Warming may increase the vulnerability of calcareous algae to bioinvasions

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ABSTRACT

Understanding the interactions between various stressors, and the resulting cumulative impacts they exert, is essential in order to predict the potential resilience of marine habitats to climate change. Crustose coralline algae (CCA) are a major calcifying component of marine habitats, from tropical to polar oceans, and play a central role as ecosystem engineers in many rocky reefs. These species are increasingly threatened by the stress of climate change. However, the effects of other stressors linked to global change, such as invasive species, have scarcely been addressed.

We have studied the interactive effects of invasive algae and global warming on CCA, combining observational and experimental approaches. CCA sensitivity to invasive algae is heightened when they are concurrently exposed to elevated seawater temperature, and the interaction between these two stressors triggers drastic synergistic effects on CCA. The reduction and eventual disappearance of these "ecosystem foundation species" may undermine ecological functioning, leading to the disappearance and/or fragmentation of the communities associated with them.

1. Introduction

Ecosystems are experiencing the sustained effects of multiple stressors related to human activities, which have resulted in new or permanently altered systems (Sala et al., 2000; Steffen et al., 2004). Marine ecosystems worldwide are affected by global stressors, including warming, acidification and the spread of invasive species (Harley et al., 2006; Molnar et al., 2008). Knowledge of the interactions between different stressors is essential to our understanding and ability to anticipate the responses of marine habitats. This knowledge is crucial for designing effective management strategies designed to enhance the potential resilience of habitats in the Anthropocene (Crain et al., 2008; Darling and Coté, 2008; Brown et al., 2013; Ban et al., 2014).

In marine environments, crustose coralline algae (CCA) are a major calcifying component of marine habitats from tropical to polar oceans at all depths within the photic zone (Littler et al., 1985; Steneck, 1986; Basso, 2012). They play a key role as "ecosystem foundation species", providing essential habitats (biogenic reefs) for a high diversity of associated organisms as well as favoring settlement and recruitment of numerous species (Kuffner et al., 2006; Nelson, 2009; Basso, 2012). In ad-

dition, due to their extensive distribution, coralline algae are important contributors to the global inorganic carbon budgets in shallow water ecosystems (Mackenzie et al., 2004; Martin and Gatusso, 2009), as they act as carbon sources and sinks (Ballesteros & Canals 1997; Cebrian et al., 2000, Krause-Jensen and Duarte, 2016), representing a substantial, global-scale, blue carbon repository (Mao et al., 2019).

The main effects of climate change-related stressors reported for CCA include the negative effects of global warming on recruitment (Kuffner et al., 2006), growth (Jokiel et al., 2008), calcification (Semesi et al., 2009; Martin and Gatusso, 2009) and necrosis (Diaz-Pulido et al., 2012, Kim et al., 2020). Recently, several authors have pointed out the potential role of invasive turf-forming macroalgae species (dense, small and usually filamentous benthic algae, typically less than 1 cm in height) in triggering impacts on the structure of native turf and erect macroalgae systems (Streftaris and Zenetos, 2006; Katsanevakis et al., 2016). However, the effects of invasive turf-forming macroalgae on CCA have scarcely been addressed.

In the Mediterranean Sea, coralline algae are the main component of emblematic habitats, such as biogenic reefs (coralligenous outcrops) and maërl, which rank among the most diverse habitats in the Mediter-

ranean and play an important role in fisheries and the carbon cycle (Ballesteros, 2006; Zunino et al., 2019). In fact, these habitats are currently protected by the EU Habitat Directive and the 1995 Barcelona Convention. On the other hand, Mediterranean ecosystems are among the most severely affected by the cumulative impacts of human activities. Two of these impacts, sea warming and the introduction of invasive species, increasingly overlap in many locations (Micheli et al., 2013; Kersting et al., 2015). Among such invasive species, *Womerleyella setacea* is a turf-forming species which can become exceedingly abundant, especially in coralligenous habitats, forming thick, persistent carpets (Airoldi et al., 1995; Piazzi & Cinelli 2001; Ballesteros et al. 2006; Battelli and Rindi, 2008) with substantial negative effects on native communities (Piazzi et al., 2002; Serio et al., 2006), benthic assemblages (Piazzi and Balata, 2009; de Caralt et al. 2013), and key structural species (Linares et al., 2012; Cebrian et al., 2012). Furthermore, despite the lack of specific studies on the impacts of positive thermal anomalies and associated marine heat waves on CCA, it has been shown, in Mediterranean waters, that extreme and unusual temperatures result in dramatic effects on many other marine benthic species in the same area (Garrabou et al., 2009; Crisci et al., 2011; Cebrian et al., 2011; Gómez-Gras et al., 2021).

Using the Mediterranean as a model system for ongoing environmental changes, we aimed to assess, for the first time, the interactive effects of invasive algae and global warming on CCA by combining both observational and experimental approaches. To achieve this goal, we first examined the relationship between invasive algae and CCA thallus necrosis of populations already affected by thermal anomalies in natural environments. Second, we experimentally tested the effect of invasive algae overgrowth and thermal stress on CCA, further exploring the biological mechanisms by which invasive algae may impair CCA by comparing it with the effects of an artificial carpet which simulates the direct physical effects – but not the allelochemical interactions – of turf algal cover.

