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NOTE

Evaluation of *in vitro* treatments against the causative agent of *Diadema antillarum* scuticociliatosis (DaSc)

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ABSTRACT: In the 1980s, a mass die-off of the long-spined sea urchin *Diadema antillarum* occurred on Florida and Caribbean coral reefs. *D. antillarum* populations largely did not recover, and in 2022, remaining populations experienced another mass mortality event. A ciliate most similar to *Philaster apodigitiformis* was identified as the causative agent of the 2022 event, which was named *D. antillarum* scuticociliatosis (DaSc). Here, we investigated possible treatments for this pathogen. We tested the efficacy of 10 compounds at final concentrations of 100, 50, 25, 12.5, 6.25, and 3.13 µM, or a 10-fold serial dilution series, against ciliates cultured from an infected *D. antillarum* specimen. Of the tested compounds, 8 induced 100% ciliate mortality at some dose after 24 h. The most effective (defined as those requiring the lowest dose to induce 100% ciliate mortality) were quinacrine and tomatine (both effective at 12.5 µM), followed by furaltadone and plumbagin (25 µM), bithionol sulfoxide and 2′4′ dihydroxychalcone (50 µM), and oxyclozanide and carnidazole (100 µM). Toltrazuril and a commercially available anticiliate product containing naphthoquinones were not effective at any dose tested. Shortened (15 min) time trials were performed using ciliate cultures reared in natural seawater to better reflect natural environmental conditions, and revealed that 2 of the compounds (quinacrine and tomatine) induced 100% ciliate mortality at 100 µM, with tomatine also effective at 50 µM. This study identified several treatments effective against the causative agent of DaSc *in vitro*, but their toxicity and utility *in vivo* remain unknown.

KEY WORDS: Anticiliate · Sea urchin · Ciliate infection · *Diadema antillarum* · Treatment · Parasite · Mass mortality

1. INTRODUCTION

In 1983 and 1984, the long-spined sea urchin *Diadema antillarum* experienced an unprecedented mass die-off event on Florida and Caribbean coral reefs (Lessios et al. 1984), the cause of which has never been established. Impacted sites experienced 93% to 100% declines in *D. antillarum* abundance, with little recovery in the ensuing decades (Weil et al. 2005, Lessios 2016, Tuohy et al. 2020). The loss of this keystone species ultimately contributed to the continued decline of reefs throughout the region as macroalgal abundance increased in response to reduced grazing pressure (Lessios 2016). Several aquaculture facilities have active *D. antillarum* rearing programs (e.g. Pilnick et al. 2021), and efforts to repopulate reefs with lab-raised individuals have observed significant beneficial declines in algae as a result (Williams

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2022), further underscoring the ecological benefits of this important grazer.

In 2022, another mass mortality affected the remaining Florida and Caribbean *D. antillarum* populations (Hylkema et al. 2023, Levitan et al. 2023), but unlike in the 1980s, the causative agent of this die-off was identified as a scuticociliate most similar to *Philaster apodigitiformis*, based on nucleotide matches of 99% and 92% within the 28S and 18S rRNA genes, respectively, and 99% within the internal transcribed spacer 2 (ITS2) (for additional details regarding the identification of *P. apodigitigormis* as the causative agent, see Hewson et al. 2023). Additionally, in some *ex situ* aquaculture facilities, populations of *D. antillarum* were impacted due to the use of natural seawater within the system (Hylkema et al. 2023). A secondary mortality event in populations of the congeneric sea urchin *D. setosum* in the Sea of Oman has also been linked to the same ciliate, indicating that *P. apodigitiformis* is spreading rapidly across the globe (Ritchie et al. 2024). Given the widespread nature and severity of these events, and the potential broad-reaching impacts for coral reef health, identifying treatments effective against this pathogen that could be employed in response to future outbreaks is of paramount importance. In this Note, we provide the results of experiments testing several compounds previously identified as effective against marine pathogens of the family Philasteridae (Iglesias et al. 2002, Sueiro et al. 2022) or other marine ciliates against cultures of the ciliate responsible for the 2022 die-off now known as *D. antillarum* scuticociliatosis (DaSc).

