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Research Article

The Immune Response of *Acanthaster planci* to Oxbile Injections and Antibiotic Treatment

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Bile salts have been recently identified as a rapid and effective method for killing *A. planci*. However the mechanistic basis of this new control method is poorly understood. This study explored the immune response(s) of *A. planci* and/or pathogenesis resulting from the injection of bile salts. To account for the possible role of pathogenesis in causing high rates of mortality, *A. planci* was treated with antibiotics to minimise the incidence and severity of bacterial infections. No significant difference in the time to death between groups with and without antibiotic treatment was reported, suggesting a limited bacterial effect on the induction of disease and death of injected sea stars. The number of circulating coelomocytes increased significantly after injection confirming the induction of a strong immune response. Five types of circulating cells were identified: (1) phagocytes, (2) small hyaline cells, (3) colourless spherule cells, (4) red spherule cells, and (5) fusiform cells. Histological analysis of *A. planci* tissues showed that the mechanism leading to rapid mortality is related to necrosis and/or apoptosis, rather than transmissible disease. Therefore, bile salts are an effective and safe method for killing crown-of-thorns sea star *in situ*.

1. Introduction

Outbreaks of the coral eating crown-of-thorns sea star (*Acanthaster planci*) are currently the single biggest threat to tropical coral reefs [1]. The increasing frequency and intensity of outbreaks over the last two decades are contributing to the rapid degradation of coral reef ecosystems throughout the Indo-Pacific [2–4]. Therefore, a key strategy to reduce and reverse coral loss is the development of effective control methods to prevent or eradicate ongoing outbreaks of *A. planci* [5].

Since the first documented outbreaks of *Acanthaster* spp. in the 1950s, the immediate response has been to collect or kill the burgeoning numbers of sea stars [6, 7]. However, attempts to control outbreaks have been ineffective in most cases. The largest and longest running control program for *Acanthaster planci* was undertaken in Japan, where approximately 13 million sea stars were collected between 1970 and 1983, at an estimated cost of US $6-7 million [8]. Despite this prolonged effort, chronic infestations of *Acanthaster* spp. still killed in excess of 90% of coral across vast areas of fringing reefs.

The most widely used method to eradicate *Acanthaster planci* is to inject sea stars with sodium bisulfate (strong oxygen scavenger) or acetic acid [9, 10]. However, current best practice is a painstaking process in which each sea star has to be extracted from the reef matrix and then injected multiple times in the oral disk and arms in order to ensure complete mortality. In addition there is a high rate of sea star survival when using the acetic acid technique [10]. Thus, present injection methods are expensive, excessively time consuming, and difficult to accomplish in large areas. Significant increases in efficiency could, therefore, be achieved by using a toxin that could be administered with a single dose and anywhere on the sea star. Rivera-Posada et al. [11, 12] demonstrated that single injections of low concentrations of oxbile and oxgall induced rapid death of *A. planci*, representing a novel and potentially efficient method for controlling crown-of-thorns sea star.

Oxbile is a digestive enzyme contained in the TCBS formula that is directly extracted from the bovine gall bladder and is generally inexpensive to produce [13]. Bile is naturally produced by most vertebrates and is totally absent in invertebrates [14], indicating a possible limitation to cope with the injection of this foreign protein that could induce a strong immune response. Improved understanding of the mechanism triggering death after bile injections requires the analysis of the immune system of *A. planci*. However, no studies on the composition, function, and specific responses of the different immune cells after an immune challenge have been published for *Acanthaster* spp. Most of the studies conducted on the immune system of echinoderms have been performed on sea urchins and sea cucumbers, and only three main types of cells have been formally identified in *A. planci*: (1) phagocytes, (2) colourless spherules, and (3) red spherules [15]. However, several studies conducted on other echinoderms revealed the existence of other types of cells, such as progenitor cells, crystal cells, and hyaline or vibratile cells [16–19]. These other types of cells have only been observed in a few instances and could possibly be present in *A. planci* but have not been identified yet.

The echinoderm immune system is an innate system [20]; thus the modulation of an immune response is only based on the variation of the population of the different cells present, as the mechanisms used remain the same. Three defence mechanisms have been identified: (1) cytotoxicity, which corresponds to the secretion or activation of toxic substances to induce lysis of pathogenic cells, (2) phagocytosis, which refers to the engulfment and destruction of foreign bodies or pathogenic cells by phagocytes, and (3) encapsulation or clotting, which corresponds to an aggregation of several types of cells around a foreign body which failed to be cleared by phagocytosis [18, 21]. The mechanism of encapsulation involves the creation of syncytia-like structures, which is the fusion of the cytoplasm of the cells present in the aggregation, and the cluster formed is eliminated from the organism [22, 23].

Echinoderms show an obvious vulnerability to bacterial disease, as many microorganisms, especially Gram-negative bacteria, have been identified as the etiological agents responsible for mass mortalities of numerous echinoderms, especially asteroids [24–26]. Oxbile could induce disease in injected *A. planci* through different mechanisms: (1) inducing dysbiosis by inhibition of normal *A. planci* microflora and promoting growth of vibrios [27–29], (2) generating an allergic reaction as peptides trigger anaphylactic reactions in injected hosts promoting more tissue damage [11], (3) inducing cell membrane and mitochondria damage leading to cell death by apoptosis and necrosis [30], and (4) by toxin formation of indigenous microflora [31].