2. Materials and methods

2.1. Field surveys

The field study was carried out in the Scandola Natural Reserve (*Parc Naturel Régional de la Corse*), Corsica, France (42.14°N; 8.37°E), in July 2012. This marine protected area (henceforth, the Scandola MPA) was established in 1975 and covers 1000 ha. It has been recognized by the United Nations as a natural World Heritage Site and was added to the world heritage list in 1983. Due to its location and level of protection, especially in the No-Take area, major stressors, such as fishing, pollution and habitat destruction, have been prevented since 1975. In the Scandola MPA, coralligenous outcrops are one of the most important marine habitats, thriving at depths of between 20 and 35 m. The main contributors to coralligenous formations are crustose coralline algae (CCA) (e.g., *Lithophyllum* cf. *stictiforme, Mesophyllum alternans,* and *Peyssonnelia rosa-marina,* among others).

The invasive species, *Womersleyella setacea* (Hollenberg) R.E. Norris, was first recorded in the Scandola MPA in 1989, although it was probably introduced earlier (Verlaque, 1989). *W. setacea* is an invasive, turf-forming macroalgae, widely distributed throughout the Scandola MPA, where it steadily develops dense and thick turfs throughout the year and reaches high biomass values (ca. 125.75 g DW m^{-2}) at depths between 25 and 35 m (Cebrian and Rodriguez-Prieto, 2012).

In addition, thanks to the Scandola MPA in situ temperature series (www.t-mednet.org), several positive thermal anomalies have been observed in the area between the summers of 2003 (when the series began) and 2011 (the last summer before sampling for this study). The thermal anomalies were reported from the surface to the depths where the coralligenous develops (Benssousan et al. 2010; Crisci et al., 2011). The higher-than-average temperature conditions were observed in the

years 2003, 2004, 2006 and 2009. In general, temperature conditions were characterized by long period of warm temperatures, T > 24 °C, along with peaks of high temperature, T > 27 °C. For instance, in 2009, at a depth of 20 m, abnormal warm temperatures (>23 °C) were recorded for almost 47 days, with a Tmax reaching almost 27 °C. Similarly, in 2006, at 5 m, a T > 25 °C was recorded for 24 consecutive days, with a Tmax of 29,05 °C (for more detailed information on temperature conditions in Scandola MPA see Garrabou et al., 2009, Bensoussan et al., 2010; Crisci et al., 2011, Gómez-Gras et al., 2021 and www.t-mednet.org).

For this study, the mean percentage of thallus necrosis (henceforth, necrosis) was selected as a descriptor to study the impact of thermal anomalies and invasion by *W. setacea* on CCA. Within the Scandola MPA, two sites, with similar rocky bottom communities, were chosen (Imbutu and Cala Solana) in 2012. Both sampling sites had depth ranges of between 20 and 35 m. For both transects, the abundance of *W. setacea* and CCA necrosis was estimated within each 5 m depth range (depths sampled: 20 m, 25 m, 30 m and 35 m at both sites, Imbutu and Cala Solana). At each depth, mean thallus necrosis and CCA abundance was assessed by means of twenty (ten per transect) quadrats of 25 × 25 cm (total sampled area 1.25m^2 per depth) divided into 25 subquadrats of 5 × 5 cm. The number of subquadrats in which *W. setacea* or CCA necrosis appeared was recorded and used as a unit of measure. The sampled area is in accordance with previous studies dealing with macroalgae abundance sampling size in the Mediterranean Sea (Sala & Ballesteros 1997; Cebrian & Ballesteros 2004;) and coralligenous biodiversity studies (Kipson et al., 2011).

2.2. Experimental design

To experimentally test the interaction between thermal stress and invasive species on CCA, *Lithophyllum* cf. *stictiforme* (Areschoug) Hauck was chosen as a target species.

Lithophyllum. cf. *stictiforme* (henceforth referred to simply as *Lithophyllum*) is widely distributed throughout the Mediterranean and usually develops in dim light environments and strong currents, from the surface to depths of 15-40 m (Ballesteros, 2006). To prevent any conditioning derived from previous thermal anomalies or invasive overgrowth on CCA physiology and survival, specimens of this species of *Lithophyllum* were collected by scuba divers from the coralligenous community of the Medes Islands at ca. 25 m depth in September 2012 (Girona, Spain 42°03′ N; 03°13′ E). A few specimens were also collected for a valid species identification by a taxonomic expert. The coralligenous habitat in the Medes Islands is similar to those found in Scandola, but in the Medes, neither thermal anomalies leading to severe effects on macrobenthic species nor algae invasion had been observed before 2012 (i.e., the time of sampling) (Linares et al., 2013; Weitzmann et al., 2013). The collected specimens of *Lithophyllum* were transported in thermostated tanks to the Experimental Aquarium Zone (ZAE) of the Institute of Marine Sciences in Barcelona within 24 h. The algae were thoroughly cleaned of epiphytic organisms without causing any damage to the thalli. Flat thalli, within a size range of between 10 and 20cm^2 (ca. 0.35 g dry weight cm^{-2}), were selected for the experiments. The algal surface area was determined from photographs using Image Seascape© software (Teixidó et al. 2011).

Womersleyella setacea was selected for the experiments because it has been ranked as one of the most harmful alien species in terms of impacts and distribution (Katsanevakis et al., 2016), forming dense and persistent filamentous turf, especially in dim-light conditions, such those present in coralligenous habitats (Piazzi et al., 2007, 2012; Cebrian and Rodriguez-Prieto, 2012). *W. setacea* was collected from Scandola MPA in October 2012 and, immediately after sampling, the algae were placed in coolers equipped with battery-powered air pumps to be transported to the experimental aquarium facilities at the Institute of Marine Sciences-CSIC (Barcelona, Spain) within the next 24 h.