2. MATERIALS AND METHODS

The ciliates used in this experiment were originally cultivated from coelomic fluid of an infected *Diadema antillarum* specimen (specimen ID: FWC2) collected from a reef in Key Largo, FL, USA, as previously described by Hewson et al. (2023). Subcultures were generated in 15 ml tubes using 500 µl of the original xenic culture, amended to 5 ml of 0.22 µm-filtered, autoclaved artificial seawater (ASW), 20 µl of yeast extract (0.1 g ml^{-1}), 250 µl of 0.2 µm-filtered *D. antillarum* tissue (0.1 g ml^{-1} ASW), and one sterile (autoclaved) rice grain. The ASW recipe consisted of 1 l of deionized water with 20.8 g NaCL, 0.56 g KCl, 4.8 g $MgSO_4 \cdot 7H_2O$, 4.0 g $MgCl_2 \cdot 6H_2O$, 0.01 g potassium phosphate dibasic, 0.001 g $FeSO_4 \cdot 7H_2O$, 2.0 g Fluval Sea Salts, and 0.48 g Tris Base $C_4H_{11}NO_3$, adjusted to pH 8.0 with HCl. Subculture tubes were incubated for 11 d at ~21°C with loosened caps to provide air flow. After this time, all subcultures were vortexed, combined, and then quantified using a Zeiss Discover.V12 stereomicroscope. Visualizations of the combined culture revealed ~1 ciliate per µl, suggesting cultures were either in log or early stationary growth stages (Hewson et al. 2023), and 900 µl (i.e. ~900 ciliates) of the combined culture was added to each well of a polypropylene 1 ml 96-well plate.

We tested 9 compounds with established efficacy against parasitic ciliates of the family Philasteridae (Iglesias et al. 2002, Sueiro et al. 2022): 2′4′ dihydroxychalcone, bithionol sulfoxide, carnidazole, furaltadone, plumbagin, oxyclozanide, quinacrine, tomatine, and toltrazuril. All compounds were dissolved in dimethyl sulfoxide (DMSO) or sterile deionized water (DI; quinacrine only) to create 20 mM stocks. Carnidazole, sourced in pill form, was crushed using a mortar and pestle prior to dissolution. All stocks were then diluted in sterile ASW to generate 1 mM working stocks, which were further diluted with ASW to final concentrations of 100, 50, 25, 12.5, 6.25, and 3.13 µM. We also tested one commercially available anticiliate product (Ich Attack; Kordon) containing naphthoquinones marketed as a treatment for the marine ciliate *Cryptocaryon irritans* in aquaria. This compound was tested at full strength and a 10-fold serial dilution series (1:10, 1:100, 1:1000, 1:10 000, 1:100 000) in ASW. Each well of the polypropylene 96-well plate received 100 µl of a treatment at a trial concentration. Controls included 1000 µl ciliate culture, and 900 µl culture plus either 100 µl ASW or 100 µl ASW containing 5% DMSO or DI (i.e. DMSO or DI diluted to the highest concentration used in the compound trials). All treatment/concentration combinations and controls were trialed in duplicate. Following addition of the treatment, each well was mixed several times with a pipette to ensure even distribution of ciliates and treatment, then the plate was covered with an adhesive plastic sheet and incubated at ~21°C. After 24 and 48 h, 30 µl samples were drawn from each well and visualized in a 1 ml glass Sedgewick Rafter counting chamber under the Zeiss stereomicroscope. The treatment was considered effective at a given concentration if 100% mortality (no moving ciliates) was observed in samples immediately upon transfer to the microscope slide, within either of the duplicate wells for that treatment.