Acanthaster planci, like most echinoderms, is highly susceptible to pathogenesis [32–35], which could provide significant advantages over existing control methods. However, the development of a lethal infection could potentially lead to the transmission of the disease to non target species such as fish, corals, and/or other invertebrates, which would represent a limitation to implementing the use of oxbile as a control method. Thus, the aims of this study are to identify and describe *A. planci* immune cells and to characterise the immune response induced by bile injections and antibiotic treatment to better understand the mechanisms involved in the induction of disease. Finally, recommendations are given about the use of oxbile as a novel control method, according to the results obtained.

2. Materials and Methods

2.1. Study Sites. The study was undertaken in March-April 2013 at 2 locations: (1) The Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University, Townsville, Queensland, Australia, and (2) Lizard Island Research Station (LIRS) located in the far northern section of the Great Barrier Reef (GBR), Queensland, Australia.

2.2. A. planci Collection and Maintenance Conditions. A total of 52 sea stars (~32 cm in diameter) were used in this study. Twenty-three *A. planci* were from Fitzroy Island and twenty-nine from Big Vickie's Reef, Lizard Island. *A. planci* collected at Fitzroy Island were immediately transported to MARFU facilities, James Cook University (JCU), Townsville, Queensland, Australia. All sea stars at both locations were kept in large holding tanks (2.7 m × 1.6 m × 0.5 m) with constant seawater flow, high level of oxygenation, and average temperature 26–28°C and left to acclimatize for 3 days. Weak and damaged individuals were discarded. All *A. planci* were located in individual tanks (Nally bins, 0.64 × 0.41 × 0.4 m) with constant water flow and high water oxygenation prior the injection.

2.3. Bile Tested. Two types of bile were used for the experiments: (1) oxgall (Difco) which corresponds to a natural dehydrated fresh bile directly extracted from the bovine gall bladder and (2) bile salts No. 3 (Oxoid) which is a refined bile widely used as a selective inhibitory agent in culture media as it is effective at a concentration 3 times lower than other bile products. Bile salts No. 3 contain 50% sodium cholate and 50% sodium deoxycholate [36]. A total of four solutions were prepared by adding 4 and 6 g of oxgall and 4 and 6 g of bile salts No. 3 to 1 litre of fresh water and stirred until the powder was completely dissolved.

2.4. Experiment 1: Bile Injections. Oxgall and bile salts No. 3 at 4 and 6 $g \cdot L^{-1}$, respectively, were tested. Ten mL of each solution was injected at the base of the arm with a 16-gauge needle in the polian vesicle area. The role of this organ is related to the storage of coelomic fluid and maintenance of hydrostatic pressure [37]. Eighteen sea stars distributed in 4 groups of 4 individuals and 2 controls were used for the experiments at each location (JCU and LIRS). Control individuals were injected with 10 mL of freshwater to determine if the osmotic pressure differential between seawater and fresh water could affect sea star survival.

2.4.1. Data and Sample Collection. Times to death of all injected sea stars were recorded, based on the activity of the podia following Rivera-Posada et al. [28].

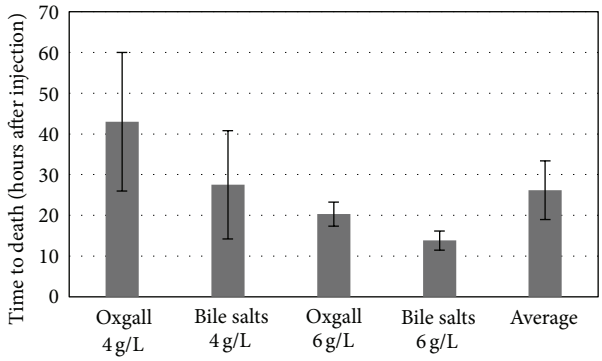


FIGURE 1: *Acanthaster planci*. Time to death after injections of oxbile.

2.4.2. Coelomic Fluid. One mL of coelomic fluid was extracted from the arm using insulin syringes.

2.4.3. Cell Count. Cell counts were performed on fresh coelomic fluid from the sea star using a Helber counting chamber with a Thoma ruling on a single round plateau of 0.02 mm depth and square size 1/400 mm^2. Coelomic fluid was extracted before and 2 hours after the injection of bile to determine increases in cell density and the type of cells responding to the insult.

2.4.4. Optical Microscopy. Volumes of 0.5 μL of fresh coelomic fluid were extracted from the first group of sea stars and centrifuged in a Cytospin (120 ×g for 10 min). The cells were then held on a slide, which was immersed in 4% phosphate buffered formaldehyde for one week. Then, cells were stained with Gram stain (GS) and Haematoxylin and Eosin (H&E) following the techniques described by Prophet et al. [38].

2.4.5. Scanning Electron Microscopy (SEM). Volumes of 0.5 μL of coelomic fluid were extracted and immediately fixed in 4% phosphate buffered formaldehyde (final stock concentration 2%). Cell suspensions were centrifuged in a Cytospin (0.5 μL at 120 ×g for 10 min) on 12 mm diameter glass coverslips (1.5 thickness) coated with poly-D-lysine (Neuvitro). Coverslips were engraved in order to mark the side were the cells were held. Then, coelomocytes were dehydrated by passing through a graded series of ethanol for 10 minutes (50%, 60%, 70%, 80%, 90%, 100%, 100%, and 100%) and transferred into hexamethyldisilazane (HMDS) (2 : 1, 1 : 1, 1 : 2 100% ethanol and 100% HMDS followed by 3 series of 100% HMDS of 10 minutes). Subsequently, the coverslips were allowed to dry overnight in a fume hood and stored in a desiccator (silicates). Finally, the coverslips were gold-coated following the procedure described by Tuan and Lo [39] and observed with JSM-5410LV SEM.

