Synergistic reduction of a native key herbivore performance by two non-indigenous invasive algae

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ABSTRACT

Native generalist grazers can control the populations of non-indigenous invasive algae (NIIA). Here, it was found that the simultaneous consumption of two co-occurring NIIA, *Caulerpa cylindracea* and *C. taxifolia* var. *distichophylla*, hinders the grazing ability of the main Mediterranean herbivorous, the native sea urchin *Paracentrotus lividus*. The ingestion of any of the two NIIA alone did not produce any difference in sea urchin righting time with respect to usual algal diet. In contrast, the simultaneous consumption of both NIIA, which grow intermingled in nature and are consumed by *P. lividus*, retarded its righting behavior. Such result reveals substantial physiological stress in the sea urchin, which resulted in reduced motility and coordination. The reported findings reveal the potential of NIIA co-occurrence to escape the supposed control exerted by the main native generalist grazer in Mediterranean sublittoral communities, which in turn can be locked in an "invaded" state.

1. Introduction

Biological invasions are progressively becoming one of the main drivers of change in marine coastal communities (Katsanevakis et al., 2013), particularly in areas where intense human activity exerts substantial pressure on nearby marine ecosystems (Halpern et al., 2008). The Mediterranean Sea represents a notable hotspot of biological invasions, possibly due to the faunal impoverishment in geological times (Krijgsman et al., 1999) and exploitation of coastal resources since prehistorical times (Morales-Muñiz and Roselló-Izquierdo, 2008). Intense shipping between the Suez Canal and the Gibraltar Strait is plausibly the main pathway for invaders into the Mediterranean, which accounts for nearly 30% of ship traffic worldwide (Dobler, 2002). Of particular relevance is the rate of invasions from the Indo-Pacific region through the Suez Canal, forecasted to increase in the near future (Katsanevakis et al., 2013). For these reasons, the Mediterranean Sea brings the opportunity to foresee the effects of the growing invasion pressure on the functioning of marine ecosystems elsewhere.

While most non-indigenous species have no noticeable effects on the recipient communities, a small proportion of them exerts a high impact on ecosystems and local economy (Williamson and Fitter, 1996; Boudouresque and Verlaque, 2002). Among these, species triggering ecosystem shifts towards undesirable alternative states are particularly harmful. Positive feedback stabilization can even hinder the reversibility of systems to pristine conditions, raising societal concern for the potential losses in ecosystem functioning and its delivery of goods and services (Pyšek and Richardson, 2010; Katsanevakis et al., 2013; Gaertner et al., 2014).

Among the most noticeable cases of positive feedback are those driven by two non-indigenous invasive algae (NIIA): *Caulerpa taxifolia* (Vahl) Agardh, 1817, and *C. cylindracea* (Sonder, 1845). Both species have prompted large changes in the structure of Mediterranean native communities since their introduction (Williams and Smith, 2007; Piazzi et al., 2016). *C. cylindracea*, a siphonous green alga original from SW Australia, has rapidly spread throughout the Mediterranean basin since its first sight in Libya in 1991, eventually reaching the Canary Islands in the Atlantic (Verlaque et al., 2004). *C. cylindracea* currently dominates different communities along Mediterranean shores from the surface down to 70 m. It grows intermingled with other algal species in its native range but tends to form monospecific, dense stands beyond its

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Fig. 1. *Caulerpa cylindracea* and *C. taxifolia* var. *distichophylla* growing intermingled onto a shallow sabellariid reef at Donnalucata, southern coast of Sicily, Italy.