Seventy-two *Lithophyllum* specimens were randomly assigned to two sets of three independent tanks (approximately 48 l each), with each set of tanks being maintained at two contrasting temperature conditions: three C-tanks under control conditions (18 °C) and three T-tanks under warming - or thermal stress - conditions (25 °C). Within each of the six tanks, the *Lithophyllum* specimens were submitted to three different invasion treatments: (i) CW, in which there was a carpet of *W. setacea* overgrowth on the specimens; (ii) AC, in which there was an artificial carpet to mimic the reduction of light penetration by *W. setacea* overgrowth while excluding any allelochemical interaction with the algal thalli; and (iii) CC, a control treatment with no carpet or other impediment to prevent light reaching the *Lithophyllum* specimens (Fig. 1). Thus, each invasion treatment (CW: *W. setacea* overgrowth; AC: artificial carpet and CC: no carpet) was applied to a total of 24 specimens of *Lithophyllum* (4 specimens × 3 tanks × 2 temperatures). In the covered treatments, CW and AC, the light intensity reaching *Lithophyllum* thalli was similar (p > 0,05) and significantly reduced in comparison with the uncovered, CC treatment. For the artificial carpet, an inert artificial material (a sponge-like, dark-colored plastic carpet) was chosen to mimic the physical effects of turf algal cover (Fig. 1). In the two covered treatments, the algae and the artificial carpet (both circle shaped, 7,5 cm in diameter and 1–2 cm thick) were held by gravity in direct contact with the thalli, simulating the way *W. setacea* turf covers substrata in natural environments.

The tanks were supplied with Mediterranean seawater (salinity: 38 ppm), which was continuously pumped from a depth of 20 m into the experimental aquarium facilities of the Institute of Marine Sciences. Each tank had an inlet pipe for the supply of fresh seawater and an outlet pipe. Both pipes remained open so that the tanks functioned as an open system. The experimental tanks were equipped with submersible pumps to facilitate water circulation. The submersible pumps provided

a continuous circular current, with a flow rate of approximately 60 l h^{-1}. The three C-tanks under control temperature conditions were maintained throughout the experiment at between 17 and 18 °C. The three T-tanks under warming conditions were maintained at 25 °C, having first been increased from 17 °C to 25 °C by one degree every two days. In all cases, seawater was heated in a buffer tank equipped with submersible resistance heaters and was regulated by temperature controllers (Aqua Medic T controller). Irradiance was obtained from 30 W cool white fluorescent bulbs, on a 12 h light/dark regime, and was calibrated by means of a Li-Cor sensor (Li-1400-501) to match the light intensity that specimens received in their natural environment (approximately 20 and 150 μmol photons m^{-2} s^{-1}) (Cebrian and Rodriguez-Prieto, 2012). After HOBO-Li-Cor calibration, temperature and irradiance of the aquaria were monitored every half hour in all aquariums using HOBO Onset Pendant © autonomous temperature and irradiance sensors to ensure that the same conditions were maintained during the entire experiment.

The response variables measured in the experimental setup were as follows: the percentage of affected thalli (i.e., those thalli displaying > 10% necrosis); the percentage of necrosis (the percentage of dead tissue on each thallus); and the photosynthetic fitness of each thallus. All the response variables were estimated every 3 or 4 days during a 40-day period. The percentage of affected thalli and the percentage of necrosis of the thalli (as percentage of dead tissue - white tissue) were estimated, both visually and photographically, with living and dead tissue being manually selected and measured using the "analysis tool" of Adobe Photoshop. The maximum photosynthetic efficiency (Fv/Fm) was measured using an underwater pulse amplitude modulated fluorometer (Diving-PAM, Walz ©, Effeltrich, Germany). Macroalgal fronds were incubated in the dark for 15 min, after which F_v/F_m measurements were estimated by applying a saturation pulse using Pulse Amplitude Modu-
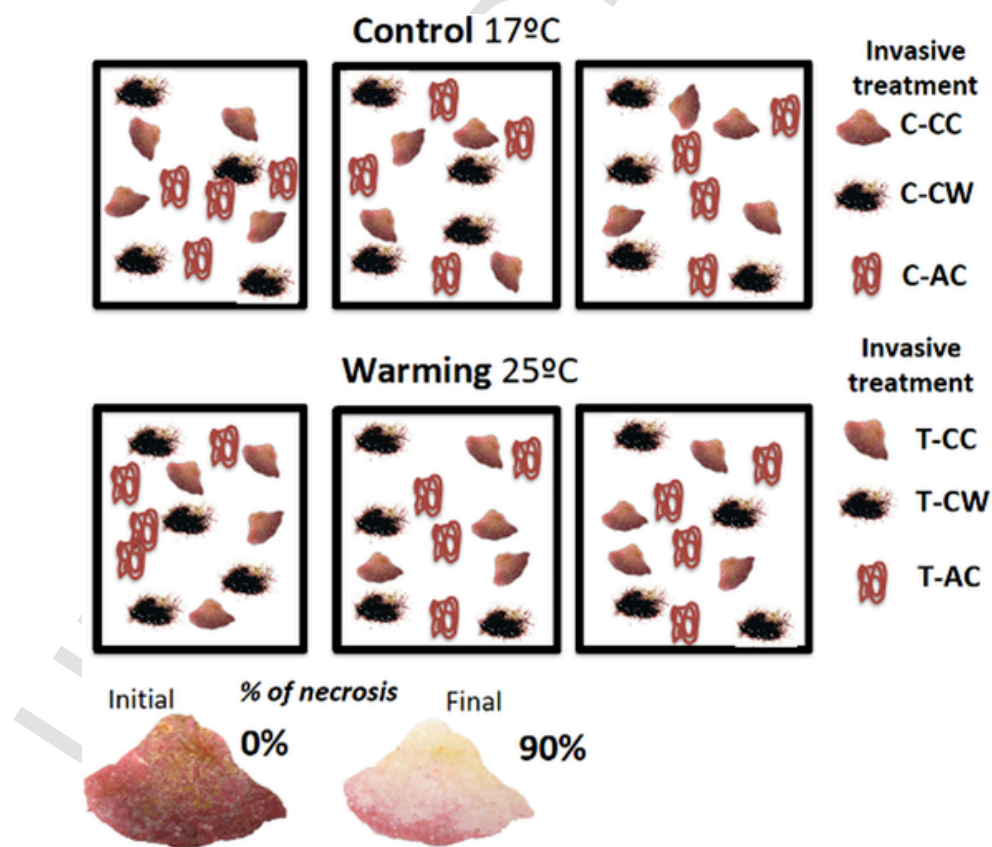