2.4.6. Histology. Sixteen tissue samples were aseptically extracted from healthy and nearly dead *A. planci*. Pieces of digestive glands and skin of 0.25 cm^2 were collected and fixed in 4% phosphate buffered formaldehyde. Samples were then embedded in agar and underwent a short cycle in the automatic tissue processor. Finally, the tissues were stained using H&E and GS following the techniques described by Prophet et al. [38].

2.5. Experiment 2: Antibiotic Treatment. The second experiment consisted of injecting 10 mL of Oxgall at 4 g L^{-1} following the same protocol as the previous experiment. However injected sea stars were treated daily with broad-spectrum antibiotics dissolved in water per 5 days in order to determine the role of bacterial infection in the mortality induced by oxbile injection. A total of 15 *A. planci* were used in this experiment. Five *A. planci* from Fitzroy Island reefs (4 injected with Oxbile and 1 control) were treated with Bactrim (160 mg of Sulfamethoxazole/32 mg of trimethoprim per tank per day), while ten sea stars from Lizard Island (8 injected with oxbile and 2 controls) were treated with amoxicillin (100 mg per tank per day). Antibiotics were administrated until the death of the animal. Control sea stars were injected with freshwater and treated daily with antibiotics for the same period of time (5 days).

2.6. Statistical Analysis. (1) A two-way ANOVA was performed to determine if the time needed to induce death is affected by the concentration and/or the type of treatment applied. As the interaction term appears not to be significant, the test was run again without the interaction term. (2) The difference between the number of cells prior to and after the injection of oxbile was tested using a paired t-test. (3) A one-way ANOVA was run to determine if the time between the injection and death was affected by the antibiotic treatment. In this case, the data obtained from the first experiment were used, considering the results obtained after injection of oxbile at 4 g·L^{-1}. Levene's test was run in order to check the assumptions.

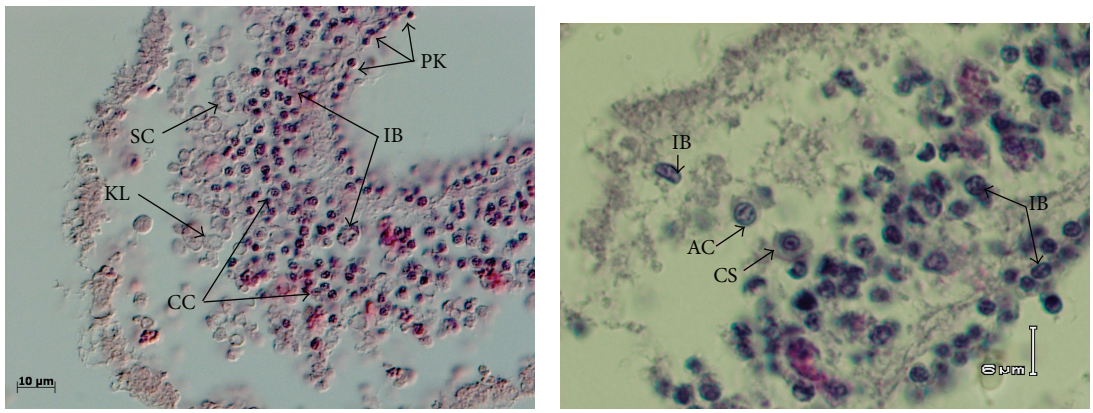
3. Results

3.1. Macroscopic Results. Injections of oxbile at a minimum concentration of 4 g·L^{-1} induced 100% mortality across all individual sea stars. In fact, all the 16 *A. planci* injected with oxgall and the 16 injected with bile salts No. 3 died within 5 days (mean 26.16 ± 7.22 SE) (Figure 1). *A. planci* presented typical signs of disease, such as loss of body turgor, loss of spines, necrosis of the skin, blisters, and high mucus production at the tips of the spine. The sea stars injected with freshwater (controls) did not show any signs of disease and all survived. Time to death appeared to be independent of the concentration and/or the type of bile used (Table 1). However, a greater variability in the time recorded was observed in individuals injected with minimum concentrations of 4 g L^{-1}, which could be related to the low dose used (Figure 1). Despite the lack of statistically significant difference, high concentrations tended to induce much more rapid and a more consistent time to mortality (Figure 1).

3.2. Histologic Observations. General organization of the tissues was significantly disrupted. Hypertrophy of glandular

Table 1: Significance of the concentration and/or type of oxbile used on the time to death.

