis of biochemical parameters, including GST, CAT, SOD, GSH, P-SH, TBARS, and ACAP, a two-way ANOVA utilizing parametric statistics was conducted. This analysis considered both the cultivation time and the treatment as factors. The assumptions underlying the analysis were assessed using the Shapiro–Wilk and Levene tests. Whenever necessary, mathematical transformations were applied to the data. Significant differences were determined at a significance level of $p < 0.05$, followed by Newman–Keuls post hoc testing for multiple comparisons.

Across the experiment results, data are presented as mean values accompanied by standard deviation. The statistical software employed for analysis was Statistica 12. Microsoft Excel was utilized for the generation of tables, while PrismGraph software was utilized for the creation of graphs. A consistent significance level of 5% was adopted for all conducted tests.

3. Results

3.1. Water quality

The parameters of dissolved oxygen, salinity, alkalinity, and ammonia did not show significant differences between the different treatments applied in this experiment (Table 1).

Temperature, pH, nitrite, nitrate, and phosphate exhibited significant differences between the treatments. For pH and nitrite, the control treatment displayed significant differences ($p < 0.05$) compared with the low, medium, and high treatments, but no differences were found between the latter three treatments. Temperature values were significantly lower in the medium and high treatments. Nitrate levels showed the lowest means in the control, low, and medium treatments, while the high treatment had the highest mean, which was not significantly different from the medium treatment.

Phosphate levels were higher in the medium and high treatments compared with the control and low treatments. The low treatment showed no significant differences compared with the control for these parameters, but its mean values were lower than those of the

medium and high treatments. The high treatment had higher mean values than the medium treatment, in line with the experimental design.

Table 1. Water quality parameters with mean values and standard deviation over the 28 days of cultivation in the treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—from 100 to 200 mg/L TSSs; high treatment—above 200 mg/L TSSs.

Parameters	Treatments			
	Control	Low	Medium	High
Oxygen (mg/L)	6.83 ± 0.67	6.65 ± 0.62	6.84 ± 0.64	6.79 ± 0.63
Temperature (°C)	20.76 ± 1.83 ^a	20.64 ± 1.51 ^a	19.40 ± 1.79 ^b	20.07 ± 1.43 ^b
pH	8.21 ± 0.05 ^b	8.31 ± 0.06 ^a	8.31 ± 0.05 ^a	8.32 ± 0.05 ^a
SS (mL/L)	0.00 ± 0.00 ^a	0.06 ± 0.12 ^a	1.78 ± 2.00 ^b	5.65 ± 4.62 ^c
TSS (mg/L)	83.04 ± 29.18 ^a	71.27 ± 44.74 ^a	189.88 ± 120.15 ^b	291.38 ± 98.26 ^c
Turbidity (NUT)	22.74 ± 18.17 ^a	33.30 ± 35.02 ^a	108.29 ± 72.43 ^b	259.22 ± 142.88 ^c
Alkalinity (mg CaCO ₃ /L)	146.11 ± 10.54	167.22 ± 25.51	173.33 ± 21.94	173.33 ± 41.53
Salinity	33.63 ± 2.39	32.38 ± 1.41	31.63 ± 1.69	32.00 ± 1.07
Ammonia (mg/L)	0.17 ± 0.13	0.09 ± 0.04	0.08 ± 0.04	0.11 ± 0.04
Nitrite (mg/L)	0.65 ± 0.60 ^b	0.07 ± 0.06 ^a	0.12 ± 0.18 ^a	0.13 ± 0.11 ^a
Nitrate (mg/L)	9.56 ± 4.42 ^a	10.87 ± 4.85 ^a	17.20 ± 5.55 ^{ab}	20.76 ± 8.56 ^b
Phosphate (mg/L)	2.64 ± 1.14 ^a	3.80 ± 0.87 ^a	6.76 ± 2.05 ^b	6.50 ± 0.84 ^b

Different letters represent a significant difference ($p \leq 0.05$) between treatments.

3.2. Hall Sensor

For mean valve opening values across each week within each treatment, no significant differences ($p > 0.05$) were observed between treatments during week 1 and week 4. However, significant differences ($p < 0.05$) emerged during weeks 2 and 3 of the experiment, as indicated in Table 2.

During week 2, a significant difference ($p < 0.05$) was observed. The control and medium treatments had a greater number of fully open oysters compared with the high treatment. In week 3, differences ($p < 0.05$) between treatments were observed across three ranges of oyster opening: closed, slightly open, and fully open. Notably, the high treatment exhibited a higher occurrence of closed oysters than the control treatment. Furthermore, the medium treatment had a higher frequency of slightly open oysters compared with the high treatment, while the high treatment had a lower frequency of fully open oysters compared with the control treatment.

Overall, it is evident that closed oysters predominated in all treatments during the four weeks of cultivation. The second most frequent opening range was fully open, followed by open. The medium open level represented the opening range with the lowest number of records in terms of percentage.