native range of distribution (Papini et al., 2013). *C. cylindracea* enhances sediment trapping and organic matter accumulation, ultimately making the environment more suitable for itself and other invaders, and prompting the shift from erect macroalgal stands to algal turfs (Bulleri et al., 2010; Bulleri et al., 2016; Piazzi et al., 2016). *C. taxifolia* var. *distichophylla* (Sonder) Verlaque, Huisman & Procaccini, 2013 is also original from Australia and it was recently introduced in the Mediterranean Sea where it is rapidly spreading (see Picciotto et al., 2016 and Bitar et al., 2017 for a complete review of its Mediterranean distribution). In Sicily, it is invasive forming sparse to dense stands onto a variety of substrates including rocky substrates, dead beds (*mattes*) of the seagrass *Posidonia oceanica* (Linnaeus) Delile, 1813 (Musco et al., 2014) and sabellariid reefs (Fig. 1). *C. cylindracea* and *C. taxifolia* var. *distichophylla* growths intermingled in shallow hard substrates (Fig. 1).

The high invasiveness of *Caulerpa* spp. is reported to depend on their biological and functional traits (van Kleunen et al., 2010), i.e. the presence of toxic grazing deterrents, wide bathymetric tolerance, rapid growth, large standing biomass, and facility of dispersion. The distribution of the species over a wide range of environmental conditions points to a weak role of abiotic factors in controlling invading *Caulerpa* species, making biological control particularly relevant (Parker et al., 2006; Piazzi et al., 2016).

Caulerpa cylindracea is selected as a food source by the Mediterranean native sea urchin *Paracentrotus lividus* (Piazzi et al., 2016) despite this alga's high levels of caulerpin, a phenazine derivate exhibiting cytotoxic activity (Schwede et al., 1986) and minor concentrations of caulerpenyne (Box et al., 2010), a sesquiterpenoid quickly converted to reactive aldehydes with oxidative activity that make algal tissues unpalatable to sea urchins (Paul and Puglisi, 2004).

In contrast, *C. taxifolia* var. *distichophylla* contains high levels of caulerpenyne (Cevik et al., 2016) and lacks caulerpine (Schwede et al., 1986).

The positive selection of *C. cylindracea* by *P. lividus*, coupled with the fact that the sea urchin is the main herbivore in Mediterranean rocky sub-tidal communities (Boudouresque et al., 1996), suggested that this native generalist herbivore could provide resistance to colonization of Mediterranean native communities by *C. cylindracea*, as well as limit the biomass of its naturalized populations (Piazzi et al., 2016). Recently, a food selection experiment evidenced positive selection for mixed *C. cylindracea* and *C. taxifolia* var. *distichophylla* by *P. lividus* (Noè et al., 2018). Nevertheless, very high levels of heat shock proteins (HSP) were found in *P. lividus* when fed with both *Caulerpa* species for three days (Gianguzza et al., 2014). Thus, unraveling the effects of the ingestion of the two invading *Caulerpa* species on *P. lividus* is of functional relevance to forecast the role of the native sea urchin in controlling the colonization of Mediterranean native communities by these NIIA.

The aim of the present work is to determine the induction of physiological stress on the native sea urchin *P. lividus* resulting from the consumption of *C. cylindracea* and *C. taxifolia* var. *distichophylla*, elicited from the righting behavior of the sea urchin (Lawrence and Cowell, 1996). It was predicted a non-linear reduction of sea urchin righting performance as result of physiological stress induced by the synergistic toxicity of both NIIA. Such prediction was based on the high production of HSP observed in the sea urchin and the major presence of chemically distinct, toxic metabolites in the two NIIA. In particular, synergism would appear if the consumption of one toxic seaweed exacerbate effects of the other one in the consumer. Otherwise, either additivity or antagonism would occur. Additivity would result from distinct toxic metabolites acting independently, their combined effects being summed on a dose basis. Antagonism would happen whereas the effects of consumption of one *Caulerpa* species diminishes that of the other one in the sea urchin (Berenbaum, 1985; Groten et al., 2001).