Source	Df	SS	MS	F	P
Concentration	1	2664.5	2664.5	2.85	0.10
Type of bile	1	968	968	1.04	0.32
Interaction	1	162	162	0.17	0.68
Residuals with interaction term	28	26947.0	962.393		
Residuals without interaction term	29	27109	934.79		



(a) (b)

Figure 2: Histological changes related to apoptotic and necrotic processes were observed in *Acanthaster planci* injected with bile salts. (a) Pyknosis (PK); intracellular bodies (IB); cell swelling (CS); karyolysis (KL); initial stages of chromatin condensation (CC). (b) Apoptotic cells (AC) with condensed and hyperchromatic chromatin and fragmented nuclei; cell swelling (CS); intracellular bodies (IB).

tissues was observed as well as a high number of cells undergoing pyknosis, a process of condensation of the chromatin that suggest apoptosis or necrosis of the cell. Intracellular bodies, karyorrhexis, and karyolysis were also observed (Figure 2). Only four individuals out of eighteen analysed via Gram stain showed the presence of bacteria within their tissues, suggesting that their presence was not the cause of the mortality induced by the injection of bile. No bacteria were observed in the coelomic fluid.

3.3. Coelomic Fluid. The first criteria confirming a change in the coelomic fluid composition was the colouration of the fluid. Coelomic fluid extracted before bile injections was colourless, while the coelomic fluid extracted after the injection acquired a red colour. The intensity of the red coloration indicates concentration of echinochrome A, an antibacterial compound commonly found in echinoderms [15]. The intensity of the red coloration increased with time, indicating an increasing reaction, triggered by the injection of bile. The number of circulating cells present in the coelomic fluid significantly increased after the injection of bile (paired t-test, $t = -3.95$, Df = 15, $P < 0.01$). Independent of the different treatments, the increase in the number of cells forms a clear pattern (Figure 3); however a high variability in individual responses is clearly noticeable and must be related to the immune system condition of each individual that can be influenced by parasites, disease, stage of life, immunosuppression, starvation, and so forth. For example,

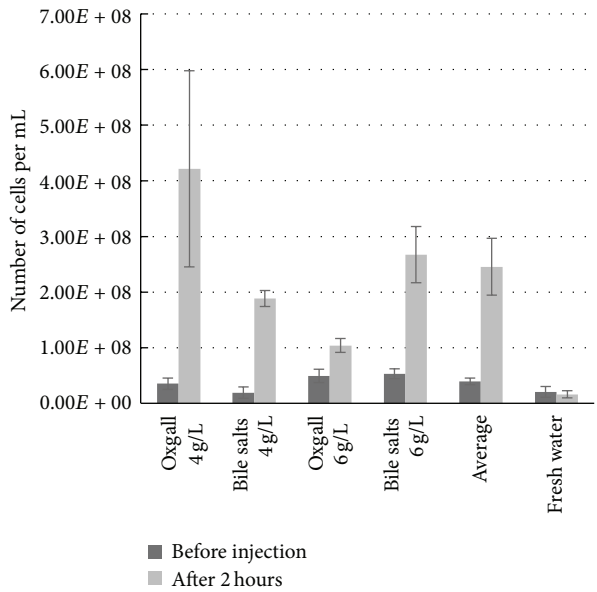


Figure 3: *Acanthaster planci.* Coelomic fluid cell counts per treatment.

undernutrition has detrimental effects on immune function and digestive tract health [40].

Four different types of cells were observed two hours after bile injections, comprising the small and large hyaline cells and two types of granulated cells. The large hyaline cells