3.3. Biochemical Analysis

The activities of the enzymes GST and CAT did not exhibit significant differences ($p \geq 0.05$) between the various treatments throughout the experiment, as depicted in Figures 3 and 4, respectively.

Table 2. The frequency distribution (in %) of valve opening values for *C. gasar* oysters over the four weeks of culture in the control, low, medium, and high treatments. Control treatment: oysters fed with microalgae. Low treatment: 100 mg/L total suspended solids (TSSs). Medium treatment: ranging from 100 to 200 mg/L TSSs. High treatment: exceeding 200 mg/L TSSs.

Opening Range of Oyster Valves						
Treatment	Week	Closed	Slightly Open	Moderately Open	Open	Fully Open
Control	1	64.54 ± 7.15	0.20 ± 0.45	0.90 ± 0.67	7.06 ± 4.89	27.30 ± 7.25
Low	1	73.23 ± 5.88	0.02 ± 0.02	1.90 ± 1.23	6.78 ± 2.93	18.07 ± 7.79
Medium	1	64.32 ± 7.81	0.24 ± 0.48	0.95 ± 0.71	7.51 ± 5.20	26.97 ± 7.89
High	1	60.86 ± 14.36	0.00 ± 0.00	0.89 ± 0.53	6.40 ± 2.80	31.84 ± 14.02
Control	2	39.35 ± 22.57	0.00 ± 0.00	0.18 ± 0.29	3.87 ± 3.48	56.60 ± 20.75 ^{ab}
Low	2	64.80 ± 26.72	0.07 ± 0.07	0.69 ± 0.60	4.45 ± 2.95	30.00 ± 25.67 ^{bc}
Medium	2	31.01 ± 10.75	0.00 ± 0.00	0.13 ± 0.28	4.37 ± 3.64	64.40 ± 9.03 ^a
High	2	65.04 ± 20.13	0.11 ± 0.13	5.39 ± 10.55	18.00 ± 25.16	11.47 ± 9.71 ^c
Control	3	66.97 ± 15.14 ^a	0.03 ± 0.02 ^{ab}	1.17 ± 1.02	3.77 ± 3.25	28.05 ± 12.88 ^a
Low	3	79.97 ± 4.93 ^{ab}	0.04 ± 0.01 ^{ab}	0.56 ± 0.71	2.99 ± 2.77	16.44 ± 7.64 ^{ab}
Medium	3	79.67 ± 4.57 ^{ab}	0.12 ± 0.05 ^a	1.23 ± 0.77	4.83 ± 2.95	14.14 ± 2.09 ^{ab}
High	3	85.10 ± 6.29 ^b	0.01 ± 0.01 ^b	0.19 ± 0.12	5.66 ± 8.08	9.04 ± 3.91 ^b
Control	4	56.99 ± 27.22	0.02 ± 0.04	1.45 ± 0.13	10.83 ± 5.04	30.71 ± 28.23
Low	4	65.06 ± 14.52	0.04 ± 0.04	0.94 ± 0.73	6.30 ± 2.66	27.66 ± 15.98
Medium	4	54.10 ± 15.54	0.04 ± 0.07	1.38 ± 0.68	10.60 ± 7.24	33.88 ± 15.81
High	4	52.43 ± 17.72	0.00 ± 0.00	0.57 ± 0.33	8.78 ± 7.76	38.22 ± 10.58

Different lowercase letters in the same row represent significant differences ($p < 0.05$) between treatments.

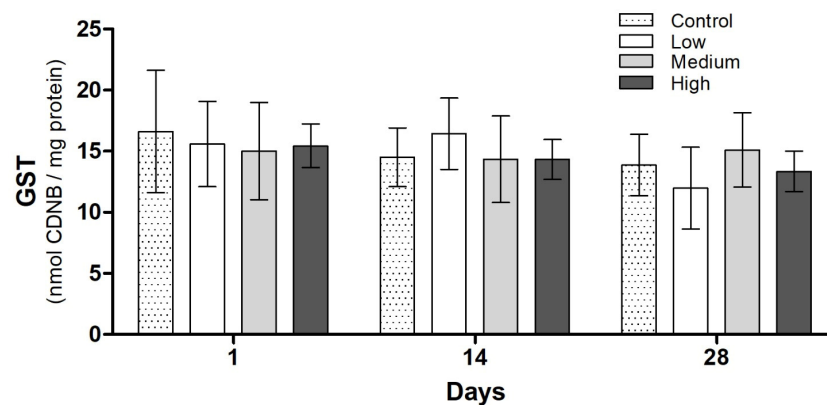


Figure 3. Specific activity of the enzyme GST (Glutathione S-transferase) in the gills of *C. gasar* oysters in the following treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—100 to 200 mg/L TSSs; high treatment—TSSs exceeding 200 mg/L over a 28-day experimental period. The bars represent the mean enzyme activity values, and the vertical lines above the bars represent the standard deviation of the values.

