Impact of Physically and Chemically Dispersed Crude Oil on the Antioxidant Defense Capacities and Non-Specific Immune Responses in Sea Cucumber (Apostichopus japonicus)

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Abstract: Currently, oil spill pollution is one of the major environmental concerns for sea cucumber (Apostichopus japonicus) aquaculture. During oil spills, spraying chemical dispersants is generally considered an efficient oil spill response. However, the impact of chemical dispersant deployment during oil spills on sea cucumbers is still less known. In this study, we treated sea cucumbers with physically and chemically (by GM-2 chemical dispersant) dispersed Oman crude oil for 24 h. For antioxidant defense capacities, our results showed that physically dispersed crude oil caused a significant elevation on superoxide dismutase (SOD) and catalase (CAT) activities, and glutathione (GSH) content, while chemically dispersed crude oil caused a significant decrease in SOD activity and GSH content with no apparent change in CAT activity. As for non-specific immune responses, our results indicated that physically dispersed crude oil up-regulated acid phosphatase (ACP) and lysozyme (LZM) activities but had no obvious impact on alkaline phosphatase (ALP) activity. Differently, chemically dispersed crude oil down-regulated ACP and LZM activities while up-regulating ALP activity. Based on the integrated biomarker response analysis, the overall impact of chemically dispersed crude oil on antioxidant defense capacities and non-specific immune responses of sea cucumbers was more severe than physically dispersed crude oil.

Keywords: oil spill pollution; chemical dispersant; sea cucumber; antioxidant defense capacity; non-specific immune response

1. Introduction

Holothurians, commonly known as sea cucumbers, belong to the phylum Echinodermata and are typical members of marine benthic communities [1,2]. They are deposit-feeding marine invertebrates widely distributed throughout tropical and temperate coastal zones [1,3,4]. Because of their nutraceutical and pharmaceutical value, holothurians are harvested worldwide, resulting in the overexploitation of natural resources [5,6]. To meet the increasing commercial demand and protect frail natural resources, the aquaculture of holothurians has proliferated and become one of the most important marine aquaculture industries in recent years, especially in the Indo-Pacific region [7]. The sea cucumber Apostichopus japonicus is a holothurian species that largely inhabits the Northern Pacific coast, such as Russia, Japan, and northern China [8]. With the surge in demand, sea cucumber is also the most numerous marine aquaculture species in China [9,10]. It is commonly cultured by pond farming, pen culture, and sea ranching in the Bohai Sea and Yellow Sea coastal areas, such as Dalian, Yantai, and Qinhuangdao. However, over recent years, with flourishing economic development and high human activities, these culture areas are often severely threatened by environmental pollution, especially from harbors, shipping, offshore
exploitation, and other accidental oil spills, leading to large economic losses and severe resource degradation.

Currently, oil spill pollution in marine ecosystems is generally considered as one of the worst catastrophic accidents that could severely threaten marine organisms and human health [11]. Crude oils once spilled into the marine environment undergo a series of physicochemical processes, e.g., volatilization, dispersion, dissolution, photo-oxidation, adsorption, sedimentation, or biodegradation, which would determine their impact on the marine ecosystem and marine economy [12–14]. Therefore, efficient oil spill responses (OSRs), such as mechanical recovery, in situ burning, chemical dispersion, and bioremediation, are crucial to counteract the overall environmental and socio-economic impact of oil spill accidents [15,16]. Among these OSRs, spraying chemical dispersants has been commonly suggested as a useful OSR strategy, especially for large oil spills. For instance, approximately 1.8 million gallons of chemical dispersants were sprayed in the 2010 Deepwater Horizon oil spill (the Gulf of Mexico, USA) [17], and over 200 t of chemical dispersants were applied in the 2010 Dalian Xingang Port oil spill (the Yellow Sea, China) [18]. Chemical dispersants could break down oil slicks into small oil droplets entering the water column, thus, facilitating their natural removal processes [19–21]. However, many scholars have proposed that the deployment of chemical dispersants could also cause, at least transiently, an increase in the concentration and bioavailability of oil-derived hydrocarbons contaminants, resulting in enhancing the impact of oil spill pollution on marine organisms [22–24]. Therefore, the potential adverse outcomes of spraying chemical dispersants during oil spills on marine organisms, especially on cultured marine species, is still a major environmental concern and needs further investigation.