Fig. 1. Experimental setup. Three different tanks were used for each temperature condition (control temperature (C) and warming temperature (T)). At each tank, 4 replicates of the invasive treatments (noncovered (CC), covered by *Womersleyella setacea* (CW), and covered by artificial carpet (CA)) were cultured. The variables measured were the percentage of individuals that displayed some signs of necrosis, percentage of tissue necrosis and the photosynthetic efficiency.

lated Fluorometry (Diving-PAM Underwater Fluorometer, Waltz, Germany) with an absorption coefficient of the leaves of 0.65, a measuring light intensity (meas-int) of 10 and an out-gain of 10 for the electronic signal gain (amplification factor). Measurements were taken from those parts of each thallus without signs of necrosis. Measuring F_v/F_m following a period of dark adaptation is a common technique for measuring stress in plants (Murchie and Lawson, 2013).

The response to the effects of single stressors and their interaction was assessed by calculating the response of the *Lithophyllum* specimens to the different variables studied (C-CW, C-AC, T-CW, T-AC and T-CC) in comparison to the response of the control (C-CC) (Coté et al. 2016).

2.3. Statistical analysis

2.3.1. Field surveys

To explore the potential effects of invasive seaweed overgrowth on the CCA already affected by thermal anomalies, the relationship between necrosis and *W. setacea* abundance was analyzed by means of a logistic regression analysis [glm (necrosis ~ invasion + Depth + Transect)]. In addition to the regression analysis, Tukey tests were performed to determine which percentage cover values were found to influence the occurrence of necrosis.

2.3.2. Experimental data

Mixed-Effects Models (MMs) are especially suitable for the analysis of the data obtained from the lab experiment because they enable both fixed and random effects to be included as predictor variables. In our case, the use of crossed and nested random effects allows us to control for the lack of independence among observational units and to handle clustered data and repeated measures across time in the same model (Bates, Mächler, Bolker, & Walker, 2015; ; Harrison et al., 2018).

The effect on "% of affected specimens" and "% of necrosis" was analyzed using a Generalized Linear Mixed Model (GLMM) with a binomial error distribution and logit link function, and the effect of temperature on the optimum quantum yield was analyzed with a GLMM with a Poisson error distribution. In both models, warming (temperature) conditions and invasion treatment were fitted as a fixed factor, with time as a crossed random factor. Additionally, a second random term, "individuals' identity nested within tank", was fitted in order to take into account that individuals were grouped within each tank and to correct for the non-independence between measurements (repeated measurements of the same individuals over time). A type II Wald X^2 test was applied to each fitted model to determine the effect of the fixed factor. Finally, for each model, a Tukey post-hoc test was applied to explore the differences between temperature conditions.

The different models were fitted using the *lme4* package (Bates et al., 2014) and the *MASS* package (Venables & Ripley, 2002) in the statistical environment R (R Core Team, 2019). p-Values were obtained by means of a Wald X^2 test using the "ANOVA" function from the *car* package (Fox and Weisberg, 2018). Finally, the function "lsmeans" from the *lsmeans* package (Lenth, R.V. and M. Hervé, 2015) was used to perform post-hoc Tukey tests.

To analyze the effect of temperature and invasive species overgrowth on our specimens of *Lithophyllum*, the percentage of affected thalli (i.e., the thalli displaying >10% necrosis), the percentage of necrosis (the percentage of dead tissue) and the photosynthetic efficiency (Fv/Fm) were analyzed using a generalized linear mixed model (GLMM) with temperature (control and warming) and invasive alga treatment (uncovered (CC), covered with artificial carpet (AC) and covered with *W. setacea* (CW)) as fixed factors. Thallus identity, nested within tanks, was used as a random factor to account for repeated measures sampling and, consequently, for the lack of independence between the observations performed at different times. Finally, pairwise comparisons were performed between the different temperature conditions. The different models were fitted using the *lme4* (Bates, Mächler,

Bolker, & Walker, 2015) and *MASS* packages (Venables & Ripley, 2002). P-values were obtained by means of a Wald X^2 test using the "ANOVA" function in the CAR package (Fox & Weisberg, 2011). Finally, the function "lsmeans" from the *lsmeans* package (Lenth, R.V. and M. Hervé, 2015) was used to perform Tukey tests for multiple comparisons.