2. Materials and methods

Sea urchins were collected from rocky reefs of Ustica Island (Tyrrhenian Sea, Mediterranean Sea, 013° 09′ 09″ E, 38° 41′ 57″ N, Fig. 2) in July 2014 from an area that has never experienced the invasion of the above-mentioned *Caulerpa* species. The collected specimens of *P. lividus* displayed a mean test diameter of 45.78 ± 2.20 [standard deviation, S.D.] mm, ranging from 40 to 50 mm as measured with calipers. Fronds of *C. cylindracea* and *C. taxifolia* var. *distichophylla* were collected from shallow rocks at Donnalucata in Southern Sicily (Sicily Channel, Mediterranean Sea, 014° 38′ 59″ E, 36° 45′ 03″ N, Fig. 2) where these algae are abundant (Musco et al., 2014). The native algae *Dictyopteris membranacea* (Stackhouse) Batters, 1902, was gathered from Capo Gallo (Tyrrhenian Sea, Mediterranean Sea, 013° 16′ 52″ E, 38° 12′ 40″ N, Fig. 2). The collected organisms were transported in aerated tanks to aquaria facilities at the University of Palermo, which were filled with natural seawater. Sea urchins were then placed in the tanks and starved for a week (Lawrence and Cowell, 1996) before the start of the experiment.

Five experimental groups were defined by the algal species provided as food to the sea urchins: (i) Food made of *C. cylindracea* (group CC); (ii) *C. taxifolia* var. *distichophylla* (CD); (iii) intermingled *C. cylindracea* and *C. taxifolia* var. *distichophylla* (CCCD); (iv) control food made of the native *Dictyopteris membranacea* (CO), which is highly palatable to *P. lividus* and regularly found in *P. lividus* gut contents (Privitera et al., 2008); and (v) no food provided (NF) in order to control for manipulation effects. Groups were made of eight *P. lividus*, fed daily (except in the NF group) during a 12-h period with 4 g of blotted wet weight (BWW) of algae. Food remnants of each algal species were removed after feeding, blotted and weighted again. The obtained measures were corrected for autogenic decay (Taylor and Brown, 2006). By the end of the rearing period, which lasted for 11 days, each individual ran one
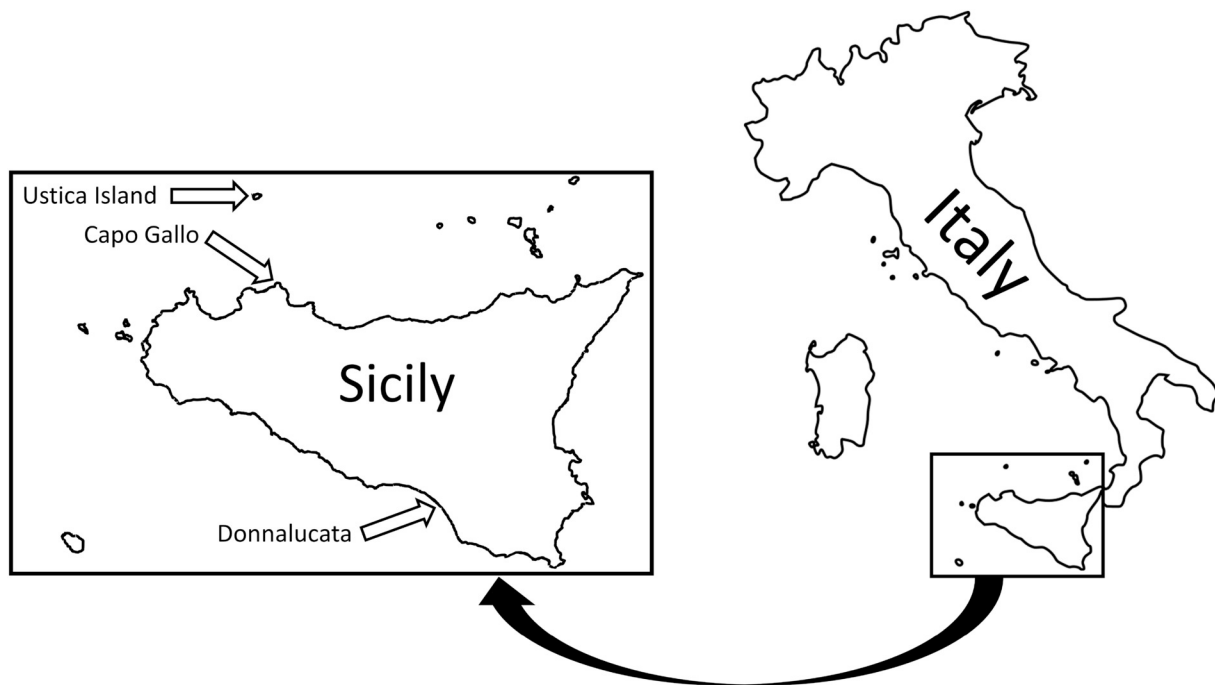
Fig. 2. Study area. Localities from which the organisms were gathered. *Caulerpa cylindracea* and *Caulerpa taxifolia* var. *distichophylla* were taken from Donnalucata; *Dictyopteris membranacea* was retrieved from Capo Gallo; and *Paracentrotus lividus* were catched from Ustica Island. All localities in Sicily, Italy.