Crude oils are complex chemical mixtures involving thousands of compounds, and the impacts of crude oils on aquatic organisms (e.g., algae, shellfish, and fish) have been widely studied over the past decades [25–29]. Previous studies have identified that polycyclic aromatic hydrocarbons (PAHs) are the main class of bioavailable compounds of crude oils responsible for toxic effects on marine organisms [23,30]. Once taken up by marine organisms, oil-derived PAHs are subjected to biotransformation processes, which concomitantly stimulate the production of reactive oxygen species (ROS) [31,32]. Typically, ROS is produced continuously by various cellular processes in living cells, which has an integral role in maintaining cell signaling and function [33]. However, marine organisms are particularly vulnerable to oxidative effects involved with the enhancement of ROS production induced by environmental contaminants, such as oil-derived hydrocarbons [5,34,35]. To maintain redox homeostasis, aerobic organisms have evolved antioxidant defense systems, such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), as the primary defense line for eliminating ROS. However, an imbalance between the production and elimination of these ROS could induce oxidative damage to basic biological molecules [34,36]. The proliferation of ROS and subsequent oxidative stress responses have been suggested as one of the primary toxic mechanisms of environmental contaminants to marine organisms [33,37]. Moreover, previous studies indicate that oxidative stress responses could also be involved in altered immune function in marine organisms and cause various diseases [33,38], subsequently threatening their development, growth, and reproduction.

As a typical echinoderm species, sea cucumber has evolved a relatively complete antioxidant defense system and non-specific immune system (e.g., lysozyme, LZM; acid phosphatase, ACP; alkaline phosphatase, ALP) to counteract environmental stresses. Previous studies have recorded that environmental stresses could induce severe oxidative stress responses and immune dysfunctions in sea cucumbers, subsequently affecting their recruitment and population growth [5,32,36,39]. As aforementioned, oil spill pollution could threaten sea cucumber aquaculture and lead to huge economic losses, limiting the sustainable development of the sea cucumber aquaculture industry. However, to the best of our knowledge, there is still little known about the effects of crude oil and the deployment of chemical dispersants as an OSR strategy on sea cucumbers. Therefore, in the present
study, we treated sea cucumbers with physically and chemically dispersed crude oil and determined various biochemical markers of sea cucumbers to assess and compare the effects of crude oil and chemical dispersant on the antioxidant defense capacities and non-specific immune responses.

2. Materials and Methods

2.1. Test Organism

Sea cucumbers (Apostichopus japonicas), with an average wet weight of 56.39 ± 9.38 g, were purchased from the Pikou Sea Cucumber Aquaculture Zone of Dalian (Dalian, China), and were acclimatized in the pre-filtered natural seawater for 14 days, under the maintenance conditions as described in our previous study [32]. During the acclimation period, sea cucumbers were fed at 19:00 every day with formulated feeds, and the residual food and feces were siphoned off at 8:00 the next day.

2.2. Exposure Solution Preparation and Chemical Analyses

Oman crude oil, a type of light crude oil, was supplied by Dalian Petro Co., Ltd., Dalian, China. GM-2 chemical dispersant, a conventional chemical dispersant, was purchased from Qingdao Guangming Environmental Technology Co., Ltd., Qingdao, China. Physically dispersed crude oil (low-energy water-accommodated fractions, LEWAF), chemically dispersed (by GM-2 chemical dispersant) crude oil (chemically enhanced water-accommodated fractions, CEWAF), and GM-2 chemical dispersant (DISP) exposure solutions were prepared according to the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) guidelines [40], with some modifications as previously described [29]. LEWAF exposure solution was prepared using fresh Oman crude oil and pre-filtered natural seawater at an oil loading rate of 5 g/L. CEWAF exposure solution was also prepared at an equivalent oil loading rate of 5 g/L by adding GM-2 dispersant at a dispersant-to-oil ratio of 1:5 (m/m). The procedure for preparing the DISP exposure solution was the same as that of the CEWAF solution without adding crude oil. The WAF and CEWAF exposure solutions were chemically analyzed for the contents of total petroleum hydrocarbons (TPH) and 16 priority PAHs using an ultraviolet-visible (UV-Vis) spectrophotometer (Epoch 2, BioTek, Santa Clara, CA, USA) and a gas chromatograph–mass spectrometer (GC–MS) (7890B GC/5977A MSD, Agilent, Santa Clara, CA, USA), respectively, the parameters of which were described in detail in our previous studies [18,41]. Furthermore, 16 priority PAHs listed by the US Environmental Protection Agency were analyzed, including naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, and benzo[g,h,i]perylene [42].