Because lengthier exposure times presumably could result in greater toxic effects or stress in treated sea urchins (e.g. Hassan et al. 2023), we also needed to assess the efficacy of treatments under shorter exposure times. Therefore, we next

trialed all compounds identified as effective within the 24 h for shorter (15 min) exposure periods. Additionally, Sueiro et al. (2022) determined that the efficacy of anticiliate compounds changed in response to the culture media of the ciliate, and treatments used in the field would likely be used in natural seawater. Consequently, these 15 min trials were conducted using ciliate cultures reared in natural seawater that had been filtered to remove endemic ciliate populations, instead of ASW. These cultures also contained reduced nutrient quantities: 10 µl yeast extract, 100 µl filtered *Diadema* extract, and one autoclaved rice grain. Using the 1 mM stocks, 10 µl of each compound was added to 90 µl of ciliate culture on the Sedgwick Rafter slide chamber (final compound concentration of 100 µM). The compounds were mixed with the culture using a pipette, and then the ciliates were immediately observed for 15 min using a Meiji Techno EMZ-13 microscope. Treatments were considered effective if all ciliates within the sample ceased all movement within the 15 min window, including exhibiting no response upon stimulus (i.e. manual movement of the cover slip). All compounds identified as effective within 15 min at 100 µM were then tested at 50 µM concentrations, and if still effective, again at 25 µM concentrations. Treatments identified as ineffective at inducing 100% ciliate mortality after 24 h (i.e. toltrazuril, Kordon Ich Attack, and controls) were not trialed in the 15 min exposures.

3. RESULTS

After 24 h, 8 out of 10 treatments induced 100% mortality in the ciliate samples at some dose (Table 1). The most effective were quinacrine and tomatine (both effective at 12.5 µM), followed by furaltadone and plumbagin (25 µM), bithionol sulfoxide and 2'4' dihydroxychalcone (50 µM), and oxyclozanide and carnidazole (100 µM). After 48 h, furaltadone also was lethal at 12.5 µM. Two treatments, toltrazuril and the commercially available anticiliate product containing naphthoquinones, were ineffective against the ciliate (i.e. full mortality of the sample was not observed) at all doses trialed. None of the control treatments induced 100% ciliate mortality within 48 h.

In the 15 min time trials, at 100 µM concentrations, 2 out of 8 treatments induced 100% mortality in the ciliate cultures reared in natural seawater: quinacrine and tomatine. Other treatments appeared to inhibit ciliate activity and possibly induce some mortality within 30 min. Bithionol sulfoxide, for example, induced a near-immediate response in dampening ciliate activity, with ciliates slowing within the first few minutes of application, but did not induce full mortality of the culture within 15 min. Quinacrine and tomatine were additionally trialed at 50 µM concentrations, with only tomatine proving effective in 15 min. Tomatine was subsequently trialed at 25 µM, but was not effective in 15 min at this concentration. The results of all *in vitro* trials are available in a U.S. Geological Survey data release (Evans et al. 2023).

Table 1. Effectiveness of different doses of anticiliate treatments at 24 and 48 h post-inoculation. Treatments were trialed at 100, 50, 25, 12.5, 6.25, and 3.13 µM concentrations, or a 10-fold serial dilution series (the commercially available anticiliate product containing naphthoquinones). X indicates a compound was effective at that concentration (i.e. 100% mortality [no moving ciliates] was observed) within 24 h, (X) indicates efficacy within 48 h, and — indicates 100% mortality was not observed within the 48 h observation window. Controls included unamended culture (Culture), culture + artificial seawater (ASW), culture + dimethyl sulfoxide (DMSO), and culture + deionized water (DI H_2O)