Regarding the SOD enzyme, a significant difference ($p < 0.05$) was observed between the treatments on day 28. No differences between treatments were evident on days 1 and 14. On day 28, SOD activity displayed higher values for the high treatment than for the control. Notably, the low and medium treatments did not demonstrate significant differences ($p > 0.05$) from each other or when compared with the high and control treatments (Figure 5).

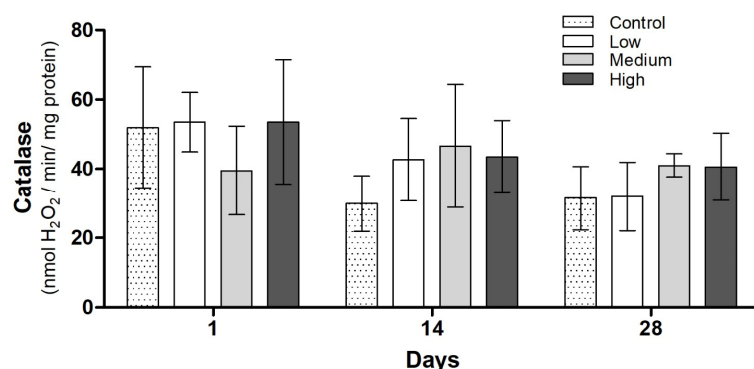


Figure 4. Specific activity of catalase ($\mu\text{M}/\text{mg}$ protein) in the gills of *C. gasar* oysters in the following treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—100 to 200 mg/L TSSs; high treatment—TSSs exceeding 200 mg/L over a 28-day experimental period. The bars represent the mean enzyme activity values, and the vertical lines above the bars represent the standard deviation of the values.

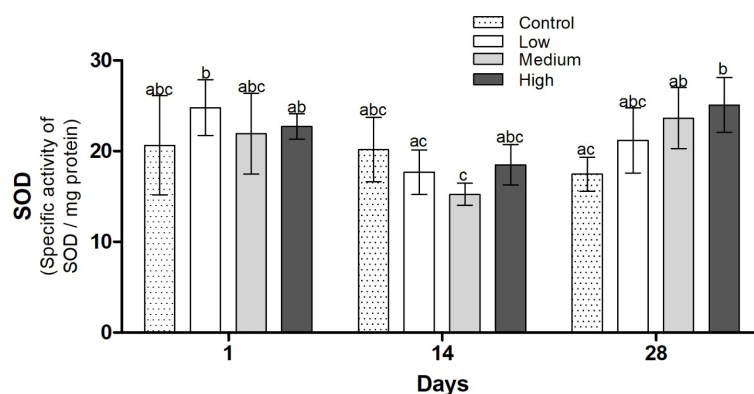


Figure 5. Specific activity of the SOD enzyme (specific SOD activity/ mg protein) in the gills of *C. gasar* oysters in the following treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—100 to 200 mg/L TSSs; high treatment—TSSs exceeding 200 mg/L over a 28-day experimental period. The bars represent the mean values, and the vertical lines above the bars represent the standard deviation of the values. Lowercase letters for different days and treatments indicate significant differences ($p < 0.05$).

The concentration of GSH did not show significant differences ($p > 0.05$) between the different treatments during the experiment (Figure 6). No significant differences ($p > 0.05$) emerged between treatments or culture days with respect to the concentration of sulfhydryl groups in the gills of *C. gasar* oysters (Figure 7). Regarding lipid peroxidation, significant differences ($p < 0.05$) between treatments were observed. No significant differences ($p > 0.05$) in lipid damage were observed between treatments on days 1 and 28. However, on day 14, a substantial increase in lipid damage was noted in the medium and high treatments in comparison to the control group. By contrast, the low treatment did not show significant differences ($p > 0.05$) on day 14 (Figure 8).

In relation to the total antioxidant capacity against peroxy radicals, a significant difference ($p < 0.05$) emerged solely on day 28 between the treatments. Specifically, the high treatment exhibited a greater mean area compared with the control treatment. By contrast, the low and medium treatments did not display differences from each other or from the control or high treatments (Figure 9). A higher mean area in the high treatment signified a reduced total antioxidant capacity.