2.3. Acute Toxicity Test

The acute toxicity exposure experiment in the present study was divided into four exposure groups: LEWAF treatment, CEWAF treatment, DISP treatment, and a control (pre-filtered natural seawater), with three replicates for each treatment. Healthy sea cucumbers were randomly assigned to the four groups using a 10 L glass exposure container with sealing films for a 24 h exposure period, according to the guidelines of the Ministry of Ecology and Environment of China [43]. Furthermore, 15 individual sea cucumbers were assigned to each exposure group, and each exposure group was replicated three times. The death and the main characteristics of skin ulcer syndrome (SUS), including general atrophy, swollen mouth, anorexia, evisceration, and ulcerative body wall, were observed during the exposure period. Additionally, the water quality characteristics of the maintenance water and exposure solutions were also measured and recorded at the beginning and end of the exposure period, including water temperature (°C), salinity (‰), pH, and dissolved oxygen concentration (mg/L). At the end of the exposure period, respiratory tree tissues from the sea cucumber individuals in each group were sheared using sterilized scissors and tweezers,
mixed fully, and then immediately frozen using liquid nitrogen for further biochemical analysis. Sea cucumbers were fed at 24 h before the beginning of the exposure period, while they were not fed during the 24-h acute exposure period. During the exposure period, the exposure solutions were not renewed.

2.4. Biochemical Analysis

In this study, the respiratory tree tissue samples were homogenized into the 4 °C pre-cold phosphate-buffered saline (PBS, 100 mM, pH 7.4, Sangon Biotech, Shanghai, China) at a mass-to-volume ratio of 1:9. The homogenate was centrifuged for 10 min with 3000 rpm at 4 °C, and the supernate was collected (10%, v/v). Then, antioxidant defense capacities (including SOD activity, CAT activity, and GSH content) and non-specific immune responses (including LZM relative activity, ACP activity, and ALP activity) of respiratory tree samples were evaluated using the UV-Vis spectrophotometer with commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Finally, to normalize the changes in each biochemical maker of each group, total protein content was also detected in the present study based on the Bradford Coomassie binding assay method [44], using the bovine serum albumin (BSA) as the external standard.

2.4.1. Antioxidant Defense Capacities

SOD activity was determined based on the water-soluble tetrazolium salt (WST-1) method [45]. Briefly, the tissue supernate (10%, v/v) was diluted to 5% (v/v) with PBS solution. Then, 200 µL of WST working solution, 20 µL of Enzyme working solution, and 20 µL of 5% (v/v) tissue supernate were added into a 96-well microplate, mixed well, and then incubated at 37 °C for 20 min. The absorbance was detected at 450 nm using the UV-Vis spectrophotometer. One unit of SOD activity was defined as the enzyme amount in the tissue supernate inhibiting the reduction reaction of WST-1 with superoxide anion by 50% and was expressed as unit per mg protein (U/mgprot).

CAT activity was measured according to the ammonium molybdate-chromogenic method [46]. In short, the tissue supernate was diluted to 1% (v/v) with PBS solution. Next, 1.0 mL of CAT Assay buffer, 0.1 mL of H₂O₂ substrate, and 50 µL of 1% (v/v) tissue supernate were added into a 2 mL tube, mixed fully, and incubated at 37 °C for 1 min accurately. Then, the incubate mixture was added with 1.0 mL of ammonium molybdate chromogenic agent and 0.1 mL of clarificant, mixed well, and settled at room temperature for 10 min. Finally, the absorbance was detected at 405 nm, and CAT activity was expressed as unit per mg protein (U/mgprot).

GSH content was quantified using the 5,5′-dithio-bis-2-(nitrobenzoic acid) (DTNB) method [47]. Briefly, 0.1 mL of 10% (v/v) tissue supernate and 0.1 mL of precipitant solution were mixed fully and centrifuged at 3500 rpm for 10 min, and the precipitate was collected. Then, 0.1 mL of the precipitate, 0.1 mL of GSH Assay buffer, and 25 µL of DTNB chromogenic agent were mixed fully and settled at room temperature for 5 min. Finally, the absorbance was detected at 405 nm, and GSH content was quantified using reduced glutathione as a reference standard and expressed as µmol per mg protein (µmol/mgprot).

2.4.2. Non-Specific Immune Responses

LZM relative activity was detected using the turbidimetric method [48], which was based on the lysis of Micrococcus lysodeikticus. In brief, 2 mg/mL of Micrococcus lysodeikticus standard suspension was diluted into 2.5 µg/mL of bacterial standard application solution using pre-cold PBS solution. Then, 200 µL of tissue supernate was added to 2 mL of the bacterial standard application solution and mixed well. The absorbance at 530 nm reduction of the reaction mixture was recorded at 20 s and 140 s. One unit of LZM relative activity was defined as the amount of LZM causing a reduction in the absorbance of 0.001 and the LZM relative activity was expressed as unit per mL (U/mL).

