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Bile salts and the single-shot lethal injection method for killing crown-of-thorns sea stars (*Acanthaster planci*)

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ABSTRACT

Given the threat posed by population outbreaks of *Acanthaster planci* to coral reefs throughout the Indo-Pacific, significant investment is being made to reduce the number of sea stars and their effects on coral assemblages, both through ongoing direct control programs and indirectly, through targeted improvements in water quality and fisheries management. In Australia, bile salts have recently replaced sodium bisulfate as the chemical used to inject, and thereby quickly and efficiently kill, individual sea stars. This study reports on results of experimental studies conducted prior operationalizing bile salts for widespread use on Australia's Great Barrier Reef, both to optimize doses of bile salts and further examine potential side-effects of administering low doses of bile salts into individual sea stars when found at high concentrations. This study showed that injecting *A. planci* with 10 mL of 8 g l^{-1} Bile Salts No. 3 or 12 g l^{-1} of Oxgall solution into the base of the arm with a new gun adapted with a 16 Gauge x1/2" needle is the most rapid and effective way to kill individual *A. planci*, which were up to 42 cm in diameter. No immediate flow-on effects on reef fish, corals, and other benthic invertebrates were observed in laboratory experiments and field surveys. Efficient control measures using bile derivatives can offer immediate relief from ongoing COTS predation, and when done in conjunction with improved land use practices that reduce nutrient input and establishment of protected areas to protect predator species, can offer benefits for the resilience of reef ecosystems.

1. Introduction

Population outbreaks of the crown-of-thorns sea star (COTS), *Acanthaster planci*, remain one of the major causes of coral loss and habitat degradation on coral reefs throughout the Indo-Pacific (Grand et al., 2014). On Australia's Great Barrier Reef (GBR), for example, outbreaks of *A. planci* are reported to be one of the major contributors to sustained and ongoing declines in live coral cover (De'ath et al., 2012). There are also renewed and ongoing outbreaks of COTS on many other reefs throughout the Indo-Pacific (Rivera and Pratchett, 2012), which are causing widespread and often very significant levels of coral loss. Despite significant investment in addressing both declining water quality and over-fishing, effective management of COTS outbreaks is limited by equivocal understanding of the proximal causes of outbreaks in different times and places (Pratchett et al., 2014); given uncertainty about the proximal causes of outbreaks, the most immediate solution (if only a stop gap measure) is to directly control outbreak populations, through hand collections of individual sea stars or *in situ* injections of toxic substances. The feasibility and effectiveness of large-scale (e.g., reef-wide) control programs has been continually questioned (e.g., Kenchington and Pearson, 1982) because it not clear that measures required to effectively protect small patches of reefs can be achieved simply by scaling up effort (e.g., number of diver hours) in proportion to reef area. There remain however; concerted efforts to kill and/or collect COTS in many locations throughout the Indo-Pacific (Pratchett et al., 2014). Logically, the quicker and the more COTS are killed in a given reef with an outbreak population, the fewer corals will be damaged (Birkeland and Lucas, 1990) and there will be reduced likelihood of successful fertilization once

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aggregations are broken up (Cheney, 1973; Bos et al., 2013). As a consequence of interrupting persistent COTS predation on corals, remnant patches can contribute to recovery via growth and sexual reproduction (Colgan, 1987). Thus, even minor improvements in efficiency and effectiveness of these programs could yield significant ecological and economic benefits.

The most widely used chemical for injecting in to COTS is sodium bisulfate (Rivera et al., 2012). However, when using sodium bisulfate, each sea star has to be extracted from the reef matrix and then injected multiple times. Significant increases in efficiency could therefore, be achieved simply by using a chemical that could be administered with a single dose and anywhere on the sea star. Rivera et al. (2012) demonstrated that single injections of low concentrations of bile derivatives, particularly Oxgall and Bile Salts No. 3 induced rapid mortality in *A. planci* (mostly within 24 h) in the Philippines and Guam. Bile is a digestive mixture produced by all vertebrates that aids in the digestion of lipids and it is composed of fatty acids, bile acids, inorganic salts, sulfates, bile pigments, cholesterol, mucin, lecithin, glycuronicacids, porphyrins, and urea (Murray et al., 1995). Oxgall and Bile Salts No. 3 are derivatives of bile collected from bovines or ovines after they have been slaughtered. Oxgall (Difco®) is bile in its simplest form, comprising natural dehydrated fresh bile directly extracted from the bovine gall bladder. On the other hand Bile Salts No. 3 (Oxoid®) is a more refined mixture of sodium cholate and sodium deoxycholate that is prepared especially for use in MacConkey Agar and Violet Red Bile Agar. Bile Salts No. 3 is reported to be effective at less than one-third of the concentration of Oxgall. The main difference between these two products is that Oxbile N3 undergo a refining process that remove lipids and reduce the pigments in the bile, thus making it a useful component of selective broths (Oxoid, 2014). Successful trials with the aforementioned products were conducted in the Philippines (Rivera-Posada et al., 2013), and have shown to have little negative effect on coral reef organisms. Being a novel substance to control *A. planci*, further trials must be conducted in order to confirm the viability of this solution as a widespread control method as there are inherent differences in size, physical conditions, nutritional status, age, parasitism, etc between populations across the Indo-Pacific.