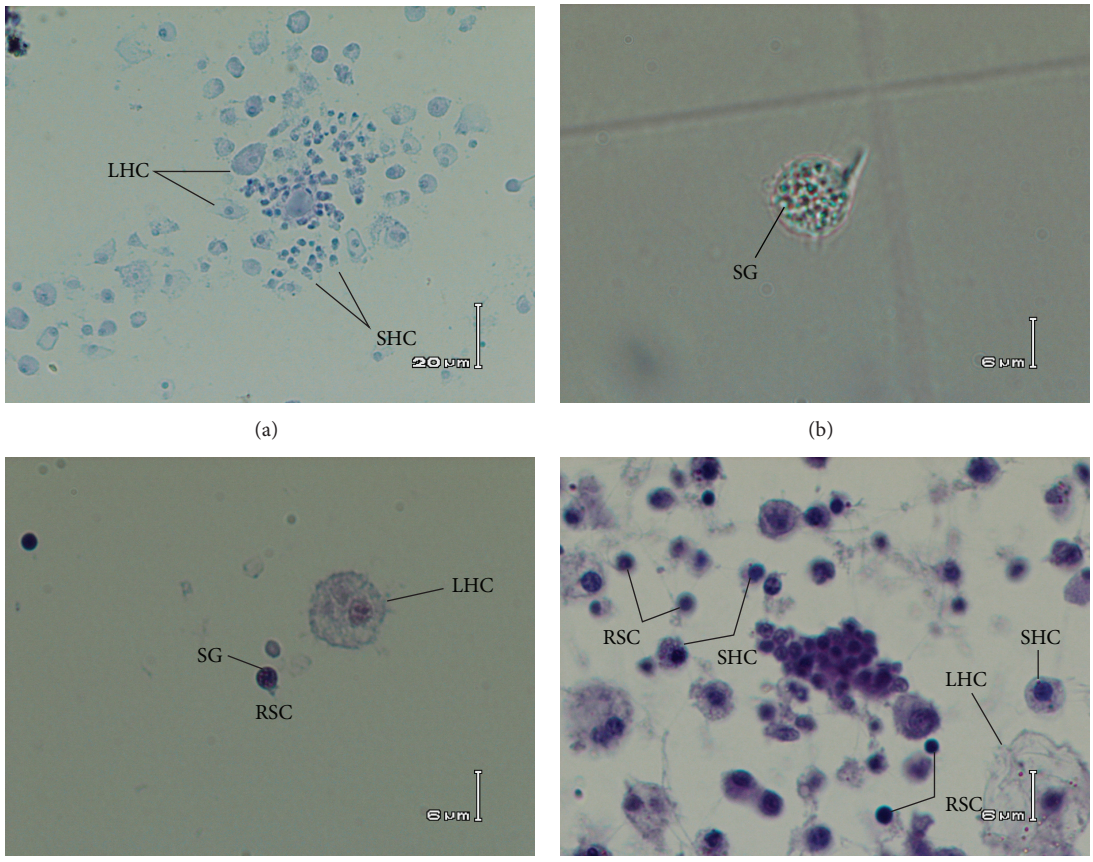


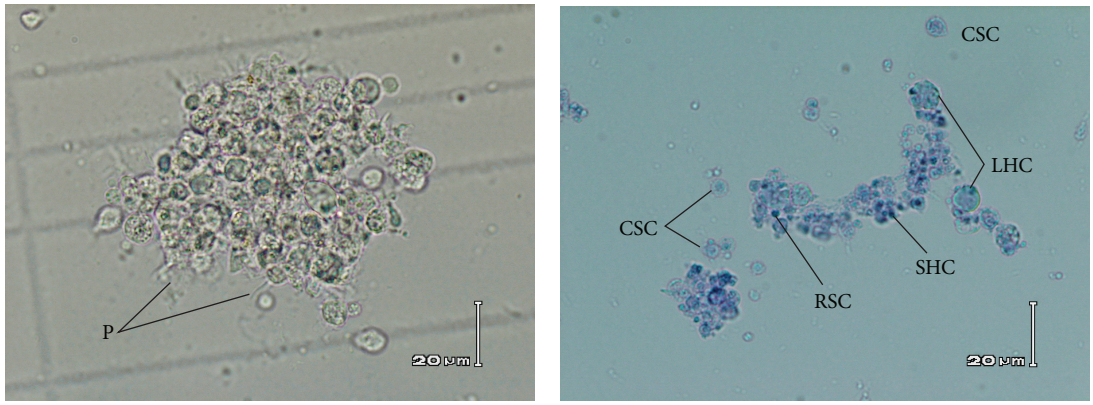
Figure 4: Circulating cells of *Acanthaster planci* 2 hours after injection. Light microscopy: (a) small hyaline cells (SHC) and large hyaline cells (LHC), Gram stain; (b) colourless spherule cell (CSC) including secretory granules (SG), fresh sample; (c) red spherule cell (RSC) with SG inclusion and SHC, Gram stain; (d) all four types of cells observed together, Gram stain.

ranged from 8 to 12 microns and displayed a clear cytoplasm, including occasionally small inclusions (Figures 4(a), 4(c), and 4(d)). The small hyaline cells were ~4 microns, showing a dense nucleus and a thin cytoplasm (Figures 4(a) and 4(d)). The two other types of cells detected were highly granulated, containing secretory granules (Figures 4(b) and 4(c)). The colourless spherule cells ranged between 5 and 7 microns and contained various types of granules. Finally, the red spherule was the smallest cell observed ~2 microns with distinct red inclusions. Red cells showed completely different staining properties as the whole cell was stained and the nucleus was not distinguishable (Figure 4(c)).

After injection of bile salts, circulating cells started to aggregate leading to the formation of large clusters (Figure 5). The size of the clusters increased drastically with time and some reached 500 microns in size 6 hours after injection. The cluster structure was dense (Figure 5(a)). Cells responsible for this phenomenon have not been clearly identified, nor the selectivity of the recruitment as the structure was compact and difficult to analyse. However, on small aggregations large and small hyaline cells and the two types of spherule cells were identified (Figure 5(b)).

Scanning electron micrographs allowed the identification of five types of cells (Figure 6): (1) large rounded cells 9 microns in size, presenting a smooth surface, probably equivalent to the large hyaline cell observed under a light microscope (Figures 6(a) and 6(b)) and (2) small rounded cells of 4 microns, showing short pseudopodia (Figure 6(a)). These cells are comparable in size and shape to the small hyaline cells described in light microscopy. (3) Cells containing granules, ranging from 4 to 6 microns (Figure 6(c)), are comparable to the colourless spherule cells. (4) Cells of 2 microns were observed in very high numbers at the surface of the cluster (Figures 6(a) and 6(b)). The size of these cells suggested a link with the red spherule cells observed in light microscopy, leading to the designation small spherule cell; however, the red spherule cells observed initially were filled with numerous spherules that were not observable on SEM images which could be related to the release of echinochrome A that gives the red coloration to the coelomic fluid after degranulation and (5) fusiform cells characterized by an elongated shape (Figures 6(a) and 6(b)).

Formation of syncytia-like structures was observed through SEM analysis. Cells were aggregated and attached between them, possibly due to pseudopodia extensions originating from specialised cells. Cells merged their cytoplasm in order to create large structures (Figure 6(d)). Precipitated salt crystals were often observed within the



(a) (b)

Figure 5: *Acanthaster planci*. Gram stain of clusters formed 2 hours after injection of oxbile. (a) Fresh sample and (b) H&E stain. Colourless spherule cell (CSC), large hyaline cell (LHC), pseudopodia (P), red spherule cell (RSC), and small hyaline cell (SHC).

clusters suggesting a role in the formation of these structures (Figure 6(b)). In this study syncytia-like structure formation was considered the classic response of the immune system to try to phagocytise or engulf large foreign particles (salt crystals and other contaminants) that could not be degraded by a single cell and it is also a mechanism widely used by many organisms to stimulate immune cells to respond to pathogens and foreign bodies [41].