ACP and ALP activities were measured according to the 4-aminoantipyrine method [49]. For ACP activity, 4 µL of tissue supernate, 40 µL of assay buffer solution, and 40 µL of
substrate solution were mixed fully and incubated at 37 °C for 30 min. Then, 80 μL of alkali reagent and 80 μL of chromogenic agent were added into the incubate mixture, mixed fully immediately, and settled at room temperature for 10 min. The absorbance of the final reaction mixture was measured at 520 nm with a 96-well microplate. As for ALP activity, 5 μL of tissue supernate, 50 μL of assay buffer solution, and 50 μL of substrate solution were mixed gently for 30 s and incubated at 37 °C for 15 min. Then, 150 μL of chromogenic agent was added to the incubate mixture and mixed. The absorbance of the final reaction mixture was measured at 520 nm with a 96-well microplate. Finally, the ACP and ALP activities were expressed as King unit per g protein (King U/gprot).

2.5. Integrated Biomarker Response Version 2 (IBRv2) Index Analysis

To further evaluate and compare the sensitivity and the combined biological effects of different biochemical biomarkers in sea cucumber following exposure to GM-2 chemical dispersant alone and physically and chemically dispersed crude oil, the integrated biomarker response version 2 (IBRv2) index was adopted in this study, which was established by Beliaeff and Burgeot [50] and modified by Sanchez et al. [51].

Firstly, the results (Xi) for all biomarkers of each treatment were divided by those of the corresponding control (X0) and log-transformed (Yi) based on Equation (1):

\[ Y_i = \log_{10} \frac{X_i}{X_0} \]  

Then, the general mean (µ) and the standard deviation (s) of logarithmic transformations (Yi) were calculated for all the biomarkers and normalized via Equation (2):

\[ Z_i = \frac{Y_i - \mu}{s} \]  

Subsequently, the value of A as the difference between the treatments (Zi) and the control (Z0) was calculated using Equation (3):

\[ A = Z_i - Z_0 \]  

Finally, the IBRv2 indexes (IBR) were obtained via the sum of the absolute values of A as shown in Equation (4) and presented using radar charts:

\[ IBR = |A| \]  

2.6. Statistical Analysis

Statistical analysis and graph plotting for all biomarkers studied in this study were carried out using GraphPad Prism version 9.3 (GraphPad Software, San Diego, CA, USA). Radar plots for IBRv2 indexes were drawn using SigmaPlot Ver 14.0 (Systat Software, Inc., Palo Alto, CA, USA). All data were presented as mean ± standard error (SEM). Statistical differences for all biomarker values studied among these treatments were determined using one-way analysis of variance (ANOVA) following the Tukey’s multiple comparisons test. If data did not meet the parametric assumption of normality (Shapiro–Wilk test) and homoscedasticity of variance (Levene’s test), the non-parametric Kruskal–Wallis H test and multiple pairwise comparisons were conducted and statistical significance was accepted when the p value < 0.05.

3. Results

3.1. Chemical Analysis and Water Quality

Based on the results of UV-Vis spectrophotometry, TPH contents of LEWAF and CEWAF exposure solutions were 4.25 ± 0.07 mg/L and 57.81 ± 1.15 mg/L, respectively. GC/MS analysis for the exposure solutions revealed that total PAHs levels (ΣPAHs) in LEWAF and CEWAF exposure solutions were 2.25 ± 0.43 μg/L and 5.68 ± 0.82 μg/L, respectively. Details for the chemical characterization (16 priority PAHs) of the exposure solutions are indicated in Supplementary Materials Table S1 and are described in our
Based on the results of UV-Vis spectrophotometry, TPH contents of LEWAF and CEWAF exposure solutions were 4.8% and 2.25 ± 0.07 μg/L and 57.81 ± 0.07 mg/L, respectively. Details are indicated in Supplementary Materials Table S1 and Table S2. Furthermore, no obvious changes were recorded in the water quality, and water parameters (including water temperature, salinity, pH, and dissolved oxygen concentration) for the acute exposure test at the beginning and end of the exposure period are depicted in Supplementary Materials Table S2.

3.2. Phenotypic Assessments

During the acute exposure period, no death cases of sea cucumbers were observed in the DISP, LEWAF, and CEWAF treatments or the control. As for the main characteristics of SUS, no observable changes were found in the control, DISP treatment, or LEWAF treatment, while sea cucumber following CEWAF exposure showed a noticeable increase in the occurrence frequency (%) of SUS characteristics, including general atrophy (35.6% ± 1.8%), eversion (15.6% ± 4.8%), and ulcerative body wall (11.1% ± 6.5%).