The purpose of this study was to test the feasibility of bile derivatives to be used as single-shot lethal injection method for killing crown-of-thorns sea stars (*A. planci*) on Australia's Great Barrier Reef. The specific aims of this study were to: (1) Determine the lethal doses of oxbile and oxgall solutions for the *A. planci* on the Great Barrier Reef, which are generally larger than those used in previous studies (e.g., Philippines, Rivera et al., 2013) and compare the rate of mortality (time until death) in *A. planci* injected in different parts of the body and with different concentrations of Bile Salts and Oxgall; (2) test the efficiency of injection guns used to administer bile solution in *A. planci*; (3) assess possible flow-on effects of injected COTS on fish, corals, and other echinoderms; (4) compare the efficacy of bile and dry acid solutions in field conditions; and (5) monitor immediate flow-on effects on fish that approach or bite injected *A. planci* in the field and assess the health of coral species in close proximity to injected sea stars.

2. Materials and methods

2.1. A. planci collection and maintenance conditions

The study was conducted at Lizard Island (14°40′S, 145°27′E), northern GBR, Australia. A total of 220 sea stars, ranging in size from 30 to 42 cm diameter were collected from back reef environments at Lizard Island. Specimens were immediately transported to the Lizard Island Research Station and kept in large holding tanks (2.7 m × 1.6 m × 0.5 m) with constant flow of ambient seawater (mean temperature = 26 °C, salinity = 33 ppt, pH = 8.3). All sea stars were left to acclimatize for 3 days. Weak or injured individuals were discarded.

2.2. Bile derivatives tested

Two types of bile derivatives were used for tank experiments to determine which solution to use in transmission experiments and field tests: (1) Oxgall (Difco®), which is a purified and dehydrated form of fresh bovine bile, and (2) Bile Salts No. 3 (Oxoid®), which is a refined fraction of bile acid salts widely used as a selective inhibitory agent in culture media. Two stock solutions at different concentrations were prepared for each bile derivative: (1) Oxgall at 6 g l^{-1} and 12 g l^{-1}, and Bile Salts No. 3 at 4 g l^{-1} and 8 g l^{-1}. Four g l^{-1} of Bile Salts No. 3 and 6 g l^{-1} of Oxgall were the minimum concentrations of each substance known to induce 100% mortality based on previous tank experiments conducted in the Philippines, albeit with much smaller sea stars (ca. 15–22 cm diameter) (Rivera-Posada et al., 2013). Due to the larger size of COTS used in this study and a marked delay in the time to death (>40 h), higher concentrations (8 g l^{-1} Bile Salts No. 3 and 12 g l^{-1} of Oxgall) were also tested. To prepare the solutions, the aforementioned amounts were added to 1 L of distilled water in a flask and stirred at room temperature until the powder was completely dissolved. The flasks were covered in aluminum foil and stored at room temperature before use.

To prepare an 8 g l^{-1} solution of Bile Salts No. 3 for field application, 4 L of distilled water was added to the 5-l plastic bottle, which attaches to the injection gun. Bile Salts No. 3 powder (32 g) was then poured into the bottle through a dry funnel. Appropriate eye protection and safety masks were used to handle the dry powder, following manufacturer's safety instructions. The cap was screwed on the bottle and then shaken vigorously for 30 s until the powder is dissolved. It is possible to use tap water or fresh seawater instead of distilled water, but any naturally occurring bacteria in the water could break down bile and make it less potent. Lead weights were also placed inside the bladders to prevent floating when contents are spent.

2.3. Tank experiment 1 – bile derivative concentration and site of injection

A total of 50 *A. planci* distributed in 10 groups (combination of bile derivative concentration and site of injection treatments) of 5 sea stars were used in these tank experiments. COTS were placed in individual 68-l plastic containers with flow-through seawater at ambient conditions. Injections of 10 ml of each solution (initially, 4 g l^{-1} of Bile Salts No. 3 and 6 g l^{-1} of Oxgall) were administered using a plastic syringe with an 18-gauge needle. Sea stars were injected in (1) the distal portion of the arm, (2) the middle of the arm, (3) the proximal portion of the arm, and (4) the central disk (Fig. 1). *A. planci* used in the double dose treatments were all injected in the central disk. Two separate measures of the effectiveness are considered in this study: i) the time until death (in hours), recorded as the time from injection until all podia (tube feet) were completely immobile (Rivera-Posada et al., 2011), and ii) the proportion of sea stars that actually died with 2–3 days.

2.4. Tank experiment 2 – effectiveness of injection guns

A total of 12 *A. planci* distributed in three groups of 4 sea stars were used for this experiment. Each *A. planci* was injected with 10 mL of 8 g l^{-1} Bile Salts No. 3 and time to death was estimated. Hyperactivity shortly after injection was used as an indicator that the sea star was correctly injected. Three different types of injection