single, independent righting trial following Lawrence and Cowell (1996). A total number of 8 trials per experimental group were performed, summing up 40 trials. Righting time (RT, in s), which is directly proportional to the level of physiological stress experienced by the sea urchin (Lawrence and Cowell, 1996), was used as response variable.

An ANOVA was performed on RT after checking for homoscedasticity via Cochran's C test. Since individuals consumed different amounts of algae, the consumed blotted wet weight (BWW) was introduced in the analysis as covariate in order to extract the effect of consuming different quantities of algae. Contrast vectors were coded following Neter et al. (1996) to test for the a priori relevant hypothesis (Ruxton and Beauchamp, 2008) on the effects of the non-native diets with respect to the native one (L_1 to L_3), as well as to control for the experimental manipulation (L_4).

3. Results

Sea urchins simultaneously fed with both NIIA displayed significantly longer RT than those fed with the native algae (L_1, Fig. 3 top, see also video clips 1 "native", and 2 "mix"), while individuals fed with single NIIA did not (L_2 and L_3) (Table 1). Manipulation, e.g. while providing the food and retiring the remnants, had not any effect on RT (L_4) (Table 1). In particular, the simultaneous consumption of both *Caulerpa* species prompted an average RT of 136.30 [mean] ± 74.15 [S.D.] s in the CCCD group, while RT was 81.60 ± 52.04 s in the CO group, 81.65 ± 66.17 s in the CD group, and 67.66 ± 25.60 s in the CC group. Importantly, the quantities of algae ingested by the animals were unevenly distributed across the experimental groups. Considering each single algal species provided, sea urchins consumed more algal BWW in the CC group (32.70 [mean] ± 2.84 [S.D.] g), followed by the CO (16.05 ± 2.47), CD (12.88 ± 2.43) and CCCD (9.80 ± 4.05 and 5.81 ± 1.97 for *C. cylindracea* and *C. taxifolia* var. *distichophylla* respectively) groups (Fig. 3 bottom).

Remarkably, RT in the CCCD group resulted to be about three times higher than that predicted by the additivity criterion, which would result in an estimated value of 50.82 s. This figure was obtained by down-scaling the observed RT for CC and CD groups to the amounts of

C. cylindracea and *C. taxifolia* var. *distichophylla* effectively consumed in group CCCD, and summing up the expected effects of both NIIS on sea urchins. The expected RT value for CCCD is thus a linear combination of scaled values for CC and CD, proportional to the amounts of algae effectively consumed in CCCD:

$$\text{Exp.RT}_{CCCD} = [(\text{BWW}_{CCCD}/\text{BWW}_{CC}) \times \text{RT}_{CC}] + [(\text{BWW}_{CCCD}/\text{BWW}_{CD}) \times \text{RT}_{CD}]$$

4. Discussion

The simultaneous consumption of the two NIIA exerted non-linear physiological stress on the native grazer, as revealed by an average righting time score higher than those of the other groups, which did not differ among them. Indeed, *P. lividus* fed with both *Caulerpa* species spent three times more time to upright than would be expected if the effect of consumption of NIIA would be additive.