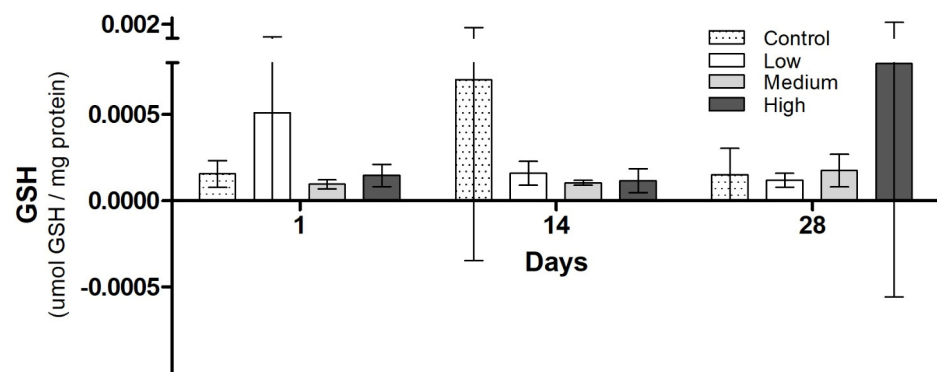


Figure 6. Concentration of reduced glutathione (GSH) (in nmol/mg protein) in the gills of *C. gasar* oysters in the following treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—100 to 200 mg/L TSSs; high treatment—TSSs exceeding 200 mg/L over a 28-day experimental period. The bars represent the mean values, and the vertical lines above the bars represent the standard deviation of the values.

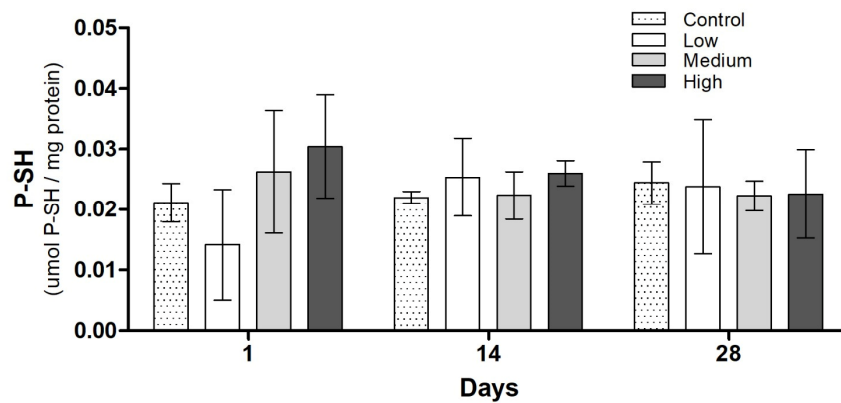


Figure 7. P-SH concentration (in nmol P-SH/mg protein) in the gills of *C. gasar* oysters in the following treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—100 to 200 mg/L TSSs; high treatment—TSSs exceeding 200 mg/L over a 28-day experimental period. The bars represent the mean values, and the vertical lines above the bars represent the standard deviation of the values.

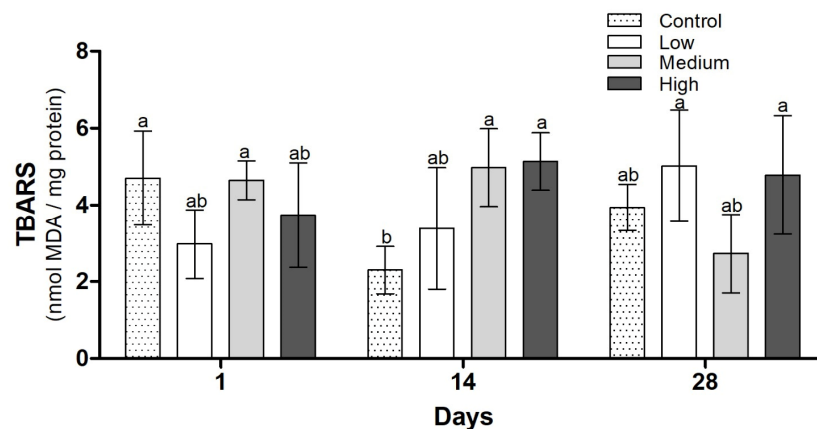


Figure 8. Lipid peroxidation (TBARS) (in mmol/mg tissue) in the gills of *C. gasar* oysters in the following treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—100 to 200 mg/L TSSs; high treatment—TSSs exceeding 200 mg/L over a 28-day experimental period. The bars represent the mean values, and the vertical lines above the bars represent the standard deviation of the values. Lowercase letters for different days and treatments indicate significant differences ($p < 0.05$).