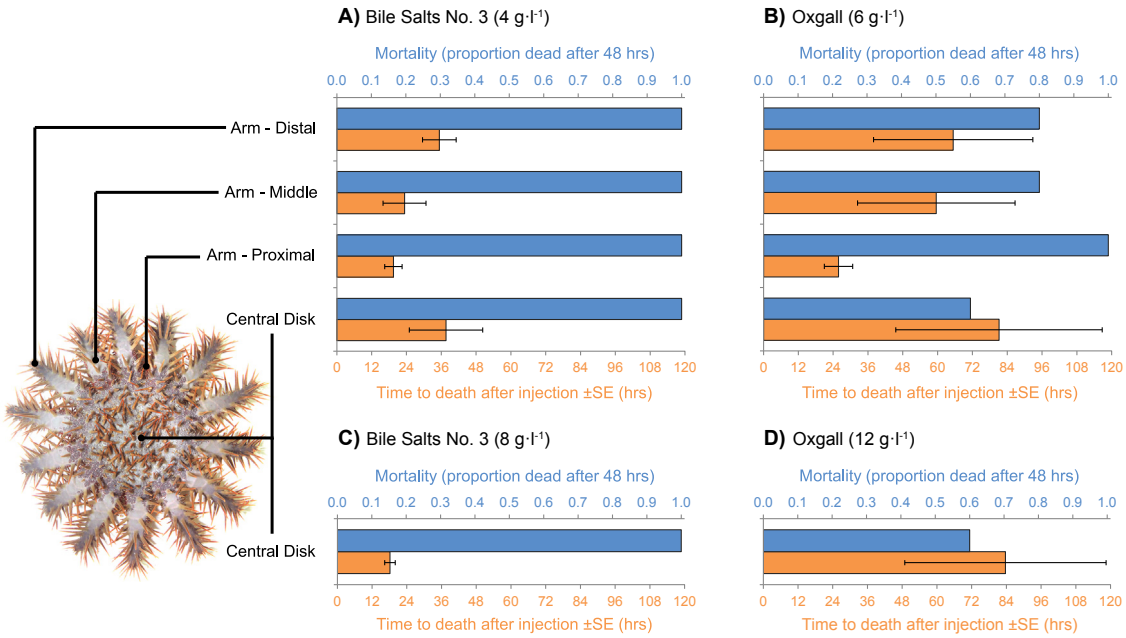

Fig. 1. Mortality rates (blue) and mean time to death after injection (orange) of: (A) 4 g l^{-1} bile salts, (B) 6 g l^{-1} oxgall, (C) 8 g l^{-1} bile salts, and (D) 12 g l^{-1} oxgall at different sites of injection. ($n = 5$ for each treatment).

guns were tested (Fig. 2): (1) DuPont™ Velpar® Spotgun®, (2) Simcro™ STV 12-ml Plastic Syringe, and (3) prototype metal injection gun. The DuPont™ Velpar® Spotgun® was fitted with a 50-cm needle, 4-mm tip, and 5-L plastic bladder, which is currently used in the field to inject sodium bisulfate (dry acid) solution. Although this gun provides good reach to cryptic *A. planci*, the width of the tip creates large holes, which raises concerns that chemicals injected could easily leak out of these openings without any effect or without killing the sea star. It is important to note that this gun was originally designed to spray herbicides and not to inject *A. planci*. Simcro™ STV 12-ml Plastic Syringe is cheap, lightweight, requires minimal maintenance, and offers the possibility to attach any size and length of syringe needle. This gun has been successfully used in *A. planci* control efforts around Japan (Kuroshio Biological Research Foundation, 2011). A prototype metal injection gun with a 50-cm spear and Luer-lock to attach a 16 Ga × 1/2″ needle was designed for more accurate injections of small amounts of solution (Fig. 2, inset). A thinner and shorter needle was used to minimize the puncture size and leakage after injection and to avoid overshooting (tip of needle exits the sea star arm and solution is not injected internally) during injection, as what usually happens with longer needles.

2.5. Tank experiment 3 – transmission experiment

Fish, corals, and other echinoderms (Table 1) were collected from back reef habitats around Lizard Island. Smaller fishes (i.e. Pomacentridae, Chaetodontidae) were collected using clove oil,

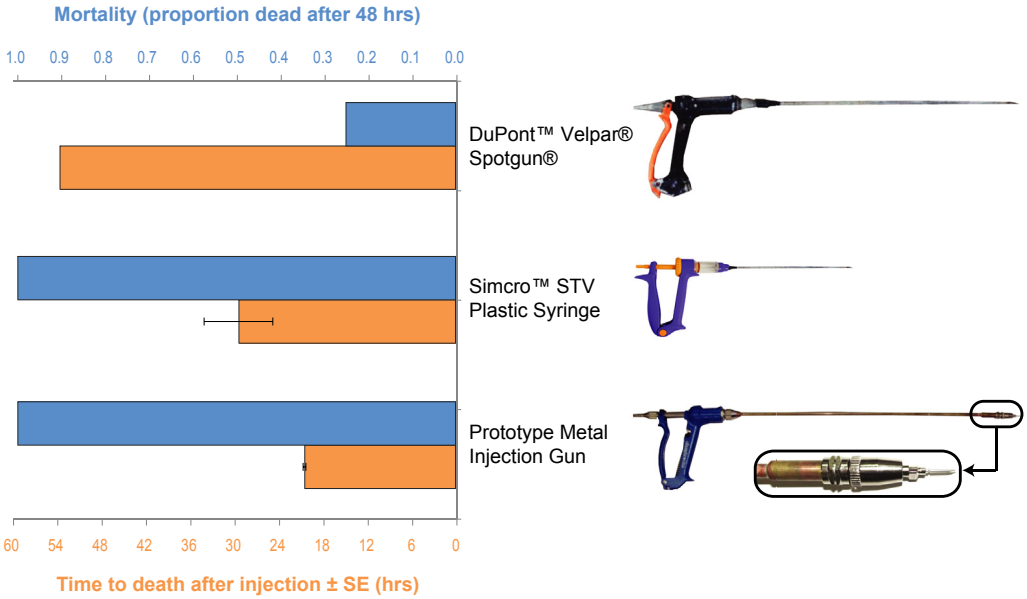

Fig. 2. Comparative efficiency (time to death after injection) of guns used to deliver 10 ml of bile salts solution (8 g l^{-1}) into the proximal region of *A. planci* arms.