The identity and concentration of active substances prompting such physiological stress in sea urchins was unknown. However, it can be safely assumed that, whatever the substance or substances, it was evenly distributed across the experimental groups since each algal species was harvested once at a unique location and time. Thus, differences in the known amount of consumed algae were proportional to those of the unknown doses of active compounds held by each NIIA, allowing for relative comparisons among experimental groups. Potential differences due to the quantities of ingested algae across experimental groups were controlled a posteriori by introducing blotted wet weigh of consumed algae as covariate in the analysis.

Caulerpa cylindracea is known to chiefly contain caulerpin (Schwede et al., 1986) and to a lesser extent caulerpenyne (Box et al., 2010). Both these metabolites are thought to deter several types of herbivores including sea urchins (Box et al., 2010). Conversely, *C. taxifolia* var. *distichophylla* contains mainly caulerpenyne (Cevik et al., 2016), which quickly breaks down into strongly unpalatable oxidizing aldehydes soon after the algae get wounded (Paul and Puglisi, 2004). In contrast to the findings of a multiple food choice experiment lasted 48 h (Noè et al., 2018) sea urchins did not consume more NIIA when offered
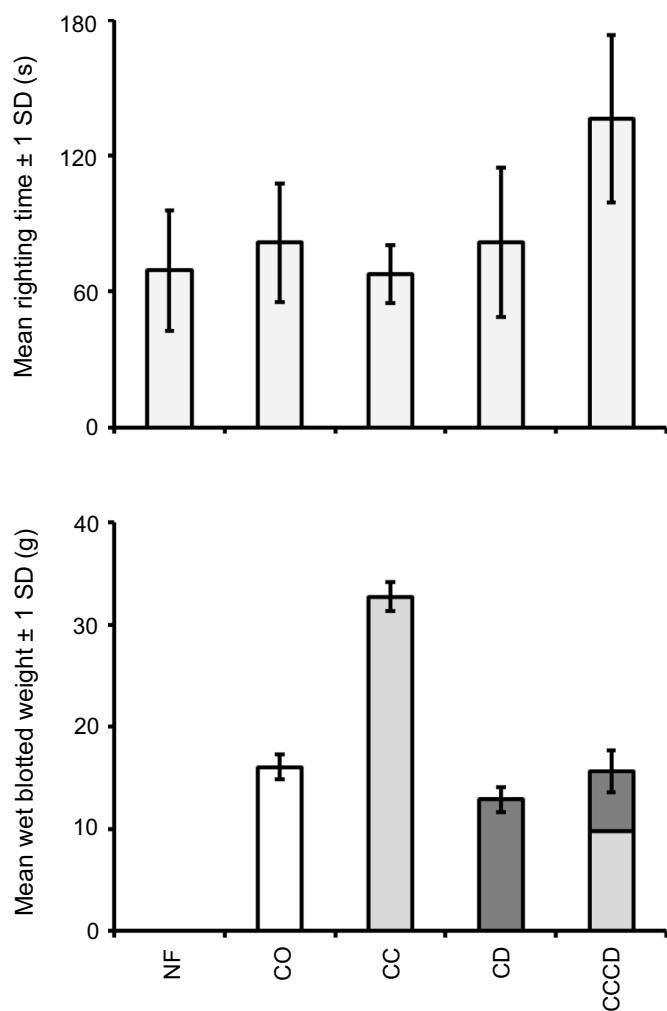
Fig. 3. Top: Average righting time of sea urchins fed with different algae, in seconds; vertical bars correspond to one unit of standard deviation. Bottom: Average amount of consumed algae by sea urchins, expressed as blotted wet weight, in grams; vertical bars correspond to one unit of standard deviation. Labels code for no food (NF); control food made of native *Dictyopteris membranacea* (CO); *Caulerpa cylindracea* (CC); *C. taxifolia* var. *distichophylla* (CD); and intermingled *C. cylindracea* and *C. taxifolia* var. *distichophylla* (CCCD).