3.4. Antibiotic Test. All *A. planci* injected with oxbile and treated with antibiotics died within 55 hours (mean 32.17 ± 3.11). The clinical signs of disease were identical to those observed in individuals without antibiotic treatment. Control individuals, injected with freshwater and treated with antibiotics, did not present any signs of disease and survived. Antibiotic treatments do not have a significant effect (one-way ANOVA, $F = 0.47$, Df = 2, $P = 0.63$; Levene's test, $F = 1.58$, Df = 2, $P = 0.23$), suggesting that bacterial disease is not the mechanism that kills *A. planci*. However, opportunistic bacteria will normally colonize any decomposing tissues.

4. Discussion

Oxbile is known to have detrimental effects when injected into individual sea stars [5, 12], but until now it was not known whether this effect reflects toxicity of the bile salts *per se* or results from induction of bacterial disease. In humans and other animal models, the retention of bile in cholestatic diseases induces liver, gastrointestinal, and renal injury and also has deleterious effects on the fetal-placental unit. Bile salts are considered cytotoxic [42, 43].

Low bile acid concentrations induce apoptosis, whereas high concentrations induce necrosis [44]. Apoptosis induction is dependent on the type of bile salt, its concentration, and/or its conjugation state [30, 43]. Bile-induced apoptosis involves 3 pathways: (1) the death receptor or extrinsic pathway, (2) the mitochondrial or intrinsic pathway, and (3) the endoplasmic reticulum (ER) stress pathway. On the other hand high bile acid concentrations cause direct damage to the basolateral membrane and cell organelle membranes, and because they are more concentrated, these compounds are particularly harmful to the outside layer of the canalicular membrane creating channels in the cell membranes leading to ion dysregulation, mitochondrial and cellular swelling, plasma membrane failure, and cell lysis, releasing intracellular contents causing tissue inflammation.

Basically, bile salts disrupt cell membranes through their detergent action on lipid components and promote the generation of reactive oxygen species that, in turn, oxidatively modify lipids, proteins, and nucleic acids causing mitochondrial dysfunction and cell death [45, 46]. Cell membrane and mitochondria damage induced by bile acids could explain why *A. planci* showed loss of skin turgor and minimal movement 2 hours after injection. Changes in the sea star mutable collagenous tissue (MCT) determine sea star movement, skin stiffness, and tensile strength through variations in the cation concentration of the proteoglycan matrix, which can be directly disrupted by bile salts [47].

In this study, low concentrations of bile were injected in *A. planci*, suggesting the possible induction of apoptosis. However, both necrosis and apoptosis were found on *A. planci* tissues collected after injections of bile salts and are likely the mechanisms involved in the induction of disease and death of *A. planci*. Pyknosis is a degenerative condition of the cell nucleus that was observed in all *A. planci* tissues 24 hours after injection and it is the hallmark of cell death induced through apoptosis and necrosis [48]. In this study it was not possible to distinguish which mechanism was dominant as both processes were observed together in the tissue samples and they can occur independently, sequentially, as well as simultaneously [49]. In some cases it is the type of stimuli and/or the degree of stimuli that determines if cells die by apoptosis or necrosis. At low doses, a variety of injurious stimuli such as heat, cytotoxic drugs, radiation, and hypoxia can induce apoptosis, but these same stimuli can result in necrosis at higher doses.

More importantly, Morgan et al. [50] demonstrated that bile toxicity increases with high levels of NaCl. Thus bile toxicity could be naturally potentiated by the presence of high