which is noteworthy because clove of its hepatotoxic properties (Javahery et al., 2012), which could make these fishes even more susceptible to toxic effects of oxbile. All organisms were distributed in three 1.5-m diameter (300 l) circular tanks, with flow-through coarse-filtered seawater and constant aeration. After an acclimation period of two days, these animals (listed in Table 1) were directly exposed to *A. planci* (ca. 30 cm diameter) that were injected with 10 ml of Bile Salts No. 3 solution (8 g l^{-1}) to assess flow-on effects. Another *A. planci* was injected and placed in the tank with the other organisms on the fourth day when the other sea star have been completely consumed or decomposed. All injected sea stars remained stationary for the most part and none were observed to feed on corals. All activities of mobile organisms in the tanks (COTS movement and decomposition, biting and consumption of remains by fishes and invertebrates, and interspecific interactions) were monitored using a GoPro® Hero3 HD video camera with a full view of the entire tank for a total of 4 h each day. Once all digestive glands, reproductive organs and connective tissue were consumed from the dead bodies, *A. planci* skeleton and spines were siphoned out of the tanks. The organisms in the control tank were not exposed to *A. planci* (see Table 2).

2.6. Field test – efficacy of solutions and immediate flow-on effects

Two adjacent patch reefs across the LIRS, with an area of less than 100 m^2 each and separated by a stretch of sand, were selected to separately test the efficacy of bile salts (LIRS Reef 001) and dry acid (LIRS Reef 002) injections (Fig. 3A). To simulate outbreak densities on these small reef patches, 50 *A. planci*, collected from nearby reefs the previous day, were placed on each patch and allowed 1 h to re-orient and disperse prior to the commencement of the field trial. *A. planci* control divers from the Association of Marine Park Tourism Operators (AMPTO) were SCUBA diving to inject *A. planci* while free-diving snorkelers helped locate the sea stars. AMPTO divers administered one 10 ml injection of 8 g l^{-1} solution of Bile Salts No. 3 into the base of the arm of each sea star using the prototype metal injection gun. Out of the 50 sea stars dropped on LIRS Reef 001, 47 were accounted for and injected in less than 12 min. *A. planci* on LIRS Reef 002 were injected using the DuPont™ Velpar® Spotgun®. Each sea star was injected 6–15 times with 10-ml doses of sodium bisulfate at 140 g l^{-1}. All 50 *A. planci* were easily located but injections took over 35 min. Moreover, the 4-l sodium bisulfate solution in the bladder was completely spent after injecting about 35 individuals. Three hours after all injections, GoPro® Hero3 HD video cameras were placed on each reef at strategic locations to monitor the activity of injected *A. planci* and its interactions with other organisms in the vicinity. Aggregations of decomposing sea stars were individually marked using bright-colored flag tapes. Mortality rates and decomposition rates were recorded. Cameras were changed twice daily (0800 and 1600 h) for four days. Further video monitoring was conducted once every week for one month. Three replicate permanent transects (10 × 1 m) on each reef patch were also established within the immediate vicinity of decomposing *A. planci*. These transects were very small relative to normal sampling protocols for coral reef fishes, but this was sufficient given the very small area of impact – all decomposing *A. planci* were within an area measuring approximately 10 m × 6 m. Injected *A. planci* were mostly hyperactive up to an hour after injection, but subsequently remain stationary prior to death. A video recording (distance of 0.5 m from the substrate) of the entire length of each permanent transect was done on day 1, day 7, and day 14 to monitor fish and macro-invertebrate populations. In addition, 20 colonies of branching corals (*Acropora*, *Pocillopora*, *Seriatopora* and *Stylophora*) were individually tagged and then photographed at regular intervals (every 1–5 days) to test for any new incidences of coral disease. These colonies were located at distances of 0–4 m from the *A. planci* aggregations.

Table 1
Pooled mortality and injury rates in fish, coral, and echinoderm species that were not exposed to *COTS* (Control) and those that were exposed to *COTS* injected with bile salts at 8 g l^{-1} (Treatment).