Treatment	Concentration (µM)					
	100	50	25	12.5	6.25	3.13
Bithionol sulfoxide	X	X	—	—	—	—
Carnidazole	X	—	—	—	—	—
2'4' Dihydroxychalcone	X	X	—	—	—	—
Furaltadone	X	X	X	(X)	—	—
Oxyclozanide	X	—	—	—	—	—
Plumbagin	X	X	X	—	—	—
Quinacrine	X	X	X	X	—	—
Toltrazuril	—	—	—	—	—	—
Tomatine	X	X	X	X	—	—
Commercially available anticiliate product containing naphthoquinones	Full strength	1:10 dilution	1:100 dilution	1:1000 dilution	1:10 000 dilution	1:100 000 dilution
	—	—	—	—	—	—
Controls	Culture	ASW	DMSO	DI H_2O		
	—	—	—	—		

4. DISCUSSION

Here, we identified 8 treatments effective *in vitro* against the scuticociliate cause of DaSc within a 24 h window. Currently, the minimum infectious dose needed to induce DaSc is not known. However, because external inoculation with a relatively small number of cells (n = 18–21) induces DaSc in *Diadema antillarum* and leads to sea urchin mortality (Hewson et al. 2023), here we considered any treatments exhibiting anything less than 100% ciliate mortality to have failed, as even one surviving ciliate could potentially still represent a viable means to infection. A 48 h exposure was further trialed to determine whether any of the treatments would become more effective with increased exposure time; however, only furaltadone increased in efficacy (i.e. a lower dose induced 100% ciliate mortality). While 24 h treatments could represent viable options for treating seawater or aquaria systems infected with *Philaster apodigitiformis*, such as those at *D. antillarum* hatcheries and/or aquaculture facilities, or possibly treating abiotic materials such as dive gear that have been exposed to contaminated water, such lengthy exposure times for treated sea urchins could potentially have unintended side effects on the animals themselves. Further, logistically the use of treatments in the field (e.g. removing sea urchins from impacted sites, treating them onboard vessels, and returning them to the reef) would necessitate shortened exposure lengths. Consequently, we also assessed whether any of the compounds identified as effective within 24 h would remain effective under shortened (15 min) time scales. Additionally, to better reflect *in situ* conditions, these 15 min exposures were conducted using ciliate cultures reared in filtered natural seawater. Two of our trialed compounds remained effective under these treatment conditions: quinacrine and tomatine.

Sueiro et al. (2022) tested 26 naturally derived (i.e. from bacteria or plants) compounds against the confamilial ciliate *Philasterides dicentrarchi* and identified plumbagin, tomatine, and 2′4′ dihydroxychalcone as particularly effective. These compounds induced 100% mortality of *P. dicentrarchi* maintained in culture media within 24 h at 100 µM concentrations (Sueiro et al. 2022). Here, we observed that plumbagin, tomatine, and 2′4′ dihydroxychalcone induced full mortality of *P. apodigitiformis* cultures *in vitro* within 24 h at final concentrations of 25, 12.5, and 50 µM, respectively. Sueiro et al. (2022) also determined that in seawater cultures, these 3 compounds were capable of inducing full mortality within 1 h (the shortest time frame assessed), and at low concentrations (30, 20, and 80 µM for plumbagin, tomatine, and 2′4′ dihydroxychalcone, respectively). The lower concentration required to induce 100% mortality at 1 h suggests tomatine was more effective than plumbagin, which was more effective than 2′4′ dihydroxychalcone. We similarly observed that at consistent concentrations of 100 µM, tomatine acted more quickly than plumbagin or 2′4′ dihydroxychalcone, and was additionally effective at 50 µM within 15 min.