3. Results

3.1. Field surveys

CCA necrosis and *Womersleyella setacea* abundance were variable among the sampled depths for both transects (Table S1). However, statistically, CCA necrosis was significantly related to *W. setacea* abundance, revealing a negative effect of invasive algae overgrowth on CCA fitness. In contrast, CCA necrosis was not significantly related to depth or transect (Table 1A; Fig. 2). Shallow-water *Lithophyllum* specimens, subjected to low levels of coverage by *W. setacea* (<20% of cover), were either completely healthy (with no signs of necrosis) or else had small patches of necrosed thalli (white tissue), never exceeding 10% necrosis (Fig. 2). At 25 m depth, in some quadrats, the abundance of *W. setacea* was found to be higher, resulting in up to 20% coverage, but the mean percentage of necrosis on the CCA specimens remained similar to that observed for the shallowest (up to 20 m depth) specimens (Tukey HSD; p = 0,912). However, when the abundance of *W. setacea* exceeded 80%, all *Lithophyllum* exhibited moderate to severe signs of necrosis (>60% of necrosis), with greater severity at depths of 30 m than at 25 m (Tukey HSD; p = 0.006) (Fig. 2). Completely dead specimens were eventually seen in a few sampled quadrats, always where *W. setacea* cover was higher than 90%.

3.2. Experimental results

In the aquaria experiment, the stress of warmer temperature significantly increased the percentage of affected thalli, while the effect of in-

Table 1
A) Logarithmic regression of the % of affected CCA specimens in the field according the *Womerleyella setacea* coverage (invasion) and depth. B) Summary table of the results from analyses of deviance (Wald ?² test) for each mixed model testing the influence of the fixed factors (temperature and Invasion) on % affected individuals, % of necrosis and yield.

A) Field experiment

	Estimate	Std. error	z value	Pr(>|z|)
Intercept	−2.076	2.6159	−0.7905	0.4291
Invasion	0.0592	0.0148	3.9852	<0.0001
Depth	−0.0574	0.1064	−0.5398	0.5892
Transect	−0.1430	0,1065	−0.2083	0.8349

B) Laboratory experiment

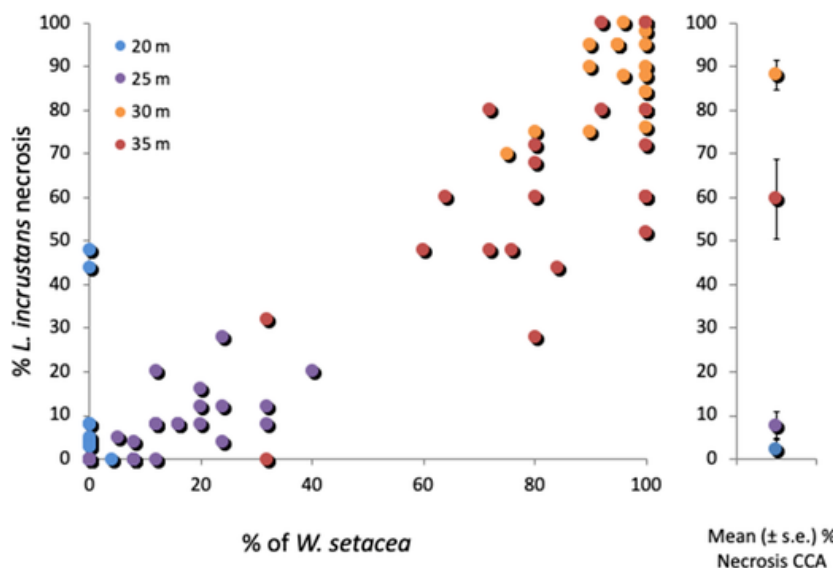|	Chisq	Df	Pr(>Chisq)
% affected individuals			
Temperature	979.85	1	<0,0001
Invasion	101.37	2	0,0203
Temp:inv	118.92	2	0,0002
% of necrosis			
Temperature	9.4972	1	0.0020
Invasion	5.9103	2	0.0511
Temp:inv	6.7840	2	0.0330
Yield			
Temperature	56.474	1	<0,0001
Invasion	27.125	2	<0,0010
Temp:inv	60.589	2	<0,0001

Fig. 2. Regression between the coverage (percent cover) of *Womersleyella setacea* and percentage of necrosis in CCA in the field according to the different depths sampled. The mean (± s.e.) percentage of necrosis per sampled depth is shown.

vasive overgrowth on *Lithophyllum* was dependent on the temperature conditions (Table 1, interaction term p = 0,002).

In the control temperature conditions, the percentage of affected specimens of *Lithophyllum* in the *W. setacea* treatment tanks (C-CW) was significantly higher than those in the C-AC (artificial carpet) and C-CC (no carpet) treatments, with the latter two presenting similar percentages of affected thalli (approximately 20%; p = 0,324; Table 2; Fig. 3A).

Table 2

Summary statistics from the Tukey test for the post-hoc pairwise comparisons between the different levels of the fixed factor (Invasion treatment) on % affected colonies, % of tissue necrosis and yield for each level of the significant fixed factor (temperature treatment).