Taxonomic groups		Species	Treatment	Control
Fishes	Chaetodontidae	*Chaetodon auriga*	0/6	0/3
	Pomacentridae	*Chromis viridis*	0/18	0/9
		Pomacentrus moluccensis	0/2	0/1
		Abudefduf whitleyi	0/2	0/1
	Balistidae	*Balistoides viridescens*	0/5	0/1
	Tetraodontidae	*Arothron hispidus*	0/2	0/1
		Arothron manilensis	0/4	0/3
Corals	Acroporidae	*Acropora spathulata*	0/2	0/1
		Acropora nasuta	0/2	0/1
		Acropora sarmentosa	0/2	0/1
		Acropora intermedia	0/2	0/1
	Pocilliporidae	*Pocillopora damicornis*	0/4	0/1
		Stylophora pistillata	0/2	0/1
	Fungidae	*Fungia fungites*	0/2	0/2
	Faviidae	*Goniastrea retiformes*	0/2	0/2
	Poritidae	*Porites lutea*	0/2	0/1
Echinoderms	Asteroidea	*Linckia laevigata*[a]	1/4	1/2
		Nardoa novaecaledoniae	0/2	0/1
		Echinaster luzonicus	0/4	0/2
	Echinoidea	*Echinometra mathaei*[b]	4/4	2/2
	Holothuroidea	*Stichopus chloronotus*	0/4	0/2
		Holothuria atra	0/4	0/2

[a] Injured.
[b] Dead.

Table 2
List of fish species approaching* and feeding on *COTS* remains injected with bile salts (LIRS Reef 001) and dry acid (LIRS Reef 002).

Family	Species	Bile salts	Dry acid
Lethrinidae	*Lethrinus atkinsoni*	1	0
	Lethrinus nebulosus	0	1
Nemipteridae	*Scolopsis bilineatus*	0	13
Mullidae	*Parupeneus multifasciatus*	0	11
Chaetodontidae	*Chaetodon aureofasciatus*	0	1
	Chaetodon auriga	4	2
	Chaetodon plebeius	0	1
	Chaetodon rafflesi	1	0
	Chaetodon rainfordi	1	0
	Chaetodon vagabundus	1	0
Pomacentridae	*Chromis viridis**	0	0
	Chrysiptera sp.*	0	0
	N. oxyodon,	1	0
	Neoglyphidodon melas	1	0
	Pomacentrus chrysurus	17	4
	Pomacentrus moluccensis	0	2
	Pomacentrus wardi	1	0
	Stegastes nigricans	2	1
Labridae	*Cheilinus trilobatus**	0	0
	Coris caudimacula	2	11
	*Labroides dimidiatus**	0	0
	Thalassoma lunare	46	107
	Thalassoma nigrofasciatum	2	1
	Unknown sp.	6	0
Balistidae	*Balistapus undulates*	0	2
Tetraodontidae	*Arothron hispidus*	0	1
Total number of species		14	14
Total number of individuals biting/consuming starfish		86	158

* Means approaching but not directly observed feeding on COTS remains.

2.7. Statistical analyses

The main parameters analyzed are mortality (proportion of individuals dead after 48 h) and time until death after injection