3.3. Antioxidant Defense Capacities Assessment

SOD and CAT activities in the respiratory tree of sea cucumbers following different treatments are depicted in Figure 1. Results showed that SOD activities were 22.73 ± 0.65 U/mgprot and 22.18 ± 0.62 U/mgprot for the control and DISP treatment, respectively, and CAT activities were 7.62 ± 0.17 U/mgprot and 7.46 ± 0.18 U/mgprot for the control and DISP treatment, respectively. Statistical analysis indicated that no obvious differences in SOD and CAT activities were found between the treatments and the control (one-way analysis of variance (ANOVA)). Furthermore, compared to these in the previous study [32], no significant differences among different treatments (one-way analysis of variance (ANOVA)).

![Figure 1.](image-url) Superoxide dismutase (SOD) activity (A) and catalase (CAT) activity (B) in the respiratory tree of sea cucumbers following acute exposure to GM-2 chemical dispersant alone (DISP, red-filled up-triangle), physically (LEWAF, blue-filled square) and chemically (CEWAF, green-filled down-triangle) dispersed Oman crude oil (n = 9). The control was sea cucumbers exposed to pre-filtered natural seawater only (control, black-filled circle). Error bars represent standard error (SEM), and intermediate lines represent mean of each treatment. Asterisks (* or ***) denote the significant differences between the treatments and the control (p < 0.05 or 0.001, respectively). Dark traits denote the significant differences among different treatments (one-way analysis of variance (ANOVA)).
As for GSH content, the effects of different treatments on GSH content in the respiratory tree of sea cucumbers are presented in Figure 2. Compared to the control (18.86 ± 0.33 µmol/mgprot), DISP exposure had no obvious impact on GSH content (19.22 ± 0.41 µmol/mgprot, \( p = 0.963 > 0.05 \)). A notable increase in GSH content was observed in the respiratory tree of sea cucumbers following LEWAF exposure (22.33 ± 0.63 µmol/mgprot, \( p < 0.001 \)), while an evident decrease was caused by CEWAF exposure (16.74 ± 0.57 µmol/mgprot \( p = 0.039 < 0.05 \)). Moreover, compared to the DISP treatment, CEWAF exposure also caused a significant reduction in GSH content (\( p = 0.039 < 0.05 \)). GSH content in the CEWAF treatment was significantly lower than that in the LEWAF treatment (\( p < 0.001 \)), as evidenced by being 0.75-fold lower.

![Figure 2](image-url)

**Figure 2.** Glutathione (GSH) content in the respiratory tree of sea cucumbers following acute exposure to GM-2 chemical dispersant alone (DISP, red-filled up-triangle), physically (LEWAF, blue-filled square) and chemically (CEWAF, green-filled down-triangle) dispersed Oman crude oil (\( n = 9 \)). The control was sea cucumbers exposed to pre-filtered natural seawater only (control, black-filled circle). Error bars represent standard error (SEM), and intermediate lines represent the mean of each treatment. Asterisks (* or ***) denote the significant differences between the treatments and the control (\( p < 0.05 \) or 0.001, respectively). Dark traits denote the significant differences among different treatments (one-way analysis of variance (ANOVA)).

### 3.4. Non-Specific Immune Responses Assessment

To further investigate the effects of different treatments on the non-specific immune responses of sea cucumbers, we determined the enzymatic activities of LZM, ACP, and ALP, as shown in Figures 3 and 4. For the relative activities of LZM (see Figure 3), DISP exposure had no obvious impact on LZM relative activity (8.35 ± 0.53 U/mL), compared to the control (8.58 ± 0.43 U/mL, \( p = 0.891 > 0.05 \)). LEWAF exposure caused a considerable increase in LZM relative activity (11.21 ± 0.62 U/mL, \( p = 0.036 < 0.05 \)), while CEWAF exposure caused a significant reduction in LZM relative activity (3.23 ± 0.34 U/mL, \( p < 0.001 \)). Additionally, LZM relative activity in the CEWAF treatment was also notably lower than that in the DISP treatment (\( p = 0.003 < 0.01 \)) and the LEWAF treatment (\( p < 0.001 \)), as evidenced by being 0.39- and 0.29-fold lower, respectively.