	Estimate	SE	z.ratio	p.value
% affected colonies				
Control (18 °C)				
C-CC/T-AC	0.672	0.470	1.431	0.3248
C-CC/T-CW	2.471	0.575	4.294	**0.0001**
C-AC/T-CW	1.799	0.569	3.162	**0.0045**
Warming treatment				
C-CC/T-AC	0.963	0.635	1.517	0.2830
C-CC/T-CW	−0.637	0.771	−0.826	0.6871
C-AC/T-CW	−1.600	0.688	−2.327	0.0522
% necrosis				
Control (18 °C)				
C-CC/T-AC	−0.663	1.347	−0.492	0.8752
C-CC/T-CW	1.076	1.640	0.656	0.7891
C-AC/T-CW	1.738	1.561	1.114	0.5055
Warming treatment				
C-CC/T-AC	−4.091	1.363	−3.002	**0.0075**
C-CC/T-CW	−4.411	1.331	−3.314	**0.0026**
C-AC/T-CW	−0.320	0.923	−0.346	0.9361
% Fv/Fm				
Control (18 °C)				
C-CC/T-AC	0.0050	0.0397	0.126	0.9913
C-CC/T-CW	−0.0693	0.0390	−1.778	0.1779
C-AC/T-CW	−0.0743	0.0390	−1.903	0.1385
Warming treatment				
C-CC/T-AC	0.2441	0.0419	5.825	**<0.0001**
C-CC/T-CW	0.3825	0.0436	8.770	**<0.0001**
C-AC/T-CW	0.1384	0.0460	3.009	**0.0076**

In the thermal stress conditions, the percentage of affected specimens was higher in all cases than it was in the control conditions (Table 1B; Fig. 3). Among the invasion treatments, the specimens subjected to the *W. setacea* treatment (T-CW) were the most severely affected, with 80% of the specimens developing necrosis during the second half of the experiment. Similar, but lower, percentages of affected specimens were observed in the specimens subjected to the uncovered (T-CC) or artificially covered (T-AC) treatments (p = 0,283; Table 2; Fig. 3A).

Regarding the *Lithophyllum* necrosis rate (i.e., the percentage of dead tissue), the invasion treatments showed different responses depending on the temperature conditions (interaction term < 0,001; Table 1). In control conditions (C-tanks, at 18 °C), no significant effect was detected for either artificial or *W. setacea* coverage (C-AC or C-CW) treatments, with mean necrosis values of 13% for the artificial carpet (C-AC) treatment and 19% for the *W. setacea* (C-CW) at the end of the experiment (Fig. 3B; Table 2). In contrast, in warming conditions (T-tanks, at 25 °C), the mean necrosis rate increased to 35% in the artificial carpet (T-AC) and to 72% in the *W. setacea* overgrowth (T-CW) treatments (Fig. 3B). Although the differences in *Lithophyllum* necrosis rates between specimens in the T-AC and T-CW treatments were not statistically significant (Fig. 3B, Table 2), they were both significantly higher (p < 0,001; Table 2) than in the treatment where CCA was uncovered (T-CC). Indeed, for the uncovered treatments in both temperature conditions (C-CC and T-CC), *Lithophyllum* necrosis was stable and close to 10% for the specimens regardless of temperature (Fig. 3B).

Regarding maximum photosynthetic efficiency, similar results were observed. In the control temperature conditions, photosynthetic efficiency was similar and stable in all three invasion treatments considered (Fig. 3C; Table 1). In contrast, at the higher temperature, photosynthetic efficiency significantly declined in the *Lithophyllum* specimens submitted to covered treatments in warming conditions: an average decline of 75% in the *W. setacea* treatment (T-CW) and an average decline of 25% in the artificial carpet treatment (T-AC) (Fig. 3C; Table 2). Statistically, the photosynthetic efficiency was significantly different between all the treatments, being the lowest in those specimens subjected to the *W. setacea* treatment (T-CW), moderate in those subjected to the artificial carpet (T-CA) and highest in those specimens with no overgrowth (T-CC) (Fig. 3C: Table 2).

When analyzing the responses of the *Lithophyllum* specimens to the different single stressors and their combinations, a synergistic response (much higher than the sum of the response for each single stressor, Fig. 4A) was found for all the variables studied (including the percentage of
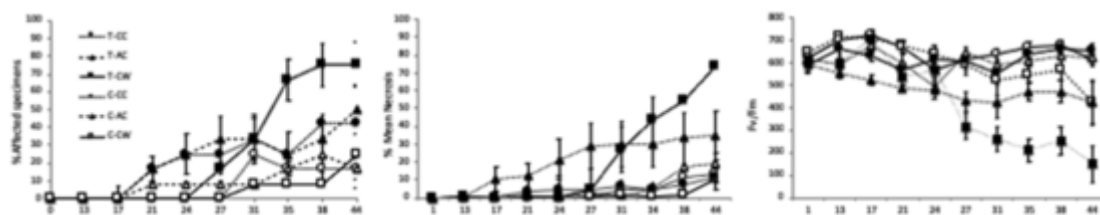
Fig. 3. A) Mean (±SE) accumulated percentage of thalli with necrosis rates over 10%; B) average percentage of necrosis and C) maximum photosynthetic efficiency of the CCA specimens subjected to thermal stress (T) and invasion treatments [including invasive algae (T-CW), artificial material (T-CA) and uncovered specimens (T-CC)] and to control temperature and invasion treatments [including invasive algae (C-CW), artificial material (C-CA) and uncovered specimens (C-CC)].
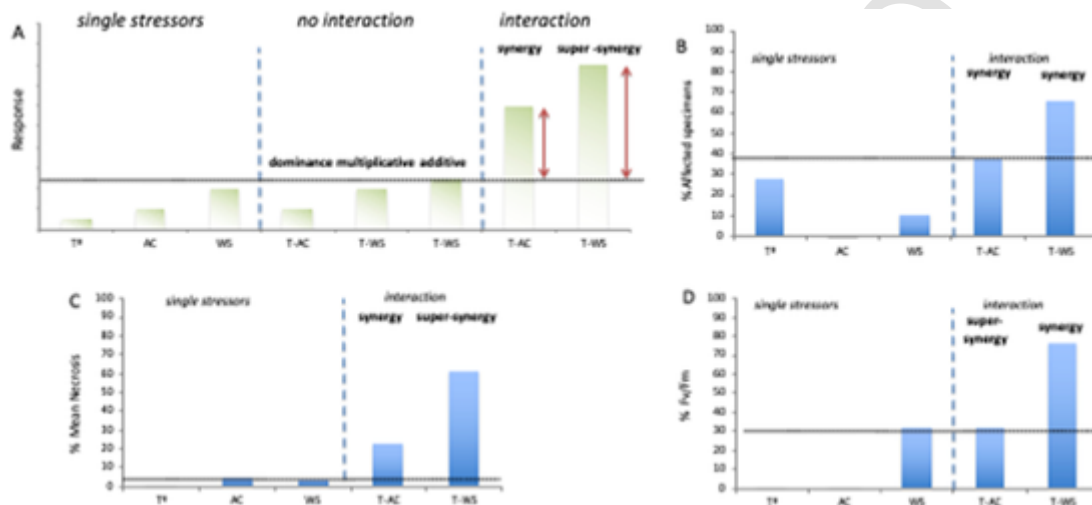