Iglesias et al. (2002) challenged *in vitro* cultures of *P. dicentrarchi* with 52 different compounds, identifying 14 that induced 100% mortality within 24 h. We tested 6 of these 14 compounds; however, only quinacrine was effective against the DaSc causative agent during the 15 min trials. One important consideration in developing treatments for DaSc is the purity of the compound. Iglesias et al. (2002) identified a commercially available tablet form of carnidazole (Harkers Spartrix tablets) as effective against philasterine ciliates, even at low doses (Iglesias et al. 2002). We used the same form of carnidazole in our trials, and while the highest dose trialed here (100 µM) was an effective anticiliate against the DaSc *Philaster* sp. within 24 h, lower concentrations of the compound appeared to promote growth of the ciliate. Visualizations of the cultures ~5 d following initial inoculation revealed large numbers of highly active ciliates, amplified in quantity in comparison to the unamended culture controls. Further, some carnidazole-treated cultures remained alive in this same 1 ml working volume 2 mo from the start of the experiment. Although the mechanism by which this treatment appeared to benefit the ciliate is currently unclear, because the tablets were not pure carnidazole, it seems likely that one of the inactive ingredients of the tablets contributed to sustaining the culture. These findings, though anecdotal, highlight the need to consider compound purity in developing treatments for DaSc, as other excipient compounds present in the treatment may have unintended impacts on ciliates, potentially exacerbating the condition.

Few studies have investigated treatments for sea urchin diseases (Taniguchi et al. 2006, Federico et al. 2023), and none have examined the effects of anticiliate compounds in sea urchins. In order to represent a viable treatment for DaSc, it is also imperative that potential treatments are not toxic to *D. antillarum* or other reef organisms. Sueiro et al. (2022) also exposed epithelioma papulosum cyprini (EPC) cells to their tested compounds to investigate their potential toxicity to fish, and determined that at a concentration capable of inhibiting growth of ciliate cultures by 50%

within 24 h (10.14 µM), tomatine treatment also resulted in 43% EPC cell mortality within 24 h. Tomatine did not exhibit any apparent toxic effects when fed to fish for 1 mo at daily doses of ~97 to ~1900 µM (Friedman et al. 2007); however, *in vitro* exposure tests have observed toxic effects in fish cells exposed to tomatine at 100 and 10 µM (Tedesco et al. 2020). Similarly, some toxicity signs have been observed in fish treated with ~100 µM of quinacrine for 3 h, though in the same study, no toxicity signs were observed in fish treated with ~200 µM of quinacrine for 3 h (Tojo et al. 1994). Those authors hypothesized this apparent discrepancy could be due to an interaction between the effects of the parasitic infection and the compound's inherent toxicity (Tojo et al. 1994), underscoring the possibility that sea urchins exhibiting varying degrees of DaSc severity could exhibit different tolerances to the same treatment.

While many of the treatments identified herein may be effective against *P. apodigitiformis in vitro*, questions remain regarding their *in vivo* toxicity and efficacy in treating infected sea urchins. For example, the ciliate responsible for DaSc penetrates into the tissues of infected sea urchins (Hewson et al. 2023), potentially rendering immersion treatments less effective. The rapid mortality of infected sea urchins following the first appearance of disease signs also raises questions regarding the ability for grossly abnormal sea urchins to recover from the condition. The potential for these identified treatments to confer a preventative benefit to healthy sea urchins that are subsequently exposed to *P. apodigitiformis* also remains unknown.

The best mechanism for administration of these treatments to sea urchins also remains to be determined. Logistically, the use of bath treatments in the field would be complicated, as *in situ* use of these compounds would be subject to a dilution effect, likely rendering them less effective, while *ex situ* dips would necessitate time-intensive collections of live sea urchins from the reef. Prophylactic treatments or injections would be similarly complicated to employ. The inherent risks these compounds may pose to the environment are also unclear and should be investigated before field implementation is attempted. Consequently, focusing treatment efforts on sea urchins in *ex situ* hatchery or aquaculture facilities for use in propagating out to impacted reefs post-DaSc events may be the most prudent treatment approach; however, even in these instances, downstream impacts may exist that are important to consider, such as the potential for the bioaccumulation of these compounds within the predators of *D. antillarum*. Nevertheless, while much remains to be investigated regarding the use of these compounds use in treating the condition in sea urchins, our findings represent a critical first step in our understanding of potential treatments for a debilitating sea urchin disease.

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