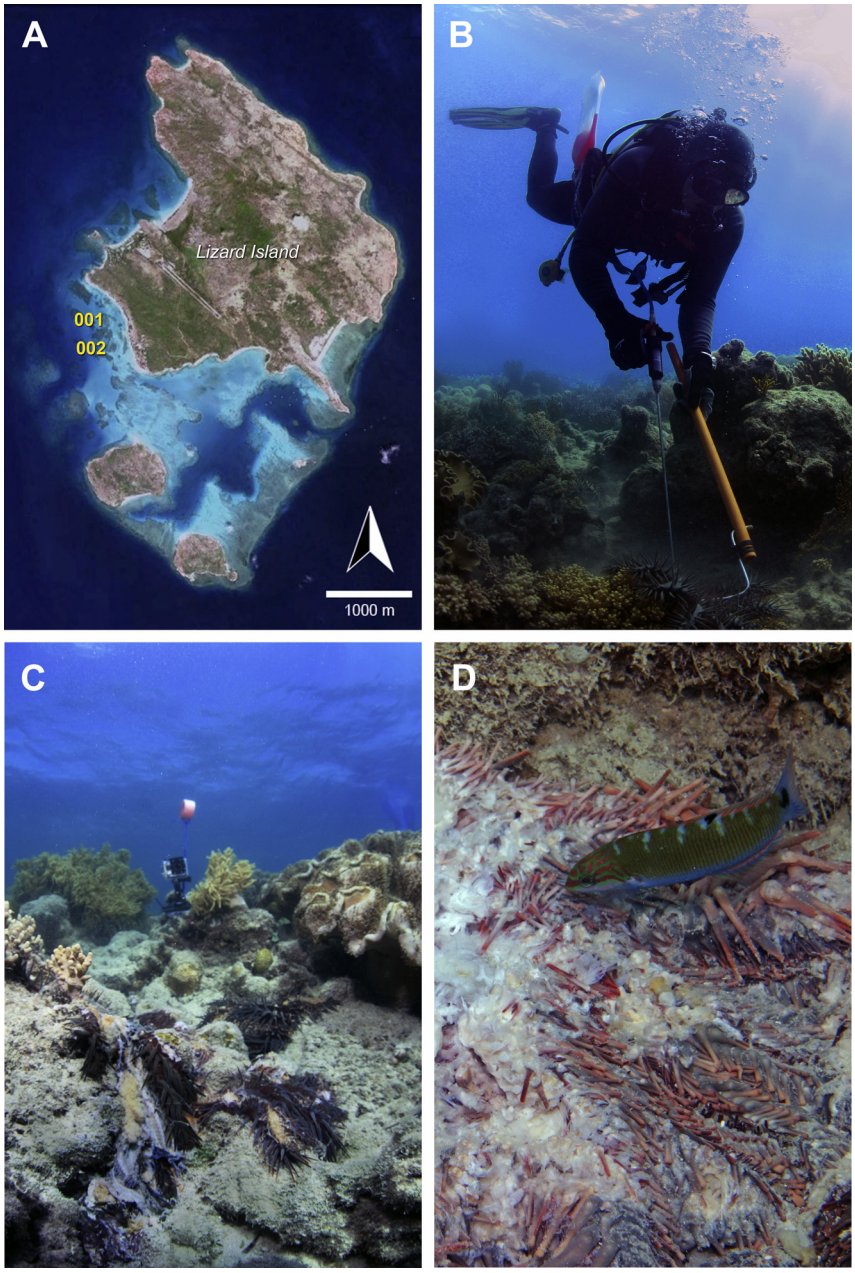

Fig. 3. Field tests of control methods: (A) Site location; (B) Injection of test solutions by AMPTO team; (C) Injected *A. planci* monitored using GoPro® cameras; (D) Crescent wrasse, *Thalassoma lunare*, feeding on COTS remains.

(Table S1). These are important factors in assessing the efficiency of any method of killing COTS and its feasibility as a control measure. Differences in mortality (proportion dead after 48 h) between bile derivatives, dosage, and sites of injection were compared using Fisher's Exact Test (Table S2; Sokal and Rohlf, 1995). Time until death in COTS injected with bile salts and oxgall at different concentrations (Table S3) were also analyzed by performing a two-way Scheirer-Ray-Hare extension of the Kruskal–Wallis test (Table S4; Sokal and Rohlf, 1995). Only those injected in the central disk was included in this analysis because double dose treatments were only injected on this part of the seastar. Variation in time to death after injection between the two bile derivatives tested and the four sites of injection (Table S5) were analyzed using Two-way (Model II) ANOVA, with both parameters as random factors (Table S6; Sokal and Rohlf, 1995). COTS that recovered and survived after the 7-day observation period was assigned a time of 168 h in order have equal sample sizes for each treatment. Data were log-transformed prior to analysis to satisfy assumptions of normality and homogeneity of variance.

3. Results and discussion

3.1. Optimal doses of bile derivatives

All COTS injected with Bile Salts No. 3 experienced 100% mortality regardless of the concentration and site of injection (Fig. 1A, C). This was significantly higher compared to sea stars injected with Oxgall (Fig. 1B, D), which only experienced 80% mortality after 48 h ($p = 0.022$, Table S2). There was no significant increase in mortality even when concentrations of bile derivatives

were doubled ($p = 1.000$, Table S2). Among the COTS injected in the central disk (Table S3), those injected with Bile Salts No. 3 (27.90 ± 6.84) died more rapidly ($H_{1,16} = 4.117$, $p = 0.042$; Table S4) compared to those injected with Oxgall (82.42 ± 23.46). There was no significant difference in time until death between the two concentrations of bile derivatives tested ($H_{1,16} = 0.099$, $p = 0.753$; Table S4). Overall, six out of the 25 sea stars injected with Oxgall initially exhibited signs of the effects of bile injections (i.e. loss of turgor and localized lesions at the site of injection) within the first 24 h, but eventually recovered after 7 days of observation. Among the COTS injected at different sites with a single dose (Fig. 1A, B; Table S5), Bile Salts No. 3 (28.95 ± 4.08 h) resulted in significantly more rapid death after injection compared to Oxgall (57.98 ± 12.95) ($F_{1,32} = 21.609$, $p = 0.019$; Table S6). Even when Oxgall concentrations were doubled, proportion of dead COTS after 48 h remained at 60% (Fig. 1D). The differences observed between Oxgall and Bile Salts No. 3 may be due to the composition of these derivatives. Bile Salts No. 3 is composed of sodium cholate and sodium deoxycholate, which are known detergents that lyse cell membranes after contact (Rolo et al., 2004). Bile Salts No. 3 undergoes a refining process that removes lipids and reduces the pigments in the bile, thus making it a useful component of selective broths and has higher potency even at lower concentrations (Oxoid, 2014).

3.2. Site of injection

There was no significant difference in mortality of COTS injected at different sites ($p = 0.891$; Fig. 1, Table S2). The highest proportion of dead COTS after 48 h (100%) was achieved by injecting COTS in the proximal region of the arm where digestive and reproductive glands are situated (Fig. 1A, B). Mortality rates were lowest (60%) when COTS were injected in the central disk with oxgall at 6 g l^{-1} and 12 g l^{-1} (Fig. 1B, D). Mortality of sea stars injected in the central disk mainly depends on which organ the tip of the syringe needle hits upon injection. Chemicals can be easily discharged by the sea star if injected in the cardiac stomach or near the mouth. Time to death was also most rapid in COTS that were injected in the base of the arm (22.68 ± 2.91 h) and slowest in sea stars injected in the central disk (59.39 ± 19.22 h), however these differences were not statistically significant ($F_{1,32} = 7.511$, $p = 0.066$; Table S6).