Fig. 4. A) Adaptation of our study to the conceptual model for defining ecological interactions between multiple stressors (modified from Côté et al., 2016). The stressors (temperature (Tª), artificial carpet (AC) and *Womersleyella setacea* (CW)) elicit a biological response when acting separately. Their combined effect could simply be equal to the effect of one of the two stressors, i.e., a dominance effect, or could be additive (the dotted black line represents the maximum level of an additive response), i.e., the sum of two stressor effects with or without a multiplicative-risk correction. Alternatively, stressors can interact synergistically (i.e., T-AC) or supersynergistically (i.e., T-CW). The effect of the individual stressors and the interaction between multiple stressors on B) the percentage of affected specimens, C) the percentage of mean necrosis and D) the photosynthetic efficiency. The dotted black line represents the maximum level of an additive response.

affected individuals, Fig. 4B; the mean percentage of necrosis, Fig. 4B; and photosynthetic efficiency, Fig. 4C) when the increased temperature was concurrent with the artificial carpet (T-AC) and *W. setacea* (T-CW) overgrowth (Fig. 4). The combined effect of increased temperature and *W. setacea* overgrowth resulted in the largest synergic interaction (Fig. 4).

4. Discussion

The present study provides experimental evidence on how synergic interactions of global stressors (e.g., global warming and invasive species) may impact coralline calcareous algae, one of the most important functional groups dwelling in tropical and temperate seas. Independently, temperature and invasive algae overgrowth have subtle, sublethal impacts on CCA, only identifiable by an increase in the percentage of specimens displaying some degree of necrosis (Fig. 4). However, when these stressors interact, we observed synergic effects that were much greater than the sum of the effects of individual stressors: specimens exposed to both stressors – increased temperature and *W. setacea* overgrowth – were much more affected than could be calculated by summing the effects on specimens exposed only to one stressor or the other (Fig. 4). During the experiment, necrosis and the reduction in the maximum photosynthetic efficiency were both higher when the invasive algae or the artificial carpet covered the CCA in the thermal stress treatment (Fig. 3). This result highlights a heightened sensitivity to invasive algae when CCA are concurrently exposed to elevated seawater temperatures. Similarly, the high necrosis rates (up to 60–100% necrosis) displayed by *Lithophyllum* cf. *stictiforme* specimens in the field

when subjected to *Womersleyella* overgrowth indicate that the two marine heatwaves (MHWs) at the study site might have heightened the sensitivity of this CCA to *Womersleyella* invasion (Fig. 2).

In contrast, neither necrosis nor a strong reduction in photosynthetic efficiency was observed in the *Lithophyllum* specimens at the control temperatures, regardless of the invasive algal treatment considered. In fact, the photosynthetic efficiency of *Lithophyllum* at the control temperature was in the range of values normally observed in marine calcareous algae (approximately 0.600; Villas-Bôas et al., 2014, Burdett et al., 2015, Lei et al., 2020). The lack of detectable stress in the specimens without artificial cover or invasive algal overgrowth at 25 °C indicates that *Lithophyllum* cf. *stictiforme* tolerates high temperatures. Similarly, previous in vitro thermotolerance experiments have demonstrated that this species can survive one year at 26 °C (Rodriguez-Prieto, 2016). The relative low impact of warm temperatures observed for CCA species in the Mediterranean (a warm temperate area), clearly contrasts with the response to thermal stress of most subtidal red algae from the temperate zone, many of which display significant necrosis rates at temperatures above 23 °C (Wilson et al., 2004). In fact, as mentioned above, experimentally, thermal stress effects were only detected by an increase in the percentage of *Lithophyllum* specimens showing subtle signs of necrosis (between 10 and 20% necrosed tissue). In line with these experimental results, our field surveys in Scandola MPA indicated low mean necrosis (<10%) rates in *Lithophyllum* specimens not covered by *Womersleyella setacea*, despite the sampling area being affected by two MHWs in previous years (Garrabou et al., 2009; Crisci et al., 2011; Cebrian et al., 2011). However, these subtle and sublethal impacts should be considered with caution, since diseases of coralline algae related to positive

thermal anomalies have been reported in the northwestern Mediterranean, where the study area is found (Hereu and Kersting, 2016; Verdura et al., 2019). Most likely, the reported CCA diseases (Quéré et al., 2015) were concomitant with the higher MHW intensity, which reached maximum surface temperatures of approximately 28 °C in some of the affected sites (Rubio-Portillo et al. 2016). These warm conditions may have affected host-pathogen interactions (Harvell et al., 2002).