ACP and ALP activities measured following LEWAF, CEWAF, and DISP exposure are shown in Figure 4. ACP and ALP activities in the control were 115.49 ± 2.13 King U/mgprot and 202.60 ± 6.17 King U/gprot, respectively. Similarly, DISP exposure had no marked impact on the ACP and ALP activities compared to the control (\( p = 0.989 > 0.05 \) and \( p = 0.563 > 0.05 \) for ACP and ALP activities, respectively), which were determined to be 115.56 ± 1.77 King U/gprot and 217.16 ± 7.88 King U/gprot, respectively. LEWAF exposure caused significantly elevated ACP activity with a value of 141.29 ± 3.17 King U/mgprot (\( p = 0.037 < 0.05 \)), with no obvious change in ALP activity with a value of 219.54 ± 8.97 King U/gprot (\( p = 0.505 > 0.05 \)).
Differently, CEWAF exposure caused an apparent reduction in ACP activity with a value of 82.75 ± 3.00 King U/mgprot (p = 0.015 < 0.05), while it caused an extremely significant increase in ALP activity with a value of 356.77 ± 8.81 King U/mgprot (p < 0.001). Moreover, CEWAF exposure also caused a notable reduction in ACP activity compared to the DISP treatment (p = 0.021 < 0.05) or the LEWAF treatment (p = 0.006 < 0.001), while it caused a remarkable elevation in ALP activity compared to the DISP and LEWAF treatments (p < 0.001).

**Figure 3.** Lysozyme (LZM) relative activity in the respiratory tree of sea cucumbers following acute exposure to GM-2 chemical dispersant alone (DISP, red-filled up-triangle), physically (LEWAF, blue-filled square) and chemically (CEWAF, green-filled down-triangle) dispersed Oman crude oil (n = 9). The control was sea cucumbers exposed to pre-filtered natural seawater only (control, black-filled circle). Error bars represent standard error (SEM), and intermediate lines represent mean of each treatment. Asterisks (* or **) denote the significant differences between the treatments and the control (p < 0.05 or 0.01, respectively). Dark traits denote the significant differences among different treatments (one-way analysis of variance (ANOVA)).

**Figure 4.** Acid phosphatase activity ACP (A) and alkaline phosphatase ALP (B) activities in the respiratory tree of sea cucumbers following acute exposure to GM-2 chemical dispersant alone (DISP, red-filled up-triangle), physically (LEWAF, blue-filled square) and chemically (CEWAF, green-filled down-triangle) dispersed Oman crude oil (n = 9). The control was sea cucumbers exposed to pre-filtered natural seawater only (control, black-filled circle). Error bars represent standard error (SEM), and intermediate lines represent the mean of each treatment. Asterisks (* or ***) denote the significant differences between the treatments and the control (p < 0.05 or 0.001, respectively). Dark traits denote the significant differences among different treatments (one-way analysis of variance (ANOVA)).
3.5. IBRv2 Index Analysis

To further compare the comprehensive changes of biochemical markers in the different treatments (relative to the control), we employed the IBRv2 index, the results of which are presented in Figure 5. The IBR indexes above or below zero (control) indicate the positive or negative regulation of a given biomarker, respectively. The IBRv2 indexes for DISP, LEWAF, and CEWAF treatments were 0.61, 3.80, and 8.72, respectively, indicating that the rank of the most affected groups could be ordered as CEWAF treatment > LEWAF treatment > DISP treatment. Moreover, based on the results, we found that compared to the control, DISP exposure had no pronounced impact on all tested biochemical markers, and LEWAF exposure caused subtle positive regulation of these biochemical markers (mainly ordered as CAT activity > LzM relative activity > ACP activity > GSH content). Differently, CEWAF exposure caused a strong deviation from the baseline for the tested biochemical markers, among which GSH content and the activities of SOD, LzM, and ACP showed mainly negative regulation (ordered as LzM relative activity > ACP activity > SOD activity > GSH content), while ALP activity showed remarkably positive regulation.

Figure 5. Integrated biomarker response version 2 (IBRv2) index for sea cucumbers following acute exposure to GM-2 chemical dispersant alone (DISP, red-filled, (A)), physically (LEWAF, blue-filled, (B)) and chemically (CEWAF, green-filled, (C)) dispersed Oman crude oil. The control was sea cucumbers exposed to pre-filtered natural seawater only (control, black dash line). SOD: superoxide dismutase activity, CAT: catalase activity, GSH: glutathione content, LzM: lysozyme relative activity, ACP: acid phosphatase activity, ALP: alkaline phosphatase activity. The results of all biomarkers are represented in the reference treatment (control). The area above 0 or below 0 indicate positive or negative regulation, respectively.