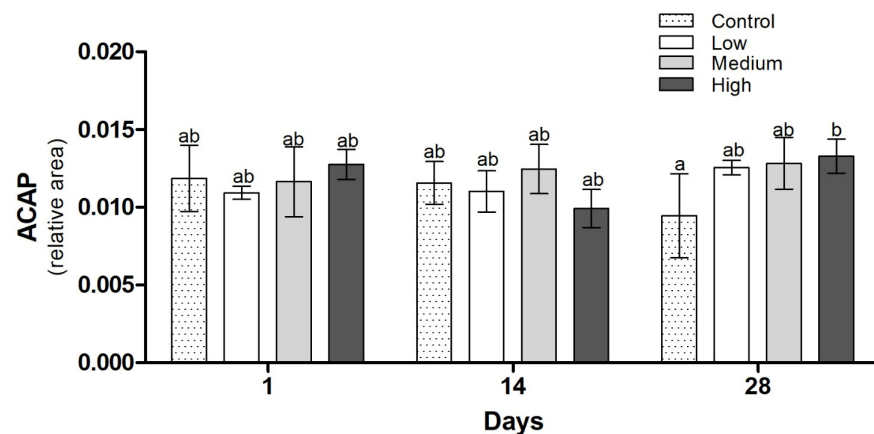


Figure 9. Antioxidant capacity (relative area) in the gills of *C. gasar* oysters in the following treatments: control—oysters fed with microalgae; low treatment—100 mg/L total suspended solids (TSSs); medium treatment—100 to 200 mg/L TSSs; high treatment—TSSs exceeding 200 mg/L over four weeks of the experiment. The bars represent the mean values, and the vertical lines above the bars represent the standard deviation of the values. Lowercase letters for different days and treatments indicate significant differences ($p < 0.05$).

3.4. Histological Analysis

Following 28 days of culture, oysters from the control treatment exhibited well-defined gill lamellae characterized by intact edges, organized cells, and the presence of infiltrated hemocytes (Figure 10).

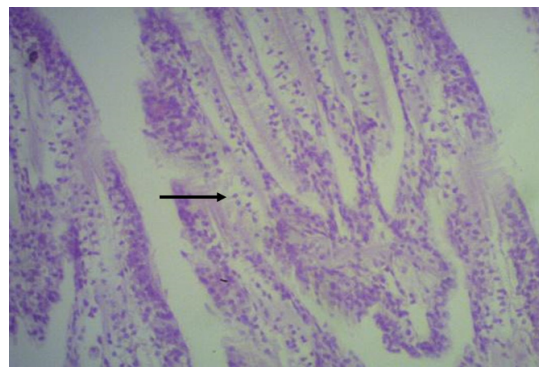


Figure 10. Gill filaments of *C. gasar* oysters after 28 days of culture in the control treatment stained with hematoxylin and eosin, magnification 40 \times .

After the same cultivation duration, oysters from the low treatment showed well-defined gill lamellae with intact edges and organized cells. The hyperplasia of the epithelium within the gill lamellae was more pronounced at the filament ends, accompanied by the presence of infiltrated hemocytes (Figure 11A). In the medium treatment, the gill lamellae structure exhibited poor organization yet intact filaments. Notably, hyperplasia of the gill lamellae's epithelium was evident, particularly at the filament ends, alongside the presence of infiltrated hemocytes (Figure 11B). In the high treatment, the gill lamellae structure was less organized, with filaments lacking consistent height and width patterns. The hyperplasia of the gill lamellae's epithelium was pronounced, and infiltrated hemocytes were observed within the gill filaments (Figure 11C).

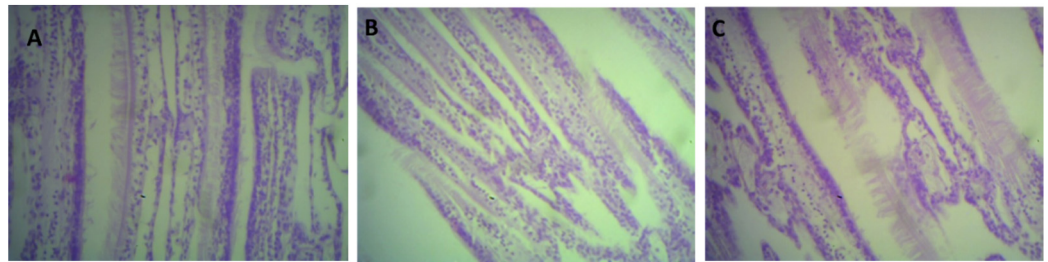


Figure 11. Gill filaments of *C. gasar* oysters after 28 days of culture in biofloc treatments, stained with hematoxylin and eosin, magnification 40 \times . (A): Low treatment; (B): medium treatment; (C): high treatment.

Concluding the experiment, the gills of oysters from both the medium and high treatments displayed notable hyperplasia of the gill epithelium and the presence of infiltrated hemocytes. In the low and control treatments, infiltrated hemocytes and filament hyperplasia were also observable, albeit with lower intensity.

For the PAS-stained samples, in the control treatment, no PAS-positive cells were observed, and the gill architecture was preserved. The gills of the treatments with biofloc presented gill structures with hyperplasia of the lamellar epithelium and isolated PAS-positive cells of a mucosecretory aspect (Figure 12).