Table 1
Summary of the ANOVA on sea urchin righting time, in seconds. *P*-values were computed by 9999 permutations of raw data. The total amount of consumed algae was introduced in the model as covariate, after correction for biomass decay. L_1 to L_3 are planned comparisons testing the relevant hypothesis. L_4 test for the effect of procedural manipulation, i.e. feeding and handling of animals.

Effect	SS	d.f.	MS	F	P
Intercept	10,363.20	1	10,363.20	3.75	0.0613
Total corrected consumption	20.11	1	20.11	0.01	0.9326
Food	25,387.79	4	6,346.95	2.29	0.0794
L_1: CCCD vs. CO	11,985.43	1	11,985.43	4.33	0.0450
L_2: CC vs. CO	316.11	1	316.11	0.11	0.7374
L_3: CD vs. CO	2.24	1	2.24	< 0.01	0.9775
L_4: CO vs. NF	75.69	1	75.69	0.03	0.8696
Error	94,073.75	34	2,766.88		

together than when offered separately, as in the present 11 days lasting study. Here, the strong effect of the NIIA mix on the sea urchin performance indicates that distinct substances held by each seaweed were involved in a synergistic toxicity response (sensu Berenbaum, 1985; Groten et al., 2001). This, in turn, resulted in physiological stress,

reflected as reduction of sea urchin motility and movement synchronization when recovering their normal position after being reversed. Since mobility and maneuverability are important attributes that allow *P. lividus* to browse, reduced motility or synchronization result in effective hindering of grazing upon erect macroalgae by the sea urchin (Agnetta et al., 2013).

At least three alternative mechanisms could produce the observed pattern. First, potentiation would occur if consumption of only one NIIA alone prompts the recorded response in *P. lividus*, while the joint ingestion of the other NIIA produces a stronger effect of the former. Second, coalism would happen if any of the NIIA alone has not any effect on sea urchin performance, but their combined ingestion has and prompts a significant response. Third, cooperation would result if distinct metabolites from different NIIA have wider overall effects on the consumer than those of any single NIIA, because they would target more organs, tissues or cellular pathways (Foucquier and Guedj, 2015). Testing these hypotheses goes beyond the scope of the present study. Undoubtedly, it would require a dose-based approach involving controlled amounts of known biologically active substances. It should possibly include the definition of the exact shapes of the curves modeling the response of sea urchins to each metabolite (Groten et al., 2001; Foucquier and Guedj, 2015). Despite such limitations, the present work fulfills the response additivity criterion for the experimental effect-based approach (Foucquier and Guedj, 2015). It also enables comparison with previously published works dealing with consumption of *Caulerpa* species by *P. lividus* where *C. cylindracea* was largely consumed by *P. lividus* with little, if any, detrimental effect on the sea urchin (Piazzi et al., 2016).