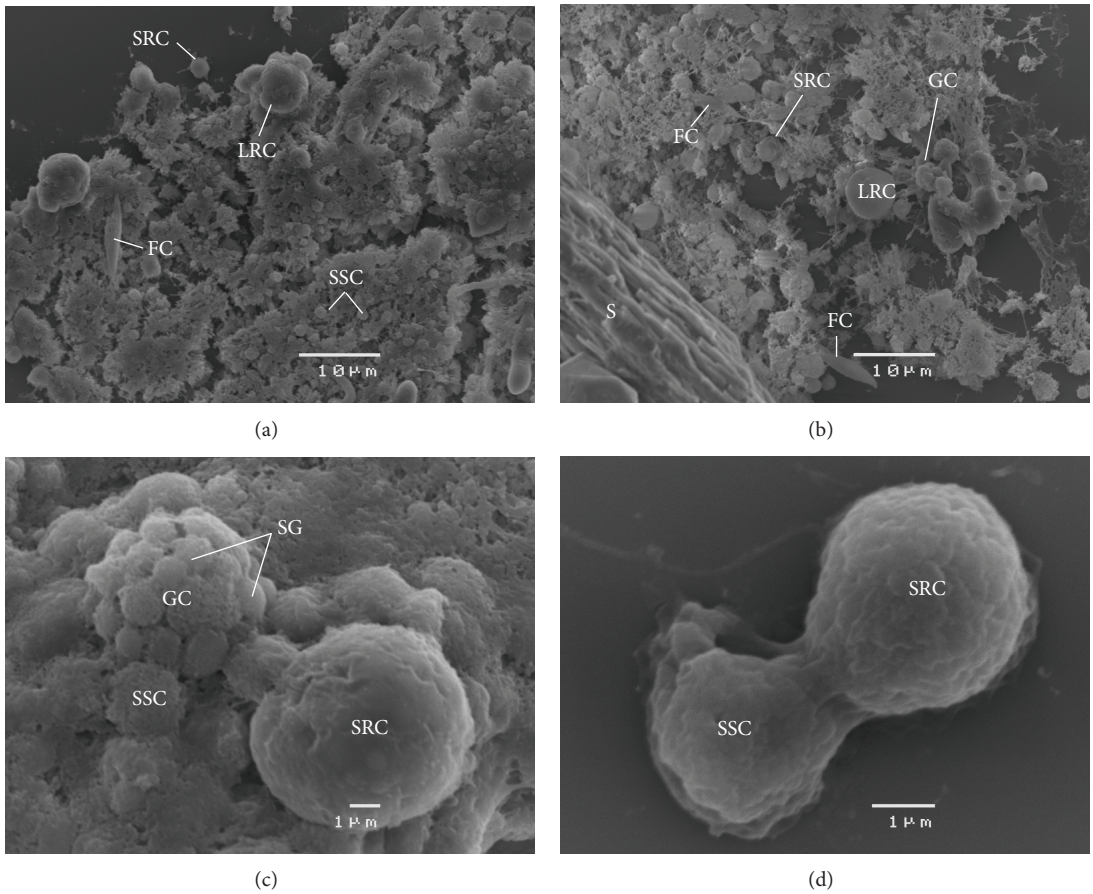


Figure 6: *Acanthaster planci*. Scanning electron micrographs of clusters formed 6 hours after injection. Fusiform cell (FC), granulocyte (GC), large rounded cell (LRC), salt crystal (S), secretory granule (SG), small rounded cell (SRC), and small spherule cell (SSC).

levels of sodium chloride in seawater which coupled with the fact that osmoconformers such as *A. planci* do not tolerate drastic changes in osmolarity [51, 52] could explain why small amounts of bile salts can induce death in injected *A. planci*.

Several indicators show that bacteria have a very limited role in the rapid mortality of *A. planci* following injections of low concentrations of bile.

(1) All individuals injected with oxbile died irrespective of the administration of antibiotics, which clearly indicates that bacteria were not the etiological agent inducing disease.

(2) There was no evidence of marked increases in the densities of pathogenic organisms in tissues of *A. planci* that died following injection of oxbile. In addition, during passage through the gastrointestinal (GI) tract, ingested bacteria that could naturally grow in *A. planci* decomposing tissues will face several challenges such as the acidic environment of the stomach, elevated osmolarity, oxygen starvation, nutrient competition, the immune response, and exposure to a number of different potentially toxic compounds such as bile and degradative enzymes [53].

(3) Previous studies showed that *A. planci* killed by injection of bile salts did not induce a transmissible bacterial disease, as corals, fish, and echinoderms that were exposed to and/or fed on diseased and dead *A. planci* did not develop disease after 12 days of exposure [5].

(4) Fish can cope with the ingestion of tissues containing traces of bile as vertebrates naturally produce bile to help the absorption of lipids from their diets.

(5) The quantity of oxbile used to control *A. planci* is remarkably low as each sea star is injected with only 0.04–0.06 mg of oxbile (10 mL at 4 $g \cdot L^{-1}$ and 6 $g \cdot L^{-1}$) which is naturally degraded in the environment. It is also important to consider the exceptional dilution and self-cleaning properties of the ocean.

(6) The coelomic fluid of individuals injected with bile was very different to that of naturally diseased individuals. Coelomic fluid of *A. planci* infected with bacteria was white (turbid); meanwhile coelomic fluid collected from sea stars after oxbile injection was orange.

The attempt to identify circulating coelomocytes highlights the lack of information about the immune system of

A. planci. Analogies between cells described in this study and cells described in the literature were found. The large hyaline cells described in this study are similar to the characterization of phagocytes described by Boolootian and Giese [16]. However, more studies are required as the role of the small hyaline cell is unclear and the cell evokes similarities to different types of cells reported elsewhere. The small hyaline cells are very similar to the progenitor cells described by Chia and Xing [18], as the cells were small in size, with short cytoplasmic extensions and a very dense nucleus [54]. Nonetheless they also resembled the small phagocytes identified in the purple sea urchin *Strongylocentrotus purpuratus* [55]. Similarly, the colourless spherule cells corresponded to the description given in the literature of cells also known as colourless morula [56, 57]. Their role has not been clearly identified. However, Arizza et al. [58] demonstrated their ability to degranulate calcium dependent cytotoxic substances in cooperation with phagocytes.

Fusiform cells were never reported in *A. planci* before, possibly due to their low occurrence as observed in other species [54] and the lack of information about *A. planci*. The presence of fusiform cells is not recurrent in other asteroids and has been suggested to increase with repetitive handling of the individual [55]. The presence of fusiform cells has often been associated with crystal cells [15, 54]; however crystal cells were not found in this study. The impossibility to detect crystal cells in this study could be related to the tendency of this type of cells to dissolve under slight osmotic changes or the fact that they are recognisable only when the hyaline cytoplasm is orientated at a specific angle [54, 59].