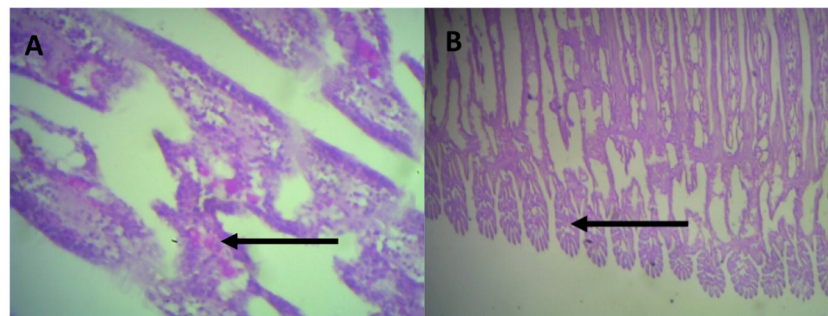


Figure 12. Gill filaments of *C. gasar* oysters after 28 days of culture in biofloc treatments, stained with PAS. (A): Magnification 40 \times ; (B): magnification 4 \times .

4. Discussion

To maintain a biofloc system with marine shrimps, the recommended TSS concentration falls within 100 to 300 mg/L [29]. Schweitzer et al. [49] indicated that a TSS reduction to 200 mg/L in biofloc systems led to decreased nitrification rates. In addition, although 44.8% of a biofloc volume is composed of particles smaller than 48 μm , which is favorable for filtering oysters [50], the guidelines for oyster cultivation suggest lower TSS concentrations. Barillé et al. [28] investigated the impact of high seston (suspended material) concentrations on food selection by *Crassostrea* oysters. They found that seston concentrations below 90 mg/L were regulated by pseudofecal production, while levels above 90 mg/L exhibited reduced filtration and rejection rates, indicative of physical constraints hindering food acquisition. Consequently, reconciling optimal solids concentrations for shrimp and microorganisms with tolerable levels for oysters in an integrated biofloc system appears to pose challenges given the conflicting concentration requirements.

Usually, elevated levels of suspended solids can potentially lead to health issues in cultured species, although tolerance thresholds vary among species [51,52]. Cultivated organisms may experience hindered gill function due to excessive particulate matter in the water, rendering them more vulnerable to hypoxia. This scenario applies similarly to oysters.

The monitoring of valve movements in bivalves serves as a common approach to discerning behavioral patterns that might signify the presence of contaminants or alterations

in environmental conditions [10,11]. For instance, Lombardi et al. [53] noted that oysters tended to keep their valves closed when exposed to unfavorable salinity levels. Various factors contribute to the modulation of valve opening and closing behaviors, encompassing hypoxia [54], food availability [55], pollutants [56,57], acoustic disturbances [58], and other environmental influences. In this context, total suspended solids may impact the valve behaviors of *C. gasar*.

Within the scope of the present experiment, a notable observation was that oysters subjected to the control treatment exhibited a higher average valve opening than those in the biofloc treatments. This disparity indicates a certain constraint on valve opening in biofloc cultures, irrespective of the TSS concentration. This implies that TSSs could be a limiting parameter in *C. gasar* cultivation. These results corroborate a study by Lima et al. [2], who tested different concentrations of settleable solids in oyster seeds and juveniles and found low survival at high concentrations (10 and 20 ml/L of settleable solids). Filter-feeding bivalves employ valve opening to filter water, respire, and feed. Accordingly, well-opened valves denote feeding periods, while partially opened or closed valves suggest decreased or halted respiratory filtration [59–61].

Schreck et al. [62] proposed that a common initial response of many marine organisms to environmental stressors is to modify their behavior, either to evade lethal consequences or minimize the metabolic costs related to maintaining physiological equilibrium under stressful conditions. If organisms are unable to escape unfavorable conditions, these circumstances can have cascading effects on various other behaviors [63]. In the context of the current experiment, during the initial days of cultivation, the TSS concentrations did not appear to significantly impact the opening behavior of the oysters. However, in the second and third weeks, the effects became prominently evident, and by the fourth week, the biochemical effects started manifesting in the high treatment.

Stressful conditions within culture environments can trigger physiological transformations [64], including changes in metabolism, the initiation of a pro-oxidative state involving modifications in the antioxidant defense system, and the potential generation of reactive oxygen species (ROS), which may or may not be accompanied by oxidative damage. Particularly in confined and intensive aquatic environments like those established in biofloc systems, the oxidative equilibrium of aquatic organisms can be adversely affected. Numerous stressors have been investigated in biofloc systems, including salinity [65], temperature [66], and pH [67], among others [18,68,69].

