Synergism between two anthropic impacts: *Caulerpa racemosa* var. *cylindracea* invasion and seawater nutrient enrichment

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ABSTRACT: Although eutrophication and biological invasions are recognized as important elements of global change, few studies have assessed how their synergism can affect structural and functional traits of marine ecosystems. The present study aimed to evaluate the effect of seawater nutrient enrichment on the spread of the introduced Chlorophyta *Caulerpa racemosa* var. *cylindracea* and the interactive effects of nutritional enrichment and *C. racemosa* invasion on the native macroalgal assemblages of a subtidal rocky bottom. To achieve these objectives, a manipulative field experiment was carried out at 24 m depth in the northwestern Mediterranean Sea by adding *C. racemosa* and supplying the water column with nutrients. The following treatments were applied: non-manipulated assemblages, nutrient enrichment, addition of *C. racemosa,* and both nutrient enrichment and addition of *C. racemosa*. Results showed that *C. racemosa* spread was enhanced by nutrient enrichment. Moreover, a significant interaction between nutritional increase and *C. racemosa* invasion was detected: the effects of *C. racemosa* invasion on native macroalgal assemblages were amplified by nutrient enrichment. Results suggested that synergism between effects of different anthropogenic impacts can have severe consequences on the integrity of marine ecosystems.

KEY WORDS: Biological invasions · Nutrients · Rocky bottom · Macroalgal assemblages · Mediterranean Sea

INTRODUCTION

Global change represents a focal concern for ecologists (e.g. Grall & Chavaud 2002, Feely et al. 2004, Schiel et al. 2004, Harley et al. 2006, Martin & Gattuso 2009). In addition to climatic changes, the global environment is undergoing modification as a result of a number of interacting processes which are directly or indirectly due to human activities. Thus, global environmental change is a complex phenomenon comprising multiple events which alter the structure and function of ecological systems, with possible occurrence of serious cascade effects. In this context, there is a need for study of synergism at a global scale between different environmental processes (Vitousek 1994, Huen-

neke 1997). Among these processes, biological invasion is recognized as an important element of global change and a major driver of ecosystem modification (Vitousek et al. 1996, 1997). However, other human induced impacts, such as changes in the atmospheric composition, alteration of biogeochemical cycles, fragmentation of habitats and erosion of biodiversity, can affect species distribution and resources dynamics in terrestrial and aquatic systems, potentially interacting with biological invasions (Dukes & Mooney 1999, Harris & Tyrrel 2001). The increase of nutrient load, for example, is considered an anthropogenic factor enhancing the dominance of non-native species in many natural systems (Hobbs & Huenneke 1992, Burke & Grime 1996). The effect of nutrient availability on bio-

logical invasions has been examined mainly in terrestrial habitats (Burke & Grime 1996, Gross et al. 2005, Leishman & Thomson 2005); in contrast, only a few experimental studies have been carried out to investgate this phenomenon in marine ecosystems (but see Steen 2003, Sánchez & Fernández 2006, Incera et al. 2009). Eutrophication is a growing problem in the marine habitats of coastal waters all over the world (Nixon 1995, Livingston 2001, Howarth & Marino 2006) and is considered one of the main causes of deteriorating coastal water quality and loss of ecosystem complexity (Soltan et al. 2001, Arevalo et al. 2007). Biotic homogenization due to the proliferation of opportunistic species has been attributed mostly to high loading of nutrients derived from industrial and urban sewage effluents (Valiela et al. 1992, Morand & Briand 1996). Furthermore, nutrient enrichment can aid the spread of introduced macroalgae through different mechanisms (Stimson et al. 2001, Torres et al. 2004). Nutrient availability is usually an important factor in determining the species composition of seaweed assemblages (Teichberg et al. 2008). An increase in nutrients in natural systems is detrimental to slow-growing plants which are adapted to nutrient-poor habitats and creates favorable conditions for faster-growing plants, such as many invasive seaweeds. Moreover, the degradation of benthic systems caused by pollution can reduce the native assemblage's resistance to invasions (Stachowicz et al. 1999). Thus, knowledge of synergetic mechanisms between nutrient enrichment and invasions of introduced seaweeds can provide valuable insights, which will assist ecologists in building predictive models and planning conservation programs (Lapointe & Beldford 2010).

The Chlorophyta *Caulerpa racemosa* (Forsskål) C. Agardh var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque (Verlaque et al. 2003) represents one of the most serious invasive species in the Mediterranean Sea (Klein & Verlaque 2008). The alga has rapidly colonized wide areas of the Mediterranean basin, forming permanent populations and interfering with the native assemblages (Piazzi et al. 2001, 2005b, Piazzi & Balata 2008, Klein & Verlaque 2008). Marine ecosystems which are essential for biodiversity conservation, such as seagrass meadows and coralligenous habitat (sensu Ballesteros 2006), have been invaded by *C. racemosa* with consequent loss of the original structure of assemblages (Ceccherelli & Campo 2002, Piazzi et al. 2007a, Antolić et al. 2008).