A number of implications follows the above reported findings. First, they highlight the need to consider species identity and their ecological traits in the assessment of invasions (MacDougall and Turkington, 2005; Bulleri et al., 2010; Bulleri et al., 2016), which ultimately points to the inadequacy of assessing the impacts of any single invasive species without taking into account possible associations with other functionally similar species already in place. Second, coupling of the two invading algae has also the potential to expand their niche through a facilitative process, by which the advantage of the reciprocal improvement of protection against grazing would outweigh the disadvantage of competition for resources as space, light, and nutrients (Bruno et al., 2003; Brooker et al., 2008). Third, the present study also supports the idea that interaction among non-indigenous species influences their invading performances (Grosholz, 2005). The synergistic avoidance of grazing obtained by the association of *C. cylindracea* and *C. taxifolia* var. *distichophylla* changes the prospect of invasion scenario for both NIIA, from the perspective of the biotic resistance hypothesis (Parker et al., 2006) to that of the enemy release hypothesis (Keane and Crawley, 2002), thereby highlighting the context-dependency of invasion assessment (Pyšek and Richardson, 2010). Specifically, it was thought that the grazing pressure exerted by *P. lividus* could provide resistance against early invasion of Mediterranean communities by *C. cylindracea* and limit the biomass of its naturalized populations (e.g. Cebrian et al., 2011; Piazzi et al., 2016). However, coupling of *C. cylindracea* and *C. taxifolia* var. *distichophylla* hinders the grazing capacity of *P. lividus* after some days, likely allowing the two invading algae to escape the control exerted by this generalist herbivore following the curtailment of the sea urchin motility and synchronization.

An intriguing, posterior hypothesis is suggested by up-scaling the possibility of escaping herbivorous control to field conditions where NIIA and native algae growth spatially segregated, as it is often the case whereas NIIA occur beyond their native distribution range (e.g. Montefalcone et al., 2007; Papini et al., 2013). In such scenario, browsing would be low or absent in patches made of joint *C. cylindracea* and *C. taxifolia* var. *distichophylla*, while maintained in patches made of native species. The net effect would then be the indirect concentration of grazing pressure over palatable native algae. This, added to the known capacity of *Caulerpa* species to modify the native environment in

their favor, adds evidence of their potential to lock Mediterranean sublittoral rocky shores in an "invaded" state through the stabilization of positive feedbacks. Indeed, it has been experimentally demonstrated that *C. cylindracea* modifies the underlying sediment, making it unsuitable for canopy-forming species (Bulleri et al., 2010; Bulleri et al., 2016; Piazzi et al., 2016). An analogous phenomenon was also described in degraded beds made of the seagrass *Posidonia oceanica*. There, the native *Cymodocea nodosa* and *Caulerpa prolifera* colonized the dead portions of the *P. oceanica* seagrass beds. Both of them were in turn replaced later on by the NIIA *C. taxifolia* and *C. cylindracea*, which "locked" the system through enhanced accumulation of sediment and organic matter (Montefalcone et al., 2007). Indirect concentration of grazing pressure on native algae, and modification of the native environment in own favor, would confer positive feedback stabilization of the ecosystem in an undesired state. For this reason, the co-occurrence of *C. cylindracea* and *C. taxifolia* var. *distichophylla* demands priority in management initiatives (Gaertner et al., 2014). Early detection and timely management actions are essential to avoid potential losses in ecosystem functioning and its delivery of goods and services (Katsanevakis et al., 2013). Several management options should be considered on depending of the status of the NIIA populations, but in general sooner detection and evaluation of the invasion leave room for more management alternatives (Pyšek and Richardson, 2010).

Further efforts should be addressed to the determination of NIIA toxicity based on controlled doses of caulerpin, caulerpenyne, and possibly other compounds. Also, the actual form of the toxicity response curve for each active substance is to be determined. In addition, the mechanisms involved in the toxicity of the invading seaweeds are still unknown. It is worth noting that the present findings involved experimentation on sea urchins from a population that never interacted with the invading seaweeds, but different trends could arise if population phenotype adapts to the exposition to grazing deterrents, e.g. through the expression of genes coding for specific detoxifying pathways. Given the extent of the potential outcomes, areas where different NIIA co-occur should be promptly monitored to early inform decision-making about alternative management interventions.

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Declaration of interests

None.

Author contribution

Contributions: TVF and LM identified the study question and designed the experiment; TVF wrote the draft; FB provided critical insight; TVF, LM, CB, FDT and PG collected the organisms; LM, CB, FDT and PG carried out the experiment; SN provided data. All authors critically revised the draft and approved the present version of this manuscript.

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