Regarding the SOD enzyme activity, it became evident that the high treatment induced the production of superoxide anion radicals (O_2^-). Although no signs of lipid damage were observed during this period, a subsequent reduction in total antioxidant capacity was noticeable. Thus, it can be postulated that within the initial two weeks of culture, the elevated TSSs in the high treatment did not prompt oxidative stress in *C. gasar* oysters. However, after this period, spanning 28 days of culture, discernible alterations in SOD enzyme activity appeared, signifying the emergence of a pro-oxidative scenario in *C. gasar* oysters. In a study by Zanette et al. [70], investigations involving distinct salinities revealed no modifications in CAT and GST enzyme activities over a span of 17 days in *C. gigas* oysters' gills. However, when exposed to diesel oil under varying salinity conditions, just one week of diesel exposure was adequate to elicit biochemical changes in the SOD and GST enzymes. This underscores the influence of the type of contaminant on the pace and nature of biochemical responses.

Organisms' antioxidant defense mechanisms can be categorized into prevention, interception, and repair [71]. When confronted with stressors, an organism's primary line of defense entails employing preventive strategies to counter the exposure. For instance, plankton employ a preventive measure during photooxidative stress by positioning themselves beneath the sea surface to avoid direct solar radiation [72]. In the case of oysters, it is plausible that, prior to engaging enzymatic defense mechanisms, they employ physical defense strategies. One such strategy might involve minimizing valve opening as a means of curtailing exposure. This adjustment could serve to limit the contact between the gills

and the surrounding water, thereby mitigating the potential adverse effects associated with elevated TSS concentrations. However, as the experiment progressed, the oysters in the high treatment ceased to display distinguishable differences in valve opening compared with the other treatments. Consequently, water ingress into the pallial cavity resumed. This marked a turning point at which enzymatic activity alterations and a decline in total antioxidant capacity manifested, indicative of a pro-oxidative scenario.

A potential influencing factor on the enzymatic defense responses of the organisms in the current experiment was the presence of a biofloc system and the subsequent utilization of biofloc by the oysters. Some studies have indicated increased antioxidant effects in cultured organisms exposed to biofloc compared with those raised in clear water conditions [18].

In the context of this study, the absence of observable shifts in antioxidant defenses and macromolecular damage in the low and medium treatments throughout the experiment could be attributed to the antioxidative properties of biofloc. In cases of lower concentrations, such as those found in the low and medium treatments, the TSS levels did not prove detrimental to the gill health of the oysters concerning the analyzed enzymatic and non-enzymatic defense mechanisms. This implies that the presence of biofloc may have conferred a protective effect against oxidative stress induced by elevated TSS concentrations.

On the 28th day of the culture period, a decline in the overall antioxidant capacity of the oyster gills became evident in the high treatment. This shift in the normal pattern compared with the control treatment during the same period signifies a departure from the expected antioxidant response. Simultaneously, an elevation in SOD activity was observed, indicating the organism's reaction to dismutase superoxide anion radicals, which are generated due to the presence of oxygen. Essentially, gas exchange persisted, but it appeared to have had detrimental effects on the oysters in the high treatment.

As a result, it is highly plausible that the reduction in the total antioxidant capacity could be attributed to other antioxidant defense mechanisms that were not directly assessed in this study. This is because antioxidant defense systems tend to collaborate [73], and their activities can be either boosted or hindered by stressors [74]. In light of this, an approach that evaluates antioxidant capacity in a broader sense, encompassing various antioxidant defenses and offering comprehensive insights into an organism's resilience against ROS-induced toxicity [45], seems better suited to elucidating the redox state of oyster gills.

Interestingly, in the context of bivalves, the presence of food sources like microalgae has been shown to mitigate oxidative stress induced by external factors [75], possibly owing to the antioxidant properties of microalgae. Notably, in the current study, there was a significant decrease ($p < 0.05$) in lipid damage in the control treatment on day 14 compared with day 1, and this reduction was also observed on day 14 in the control treatment compared with the medium and high treatments, aligning with the findings of Li et al. [75].

Pathologies affecting bivalve gills span from inflammatory responses to tissue necrosis. Hyperplasia, a defensive inflammatory pathology, represents an immune reaction in bivalves [76]. Interestingly, such hyperplasia was observed in treatments involving biofloc, irrespective of the concentration of solids. However, the morphological changes in the gills of *C. gasar* oysters did not display notably more pronounced alterations compared with those in the control treatment. As Au [77] suggested, gill histopathological changes are generally indicative of responsiveness rather than specificity to pollutant exposure, implying that they can signify a broad spectrum of contaminants, signifying environmental toxicity.

In terms of the gill lamellae structure of *C. gasar* oysters exposed to biofloc, the mild effects observed in the medium and high treatments suggest the influence of higher solids concentrations on the bivalves. Nevertheless, these effects did not compromise the overall integrity of the gill structure. Notably, the presence of biofloc in the low, medium, and high

treatments did not result in significant damage to the gill morphology of *C. gasar* oysters during the 28-day culture period under the conditions of this experiment.