3.3. Injection gun

The newly developed hybrid gun was the most consistent and effective of all three-injection guns, killing all COTS in 20.49 ± 0.18 h (Fig. 2). The Simcro® plastic syringe was also effective, killing all sea stars in 29.45 ± 4.66. However, the long needle fitted to this gun can overshoot during injection and release solutions outside the sea star's body. The classic metal DuPont™ Velpar® Spotgun® only killed one individual, which lasted 53.78 h before dying. This finding confirmed that the large holes created by the traditional spray gun allow chemicals to leak back into the ocean and it is one of the causes of high rates of COTS survival during control efforts.

A. planci injected with 4 g l^{-1} Bile Salts No. 3 experienced 100% mortality regardless of where the sea star was injected (Fig. 1A, C). In contrast, sea stars injected with 6 g l^{-1} Oxgall (Fig. 1B) only experienced 80% mortality ($FET_{1,\ N=40}$; $p = 0.053$). While there was a significant difference between Bile Salts No. 3 and Oxgall in the overall proportion of sea stars that died within 2–3 days ($FET_{1,\ N=50}$; $p = 0.011$) the concentration of these chemicals had no apparent effect on the proportional mortality of injected sea stars (Fig. 1C, D; $FET_{1,\ N=10}$; $p = 0.053$). Six out of 25 Sea stars that were injected with oxgall initially exhibited signs of the effects of bile injections (i.e. loss of turgor and localized lesions at the site of injection) within the first 24 h, but ultimately recovered after 7 days of observation. Even when Oxgall concentrations were doubled, mortality rates were at 60% (3 out of 5).

The time until death was significantly affected by both the substance used and the dose ($F_{1,32} = 4.335$, $p = 0.045$; Table 3); COTS injected with oxbile N3 died in 28.95 h ± 4.08SE compared to 57.98 h ± 12.95SE for those injected with Oxgall. Time until death was also substantially reduced by doubling the dose of each of the bile derivatives: At 8 g l^{-1} of oxbile N3, all *A. planci* died within 24 h (Fig. 1C), whereas at 4 g l^{-1}, some individuals persisted for over 48 h (Fig. 1A). The differences observed between oxgall and oxbile N3 could be related with the fact that oxbile N3 is composed of sodium cholate and sodium deoxycholate that are two well known detergents that lyse cell membranes after contact.

3.4. Tank test

After 8 days of exposure to dead *A. planci* injected with the higher concentration of Bile Salts No. 3 (8 g l^{-1}), and despite complete consumption of sea stars remains, none of the fishes, or corals exhibited any signs of ill-health. The remains of the sea stars were consumed mainly by the pufferfishes (*Arothron* spp.), but also triggerfishes (*Balistoides viridescens*), butterflyfish (*Chaetodon auriga*) and damselfishes (*Pomacentrus moluccensis*). Most notably, each individual pufferfish (*Arothron* spp.) consumed up to 0.9 *A. planci* during the course of this experiment. Oxbile contained within the tissues of dead and dying *A. planci* is likely to be readily decomposed by free-living marine bacteria (Maneerat et al., 2005), thereby reducing the amount of bile ingested by fishes, especially when feeding on the remains of sea stars that have been dead for hours to days.

There were no adverse effects on behavior or health following substantial ingestion of *A. planci* killed using Bile Salts No. 3 at 8 g l^{-1}. These findings support observations made during similar trials conducted in the Philippines (Rivera-Posada et al., 2013). However, the fishes used in the current experiment (especially, pufferfishes and triggerfishes) were generally smaller than those caught in the Philippines (using fish cages), such that any toxic effect from ingestion of oxbile would be expected to have been even more apparent.

Among the echinoderms, there were high levels of injury and mortality recorded across both control and treatment tanks, for the

Table 3
Cost-benefit comparison of popularly-used solutions in *A. planci* control programs.

Solution	Average mortality rate (h)	Lethal concentration (%v/v or g l^{-1})	Total Volume per individual (ml)	Price range (AUD)	Number of injections per individual
Bile salts[a]	24	8 g l^{-1}	10	$80 kilo	1
Dry acid[b]	16	140 g l^{-1}	80–150	$15 kilo	8–15
Acetic acid 99%[c]	72–108	15%	10–15	$26 l^{-1}	6

[a] This study.
[b] Lassig, 1995.
[c] Yamamoto and Otsuka, 2013.

blue sea star, *Linckia laevigata* and the burrowing urchin, *Echinometra mathaei* (Table 1). These injuries were inflicted by triggerfish (*B. viridescens*) in the tank, which are known to be one of the most important predators on sea urchins on the Great Barrier Reef (Young and Belwood, 2012). Triggerfish also consumed all *E. mathaei* in both control and treatment tanks within 48 h of their introduction. For *L. laevigata*, deep lesions on the tips and side on the arms were seen from Day 5, but video monitoring revealed these were caused by feeding on these sea stars by both *B. viridescens* and *Arothron manilensis.*

3.5. Field-based test

The small-scale field trial at Lizard Island enabled direct comparisons of the efficacy of bile salts versus sodium bisulfate, when each injected into approx.50 sea stars arranged in very close proximity. One apparent benefit of the sodium bisulfate method, was that it was immediately obvious if and when a sea star had been injected; not only where the large number of injection sites (up to 30 per sea star) administered using the large bore, spraying tip was immediately obvious, but the sea star did not move after they had been treated. In contrast, *A. planci* injected once with 10 ml of Bile Salts No. 3 solution administered with the hybrid gun were extremely mobile immediately after the injection, and the site of the injection was barely visible. Rapid initial movement of *A. planci* injected with oxbile (for up to 1 h) was also recorded in aquaria, and appears to be an immediate reaction to oxbile (Rivera-Posada et al., 2012, 2013). In the field, injected sea stars travelled 1–2 m and sought shelter within the reef matrix.