4. Discussion

During large oil spills, chemical dispersants are generally deployed as an efficient OSR strategy to quickly disperse oil slicks and reduce the amounts of oil slicks reaching coastal regions, which could consequently alleviate the overall impact of oil slicks on coastal
ecosystems. As a typical echinoderm species, sea cucumber has been reported as being relatively sensitive to environmental stresses compared with other marine species, such as ascidians and mollusks [5,9,52]. Nevertheless, the responses of sea cucumbers to crude oils and chemical dispersants are still not well understood. In this study, we investigated and compared, for the first time, the effects of physically and chemically dispersed crude oil on the antioxidant defense capacities and non-specific immune responses of sea cucumbers. During the acute exposure period, based on the results of the main characteristics of SUS, we found that both GM-2 chemical dispersant alone or physically dispersed crude oil exposure caused no observable changes in phenotypes or death of sea cucumbers. However, chemically dispersed crude oil exposure caused a remarkable increase in the occurrence frequency of SUS characteristics, such as general atrophy, evisceration, and ulcerative body wall, but also without any death cases of sea cucumbers recorded. Based on the chemical analysis, the TPH concentration of chemically dispersed crude oil exposure solution was $57.81 \pm 1.15$ mg/L, which was 13.60-fold higher than that of physically dispersed crude oil exposure solution ($4.25 \pm 0.07$ mg/L). Therefore, we inferred that acute exposure for 24 h of crude oil with TPH concentration below 57.81 mg/L would not cause the death of sea cucumbers. The high occurrence frequency of SUS characteristics observed in sea cucumbers following chemically dispersed crude oil exposure was due to the addition of GM-2 chemical dispersant, which could significantly increase the concentrations of dissolved hydrocarbons into the water column available to sea cucumbers compared with the physically dispersed crude oil exposure.

Biochemical analysis results of the present study indicated that physically or chemically dispersed crude oil exposure induced different extents of oxidative stress in sea cucumbers, which was in agreement with the overall knowledge on ROS production induced by oil-derived hydrocarbons [22,53,54]. Numerous studies have documented the occurrence of disruption in oxidative homeostasis of marine organisms following exposure to oil-derived hydrocarbons, e.g., copepod (Tigriopus japonicus), mussel (Mytilus galloprovincialis), and sea urchin (Strongylocentrotus intermedius) [55–57]. Indeed, although being natural by-products of aerobic metabolism, ROS are also highly unstable molecules often associated with oxidative stress, such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), or hydroxyl radicals (OH•) [58,59]. Nevertheless, as mainly toxic constituents of crude oil, PAHs could enhance ROS production during their biotransformation reactions and metabolism, which would disrupt the balance of production and/or elimination of ROS and inevitably cause an increase in oxidative stress in the internal environment of marine organisms [60–62]. Our previous study has reported that both physically and chemically dispersed crude oil with an equivalent oil loading rate caused a pronounced accumulation of the ROS levels in sea cucumbers, and the ROS level following exposure to chemically dispersed crude oil was 1.08-fold higher than that exposed to physically dispersed crude oil [32]. Under oxidative stress, ROS accumulation in cells could mediate the activation of the antioxidant defense system to maintain oxidative homeostasis and protect marine organisms from oxidative stress-induced toxic effects [36,63].

As important endogenous antioxidant enzymes, SOD and CAT are the first defense lines of cellular protection, which could catalyze O$_2^-$ to H$_2$O$_2$ by SOD and decompose H$_2$O$_2$ into harmless byproducts H$_2$O and O$_2$ by CAT [58,64,65]. In the present study, a significant elevation in SOD and CAT activities in the LEWAF treatment indicated that physically dispersed crude oil exposure induced a high activation of SOD activity to clear excessive ROS and an H$_2$O$_2$-induced consequent activation of CAT activity to eliminate free radicals from the body of sea cucumbers. However, concerning a significant decrease in SOD activity and no noticeable change in CAT activity in the CEWAF treatment, we inferred that chemically dispersed crude oil exposure-induced ROS accumulation was more than the clearing ability of SOD and concomitantly inhibited the SOD activity, which subsequently resulted in no sufficient H$_2$O$_2$ content to induce the activation of CAT activity. Moreover, GSH, a non-enzymatic antioxidant, also possesses the ability of eliminating ROS or free radicals to protect the body from oxidative stress. It generally functions as a substrate of
glutathione S-transferase (GST) and glutathione peroxidase (GPx) to decompose H$_2$O$_2$ into H$_2$O and O$_2$ [66,67]. A significantly elevated GSH content in the LEWAF treatment suggested an increased utilization of sea cucumbers in response to oxidative stress induced by physically dispersed crude oil exposure. Conversely, a lower GSH content in the CEWAF treatment might be partly attributed to the enhancement of ROS production caused by chemically dispersed crude oil exposure, which consumed large amounts of GSH and led to a more inadequate antioxidant response of sea cucumbers. Overall, our results unraveled that under physically dispersed crude oil acute exposure, a relatively low concentration of dissolved hydrocarbons triggered oxidative stress in sea cucumbers could be effectively alleviated and/or counterbalanced by improving the antioxidant defense capacities to eliminate ROS. However, under chemically dispersed crude oil acute exposure, excessive ROS production stimulated by a relatively high concentration of dissolved hydrocarbons might provoke much more severe oxidative damage, which would exceed the eliminating ability of the antioxidant defense system. Consequently, it could reversely inhibit the activities of antioxidant enzymes and cause insufficient antioxidant defense capacities in sea cucumbers. Consistent with our findings in sea cucumbers, other studies have also reported that compared to physically dispersed crude oil, chemically dispersed crude oil exposure could produce more excessive ROS and compromise the abilities of the antioxidant defense system in marine species, resulting in extensive oxidative damage [32,68,69], which could subsequently contribute to cell dysfunctions and tissue injuries.