Although we cannot elucidate the actual mechanism by which *W. setacea* overgrowth affects *Lithophyllum* cf. *stictiforme* specimens under thermal stress treatments, the higher percentage of specimens affected by the invasive alga *W. setacea* than by the artificial treatment points to an allelopathic effect of algae overgrowth. It is well known that many benthic algae species produce toxic secondary metabolites, which act as agents against herbivory and antifouling (Paul et al., 2001; Sudatti et al., 2020). Although the secondary metabolites of *W. setacea* have not been studied to date, chemical interactions should not be disregarded. In fact, the highest diversity of secondary metabolites is found among rhodophytes and algae from the family Rhodomelacea (which includes *Womersleyella* spp.), which are particularly rich in highly toxic halogenated compounds (Paul et al., 2001; Sudatti et al., 2020 and references therein).

On the other hand, although the artificial carpet treatment did not result in a significant increase in the mean necrosed tissue compared to those specimens left uncovered, it significantly increased and impaired photosynthetic efficiency. Photosynthetic efficiency, which can be considered a fast response variable linked to specimen fitness, may decrease even before thallus mortality can be observed (Wilson et al., 2004; Kersting et al., 2015). Similarly, experiments carried out for other CCA species clearly show a reduction in photosynthetic efficiency as a function of sediment coverage (Figueiredo et al., 2015; Nilssen et al., 2015), probably due to reduced gas exchange (Figueiredo et al., 2015; Harrington et al., 2005; Wilson et al., 2004). A decrease in the maximum photosynthetic efficiency also indicates a significant physical effect of the artificial carpet and *W. setacea* cover treatments. There may be multiple mechanisms by which CCA is affected by physical cover (artificial or by the turf formed by species such as *W. setacea*). In fact, similar impacts have been observed for other photosynthetic organisms such as corals. As described for corals, the thick turf probably physically stresses CCA by limiting light and constant water exchange (Vermeij and Sandin, 2008; Arnold et al., 2010; Kersting et al., 2015) and, as a result, the availability of nutrients and energy that the algae need. Likewise, it is known that structurally complex turf increases sedimentation and passive entrapment of particulate material (Airoldi, 2000, De Caralt and Cebrian, 2013). Whatever the mechanisms by which invasive algae overgrowth impairs CCA in a warming scenario, this is an especially worrying issue considering the expected increase in MHWs (Oliver et al., 2018) and the growing trend in the abundance of turf algae (native and exotic) in temperate and tropical coastal ecosystems (Airoldi, 1998; Vermeij et al. 2008; Vermeij et al., 2010).

In contrast to other species dwelling in coralligenous outcrops and maërl (e.g., invertebrates such as azooxanthellate corals, gorgonians, and sponges), the bathymetric distributions of CCA is restricted by their light requirements. Bearing in mind that most invasive algae thriving in Mediterranean waters display similar light requirements as those of CCA, the latter's persistence is threatened in areas exposed to invasion (Cebrian and Ballesteros, 2009, 2010; Cebrian and Rodriguez-Prieto, 2012). This limitation, together with the negative effects that future acidification has on these calcareous formations (Hall-Spencer et al., 2008; Linares et al., 2015) and especially in the case of CCA (McCoy and Kamenos, 2015), will possibly exacerbate the shift from dominance by calcifying organisms to that of the fleshy, turf algae observed on many reefs today (O'Brien and Scheibling, 2018).

Our results have shown that the interaction of two of the most widespread global change stressors (warming and invasive species) triggers drastic synergistic effects on temperate CCA. These findings will be important for modeling future rates of carbonate production by reefs and associated ecosystems. The reduction and eventual disappearance of these "ecosystem engineers" may undermine ecological functioning, leading to the disappearance and/or fragmentation of populations and communities associated with them (Airoldi et al., 2008). However, the increasing threat to calcareous coralline algae populations worldwide may be detrimental not only to the overall marine biodiversity but also to the potential long-term C storage and C-sink estimates (Krause-Jensen et al., 2018).

Since this study was performed within one of the oldest Mediterranean MPAs, some concerns arise as to how to manage the impact of invasive algae and their interaction with warming. The role of MPAs in preventing biological invasions remains unclear (Giakoumi and Pey, 2017). Although in some cases, complementary management actions, such as species removal in the case of invasive fish populations, can be implemented (Giakoumi et al., 2019a), measures to control invasive algae can be time consuming and require considerable effort, while meeting with low success (Giakoumi et al., 2019b). In this case, preventing the establishment of invasive species through potential regulations, monitoring and awareness among all users should be prioritized (Otero et al., 2013).

In conclusion, we have shown that understanding how global change-related disturbances interact is critical in the current context of rapid environmental changes and the increasing geographical overlap of stressors. Overall, our study will enhance our capability to predict changes in community structure and functions in a changing ocean and in the ocean in the Anthropocene era.

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CRediT authorship contribution statement

All authors conceived the idea and designed the study and collected the field data; EC and CL performed the laboratory experiment and lab work; EC conducted statistical analyses and wrote the first draft; and all authors contributed critically to writing subsequent drafts and gave their final approval for the publication.

Uncited references

Canals and Ballesteros, 1997
Dumay et al., 2002
Nannini et al., 2015
Rindi et al., 2019

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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