All *A. planci* (47/47 injected with bile salts, and 50/50 injected with sodium bisulfate) died within 24 h of being injected, but the sea stars injected with sodium bisulfate tended to decompose much more quickly than those injected with bile. By day 4 there was little evidence of any dead *A. planci* on the patch reef where we used bile salts and sodium bisulfate, except for small piles of spines and skeletal elements. Given the rapid decomposition of sea stars injected with bile salts, we suggest that any residual oxbile is likely to be rapidly broken down by free-living marine bacteria.

Observed differences in the rate of decomposition was not only attributable to the predation on the dead and dying *A. planci*; rates of predation (mostly by pufferfishes and butterflyfishes) were higher for sea stars injected with sodium bisulfate, compared to bile salts. In particular, there was one very large pufferfish (*Arothron hispidus*) on the reef where we used sodium bisulfate that was seen to eat entire arms from a dying sea star in a single bite. On the nearby reef where oxbile was tested, there were both pufferfishes (*Arothron* spp.) and triggerfishes (*B. viridescens*) present, but no direct feeding on the dead and dying sea stars were recorded during 6 h of video recording each day from Day 0 to Day 4. However, several species of butterflyfishes and damselfishes were recorded picking at the remains, mostly from Day 2 to Day 4. Glynn (1984) suggested that exposure of internal organs can considerably increase the likelihood of attacks by a broader array of predators or scavengers and reported that internal tissues of *A. planci* were acceptable as food to fishes even if it is not part of their ordinary diet. Finally, there were no incidences of coral disease or partial mortality recorded on individually tagged coral colonies within the following month after the injections.

4. Conclusions

Oxbile provides a relatively effective medium to control *A. planci,* requiring only a single injection, preferably at the base of one arm. At 8 g l^{-1} of Bile Salts No. 3 (Oxoid®), *A. planci* die rapidly regardless of the site of injection, though it is possible that when injected into the oral disk, the sea star can rapidly expel the oxbile through the stomach and mouth. Thus, *A. planci* should be injected at the base of an arm in the polian vesicle area were the coelomic fluid is stored. Bile salts disrupt cell membranes and induce osmotic shock through their detergent action (Rolo et al., 2004). Thus, injection of oxbile in this area will ensure a rapid distribution of the solution throughout the sea star and will affect directly the organ in charge of maintaining hydrostatic pressure (Lawrence, 2001). The resulting death of *A. planci* is caused by cell membrane and mitochondria damage (by creation of channels) coupled with a dramatic immune response to the tissue damaged caused by bile salts (Rivera-Posada et al., 2011; Grand et al., 2014).

The benefit of this new method was extremely apparent following the first field trial, whereby divers from the Association of Marine Park Tourism Operators (AMPTO), killed *A. planci* at a rate of 5–6 sea stars per minute using single injections of bile salts, compared to just 1 sea star per minute with sodium bisulfate. Moreover, there was no flow-on effects of this chemical, even among fishes (*Arothron* spp.) that consume large quantities of *A. planci* remains following injection of higher doses of bile salts, either in aquaria or in the field. Given rapid mortality and no apparent increase in concentrations of bacteria among tissues of sea stars killed using oxbile, the risk of direct transmission of disease (e.g., to corals) appears very minimal. Similarly, the risk of toxicity from excess oxbile consumption by organisms that consume *A. planci* remains (e.g., *Arothron* spp.) is very low, especially among vertebrates that naturally produce and can readily excrete bile. In addition, the low quantity of bile (0.08 mg per sea star) used to control *A. planci* (Table 3) will be rapidly degraded by marine bacteria that use bile as energy source (Maneerat et al., 2005; Bode, 2012). Moreover, sodium cholate and sodium deoxycholate which are the main ingredients of oxbile N3 are used as feed supplements in the prawn, shrimp, fish and poultry industries. Bile acids and cholesterol are precursors of sex hormones, adrenal cortex hormones, and skin-shedding hormones in crustaceans and are routinely added to prawn feeds for this purpose. Bile acids are also potent olfactory stimulants to several fish species and improve fat utilisation and promote growth (NZP, 2014).

The next step is to undertake a much larger-scale field trial, where several thousands of *A. planci* will be injected with 10 ml Bile salt No 3 (Oxoid ®) solution at 8 g l^{-1} within the confines of a single isolated reef. The purpose of this is primarily to test whether there are likely to be any flow-on effects for other reef organisms, due to either i) the large quantity of bile salts solution that will be used within a relatively localized area (e.g., any evidence ill-health among the diverse range of organisms that may consume *A. planci* remains) or ii) the sheer quantity and biomass of dead an dying sea stars that will result from improvements in the efficiency of the control method.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ocecoaman.2014.08.014.

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