Previous studies have also suggested a link between oxidative stress and altered immune function in marine invertebrates undergoing environmental stresses [33,37,70]. Except for the antioxidant defense system, the adaptation ability of marine invertebrates to environmental stresses as well depends on their immune system [71,72]. Similar to other marine invertebrates, sea cucumber lacks an adaptive immune system and depends overwhelmingly on non-specific immune responses [73], the main enzymes of which include ACP, ALP, and LZM, etc. Our results showed that physically dispersed crude oil exposure significantly up-regulated ACP activity but had no obvious impact on ALP activity in sea cucumbers, while chemically dispersed crude oil significantly down-regulated ACP activity but caused a significant increase in ALP activity of sea cucumbers. As typical hydrolytic enzymes, ACP and ALP function in modulating phagocytosis and degrading exogenous or endogenous proteins, carbohydrates, and lipids [74,75], which play a pivotal role in cellular immune responses in sea cucumbers undergoing various environmental stresses. The dysregulations of ACP and ALP activities observed in this study revealed that oil-derived hydrocarbons exposure could induce the dysfunctions of the non-specific immune system in sea cucumbers, which might cause immune injury and physiological disorder. Furthermore, LZM is a type of alkaline globulin widely distributed in the body of sea cucumbers. It could hydrolyze the peptidoglycans in bacterial cell walls of bacteria [76], such as *Vibrio splendidus*, of which previous studies have documented as the primary pathogen causing the SUS in sea cucumbers [77]. In this study, a significant elevation in LZM activity in the LEWAF treatment indicated that physically dispersed crude oil exposure up-regulated the LZM activity to enhance the adaptation of sea cucumbers to stress. Differently, a significant decrease in LZM activity under the CEWAF treatment suggested that chemically dispersed crude oil exposure might impair the ability of sea cucumbers to resist exogenous stress, which could further lead to an increase in the occurrence frequency of SUS characteristics observed in this study. Similarly, several studies have also reported that oil-derived hydrocarbons could induce immune dysfunctions in marine invertebrates on the biochemical or physiological levels [33,37,70], threatening their physiological processes or even survival. However, the underlying mechanisms are still not well-known and need further exploration.

Overall, through the IBR analysis, we quantificationally compared the effects of physically and chemically dispersed crude oil on the antioxidant defense capacities and non-specific immune responses, which certified the IBRv2 index as a valid tool to comprehensively evaluate the biochemical and physiological responses of marine organisms.
subjected to environmental contaminants. Our findings were consistent with the findings for other aquatic species and oil types, suggesting that adding chemical dispersants could enhance the toxic effects of crude oil on antioxidant defense capacities and immune responses [32,70,78], which should be predominantly ascribed to the ability of chemical dispersants that could increase the concentration and bioavailability of dissolved oil-derived hydrocarbons in the water column. In terms of oil spill risk assessment, our results further complement the knowledge about the underlying toxic effects of oil-derived hydrocarbons contaminants on sea cucumbers. The results could also help us highlight the importance of considering the impact of chemical dispersants deployed as an OSR strategy in the OSR planning and decision-making during oil spill accidents, of which it is remarkable when comparing the biochemical markers at an equivalent oil loading rate with and without the addition of chemical dispersant. Furthermore, our findings could contribute to a better understanding of the impact of oil spill accidents on sea cucumber aquaculture. Future research should focus on the underlying toxic mechanisms involved in oxidative stress and immune responses of sea cucumbers following exposure to oil-derived hydrocarbons contaminants. Moreover, to better understand the underlying toxic mechanism of oil-derived hydrocarbons exposure, determining the bioaccumulation and metabolic processes of petroleum hydrocarbons in sea cucumbers following oil exposure is also suggested for future research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse10101544/s1, Table S1: Contents of 16 priority PAHs (ΣPAHs) listed by the US Environmental Protection Agency (EPA) in the physically (low-energy water-accommodated fractions, LEWAF) and chemically (chemically enhanced water-accommodated fractions (dispersed by GM-2 chemical dispersant), CEWAF) dispersed Oman crude oil exposure solutions. Table S2: The water quality characteristics of the maintenance water and exposure solutions.

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