The main cell involved in the immune response after bile injections was the red spherule cell, also known as morula, small pigment cell, amoebocyte, or granulocyte [19, 20, 57]. They were present in high numbers at the surface of the clusters formed after injection. Red spherule cells contain a red pigment, echinochrome A, which show similarities to histamine, displayed antibacterial properties, and triggers the inflammatory response as part of the immune response to foreign pathogens. Boolootian and Boolootián [15] mentioned that the red spherules in *A. planci* were darker than the red spherules observed in other echinoderms, suggesting a high concentration of these compounds in the secretory granules of red cells. As the red spherule cells are the only cells responsible for the development of the yellow/red colouration observed after immune challenges [60], it is clear that the increasing red colouration of the coelomic fluid after injection of bile was due to the number of circulating red spherules arising, probably coupled with an important degranulation in the vicinity of the bile compounds. The substantial increase of antibacterial compounds could have led to an adverse immune response, a phenomenon observed in the sand dollar *Mellita quinquiesperforata* [61]. Indeed, they described a process of sensitization associated with an important release of antibacterial compounds, similar to the sensitization triggered by the histamine in vertebrates, suggesting that this mechanism is a precursor to the vertebrate allergic reactions.

Moreover, the introduction of contaminants in asteroids, such as heavy metals, has been shown to significantly increase the phagocytic activity and the production of reactive oxygen species (ROS) [62, 63]. The presence of high concentrations of antibacterial compounds and highly reactive metabolites in the coelomic fluid could have led to a severe adverse effect against the host with a strong reactivity at high energetic cost [64, 65]. This coupled with the fact that bile induces cell membrane and mitochondria damage leading to osmotic shock allows us to conclude that oxbile injections induced a strong immune reaction that triggers a marked increase in coelomocytes in association with direct tissue damage. The toxic reaction triggered by the injection of oxbile could then be considered as the main reason for the mortality of *A. planci*.

The injection of low concentrations of bile salts represents a clear improvement to current best practice which inject each sea star up to 12 times with sodium bisulfate at 140 $g \cdot L^{-1}$ [4]. This study contributes to a better understanding of the mechanisms involved in the induction of disease after injections of bile and gives clear insights into the non transmission of disease to other marine organisms. After injection, the low amount of bile was distributed in *A. planci* tissues triggering an immune response. High amounts of immune cells (mainly red spherules) were rapidly released in an effort to fight the insult. Syncytia-like structures were observed as one of the main mechanisms used to engulf bile salts in an attempt to eliminate foreign particles from their body [21–23]. Importantly, as a consequence of these findings, once bile salts are expelled or exposed to the marine environment after *A. planci* dies, the bile salts undergo rapid transformation and degradation through natural processes [65]. Indeed, many naturally occurring marine bacteria have been found that have the capacity to metabolise and degrade bile salts present in the digestive tracts of marine organisms, thus limiting toxicity and the ecological impacts in the environment [65–67].

Oxbile is a rapid and more efficient method to control *A. planci* outbreaks but its application requires the implementation of safe procedures and further studies. Large-scale field test is required to quantify the potential risk associated with mass mortalities. Densities of crown-of-thorns sea star (*Acanthaster planci*) can reach up to 156,650 sea stars per hectare [4] and the frequency and intensity of *A. planci* outbreaks are increasing [3, 68]. Thus it is highly probable to find massive aggregations of *A. planci* in the near future. In addition the new single injection control method with oxbile will increase operational efficiency tenfold. Therefore to achieve reliability, precise information about the effects of oxbile and large amounts of decomposing sea star tissue on marine organisms in the field is recommended. Previous studies showed that oxbile did not induce disease in animals exposed to diseased and dead *A. planci* [5]. However, the effects of massive amounts of decomposing sea star tissue in the reef have not been studied. Organisms in close contact with dead tissue are exposed to infectious hazards; that is, where dead bodies have contaminated water supplies, gastroenteritis has been the most notable problem in humans [69]. Thus during large scale control efforts the large amounts of death sea star could pose a risk for people fishing or

swimming in the area and certainly could have a negative effect on already stressed marine organisms such as corals. Feeding injuries, bleaching, cyclones, high water temperature, acidification, sediments, and other stressors can greatly affect corals immune defense response. Information of what species are most at risk and what precautions should be taken, that is, maximum number of *A. planci* that can be safely killed in a reef with no risks to induce disease in other reef organisms or humans living in areas subject to control efforts, should be considered.

Finally it is paramount to consider that marine systems differ from terrestrial environments with respect to the types of control agents available, the degree of pest-population reduction needed for effective control, the spatial scale over which biological control must operate effectively, the practicality of implementation, and the nature and degree of concern over safety as there is no boundaries that can limit the dispersal of an infectious disease. Once there is a bloom of a particular disease it can be easily disseminated by ocean currents. One of the best examples is the catastrophic mass mortality of *Diadema antillarum* in the Caribbean. Most populations across the Caribbean were eventually affected by this spreading disease, which traveled in a pattern consistent with major surface currents. The disease spread at a rate of roughly 2000 km/yr to the east and nearly 3000 km/yr to the west [70]. Eventually, the entire Caribbean was involved, 3.5 million square kilometers of ocean habitat were affected, and no population of *D. antillarum* escaped massive mortality.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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