However, a noticeable variation was observed in mucus production in the gills of oysters subjected to biofloc treatments. Through the use of the PAS method, the presence of PAS-positive cells was discerned in the brachial filaments of oysters from the biofloc treatments, indicative of the activity of mucosecretory cells in these treatment groups. This observation underscores the potential influence of biofloc on the mucous production of the oysters' gills.

A study conducted by Salas-Yanquin et al. [78] provided relevant insights into the relationship between mollusks and the production of mucus in response to environmental conditions. Their analysis of different ash concentrations in water and the subsequent increase in mucus production in mussels highlighted the ability of mollusks to separate ingested material from material to be expelled as pseudofeces, often involving substantial amounts of mucus.

In the context of the present experiment, the observed increase in mucus production associated with the biofloc treatments aligns with the presence of cells displaying mucus production. This phenomenon may be attributed to the requirement for greater pseudofeces production in bivalves within the biofloc treatments. The bioflocculation process could potentially necessitate enhanced pseudofeces production by bivalves, contributing to the selection of food items.

The findings of Garrido et al. [79] further support this notion. Their observations of the food particle selection process in bivalves through endoscopy revealed that particles reaching the mantle cavity are intercepted by gill filaments and transported toward the food groove in small mucous aggregates facilitated by the frontal cilia. Particles slated for rejection, to be eliminated as pseudofeces, are expelled from the mantle cavity through mucous channels that actively engage in mucus secretion. This process's demand for high mucus production could explain the presence of a greater quantity of mucosecretory cells within the gills of oysters exposed to biofloc treatments in the present experiment.

Another explanation for the presence of mucocytes could be the interaction with biofloc, since the diversity of microorganisms in this system is high, especially when compared with the control treatment. The production of mucus in aquatic organisms is for protection against pathogenic organisms.

David et al. [80] analyzed changes in the gills of *Mytella falcata* collected in polluted regions of the Santos estuary. Among other histopathological changes, they observed morphological alterations in the epithelium and an increase in the number of mucous cells, possibly as an attempt to prevent the entry of pollutants through the gill filaments to the entire organism. Zannella et al. [81] reinforced that the production of antimicrobial substances, mainly peptides or polypeptides, is an ancient mechanism of innate immunity. These substances are produced by different types of cells and secretions and are synthesized at the time of infection.

5. Conclusions

The initial defense response of *C. gasar* oysters to elevated concentrations of biofloc involved the regulation of valve closure behavior, followed by subsequent biochemical reactions. Hemocytes were consistently present in the gills, serving as an active defense mechanism for the oysters across all treatments in this study. Furthermore, the gill morphology of the oysters remained relatively stable in the biofloc treatments, with no significant structural changes. Exposure to total suspended solids (TSSs) concentrations comparable to those in the high treatment prompted alterations in the valve opening behavior of *C. gasar* oysters from the second week of exposure onward. Prolonged exposure exceeding 14 days to such TSS concentrations could impact the antioxidant capacity of *C. gasar* oysters while also influencing the modulation of superoxide dismutase (SOD) enzyme activity under similar conditions.

Total suspended solids (TSSs) concentrations below 200 mg/L, under conditions comparable to those in this study, did not induce oxidative stress, alterations in behavior, or histological changes in *C. gasar* oysters cultured over a 28-day biofloc period. However, higher TSS concentrations exceeding 200 mg/L prompted shifts in valve opening behavior and led to oxidative stress in *C. gasar* oysters, making them unsuitable for cultivation.

Author Contributions: Conceptualization, L.C.d.O.C.; L.H.P.; B.G.; J.N.J.; M.P.; S.B.; L.R.; J.V. and M.H.; methodology, A.C.; M.H.; J.S.; L.B.; L.R.; V.F.; B.G.; J.N.J.; L.M.; J.V. and L.H.P.; formal analysis, L.C.d.O.C.; L.M.; L.R.; V.F.; L.B. and J.V.; investigation, L.C.d.O.C.; A.C.; M.H.; L.B.; J.S.; B.G.; S.B.; J.N.J. and L.M.; resources, L.H.P.; M.P.; S.B.; J.V. and L.R.; data curation, L.C.d.O.C.; L.M.; V.F.; B.G.; J.N.J. and L.R.; writing—original draft preparation, L.C.d.O.C.; M.H. and A.C.; writing—review and editing, A.C.; M.H.; B.G.; J.N.J.; L.M.; J.V.; V.F.; L.B.; M.P.; S.B.; J.S. and L.H.P.; supervision, L.H.P. and M.P.; project administration, L.H.P.; funding acquisition, L.H.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by ASTRAL Project—H2020 grant Agreement 863034.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The Authors are grateful to the ASTRAL project that has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement No 863034. Special thanks to Brazilian Council of Research (CNPq) and Coordination for the Improvement of Higher Level or Education Personnel (CAPES). L.H. Poersch received productivity research fellowship from CNPq.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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