The interaction of *Caulerpa racemosa* with other kinds of human impact has been investigated in relation to the increase of sediment deposition, showing important synergetic effects (Piazzi et al. 2005a, 2007b), but no data are available on the consequences of increased water nutrient load on *C. racemosa* invasion. However, several observations suggest that the spread of this invasive alga is related to different types of human pressures. For example, along the Mediterranean French coasts, *C. racemosa* cover is higher in marine areas close to harbors, and in areas affected by industrial and urban pollution, than in undisturbed areas (Klein et al. 2005). The same pattern is observed along the urbanized coasts of Tuscany, Italy, where the highest *C. racemosa* biomass values in the Mediterranean Sea have been recorded (Piazzi et al. 2001). Correlative and experimental studies highlight a positive influence of increasing nutrient availability on the spread of the co-generic invasive seaweed *Caulerpa taxifolia* (Vahl) C. Agardh (Chisholm et al. 1995, 1997, Ceccherelli & Cinelli 1997), suggesting similar effects on *C. racemosa*. The present study aimed to evaluate the role of seawater nutrients in the spread of *C. racemosa* and to detect possible interactive effects of nutrient enrichment and *C. racemosa* invasion on the native assemblages. To this end, a manipulative field experiment was carried out by adding *C. racemosa* and supplying water column with nutrients. The following hypothesis were tested: (1) an increase of nutrient load can enhance the growth of *C. racemosa*, (2) both nutrient enrichment and *C. racemosa* invasion may cause modifications in the structure of macroalgal assemblages of the subtidal rocky bottom, and (3) the concurrent presence of these 2 stressors may lead to greater effects than the sum of the effects of each stressor acting alone.

MATERIALS AND METHODS

Study site and experimental design. The study was carried out on a rocky subtidal bottom 24 m deep located along the Tuscan coast south of Leghorn (43° 28′ 24″ N, 10° 19′ 42″ E) where the substratum is colonized by coralligenous assemblages below 20 m depth. *Caulerpa racemosa* has been present at the study site since 1996, showing a patchy distribution extending from the surface to 40 m depth (Piazzi & Balata 2009).

Field experiments took place from July to October 2009, which represents the main growth season of *Caulerpa racemosa* (Ruitton et al. 2005). The following treatments were considered: non-manipulated assemblages (hereafter indicated as control), nutrient enrichment (nutrient-enriched), addition of *C. racemosa* (invaded) and both nutrient enrichment and addition of *C. racemosa* (nutrient-enriched+invaded). For each treatment, 4 areas of about 100 m² and 10′s of m apart were selected at random along 3 km of coastline. In each area, 3 replicate experimental plots of 400 cm² were located at random, several meters distant from each other.

Manipulative field experiment. In the areas assigned to invaded and nutrient-enriched+invaded treatments, 15 fragments of the invasive alga, 12 cm in length, were anchored in each plot using 2 wire bridges fixed to the substratum by means of 15 cm long nails. Alga fragments were collected in the adjacent patches inside the study site and attached within 1 h. At the beginning of the study, the mean cover of *Caulerpa racemosa* was about 70% of the experimental plot surface, thus comparable to that measured for naturally invaded assemblages in the same habitat (Piazzi & Balata 2008, 2009).

In the areas assigned to nutrient-enriched and nutrient-enriched+invaded treatments, two $20 \times 5 \times 5$ cm nylon mesh bags (1 mm mesh size), each containing 200 g fertilising pellets (Osmocote®, Scotts), were placed along the 2 opposite sides of each plot and fixed to the substratum with nails. The NPK composition of the pellets was 15-9-9, which is one of the combinations of nitrogen (15%, made up of 7% ammonia and 8% nitrate), phosphorus (9% in the form of P_2O_5) and potassium (9% as KNO_3) commonly utilized for fertilizing terrestrial plants. After 2 mo, before the complete dissolution of pellets, the bags were replaced with new ones in all treated experimental plots in order to maintain a constant water nutrient concentration throughout the study period. The quantity of fertiliser pellets per surface unit was higher than that used in similar experiments carried out previously on the same site so as to induce a response in macroalgal assemblages over a short time period (Balata et al. 2010).

To verify the efficiency of experimental manipulation, concentrations of seawater nutrients were measured after 1 mo from the beginning of the study in 2 areas selected at random among those with and without added nutrients. In each area, 2 replicate water samples were taken from each of the 3 experimental plots using 60 ml sterile syringes. Samples were immediately filtered (0.45 µm mesh size) and stored at –20°C pending laboratory nutrient analysis. Seawater concentrations of ammonia (N-NH$_4^+$), nitrite (N-NO$_2^-$), nitrate (N-NO$_3^-$) and phosphorus (P-PO$_4^{3-}$) were determined using a continuous-flow AA3 Auto-Analyzer (Bran-Luebbe) and expressed in µmol l^{-1}. For each nutrient component, the statistical significance of concentration values was analyzed by 2-way analysis of variance (ANOVA) with Nutrient (nutrient-enriched vs. not enriched) as fixed factor and Area (2 levels) as random factor nested in Nutrient. The concentrations of ammonia, nitrate and phosphorus were significantly higher in nutrient-enriched plots (Table 1), confirming the effectiveness of the tested method for nutrient enrichment of the experimental plots. Differences between areas were significant only for nitrite concentration, which was not significantly different between nutrient-enriched and non-enriched plots.

***Caulerpa racemosa* growth.** The maximum diameter (cm) of *Caulerpa racemosa* patches was measured every 40 d throughout the study period. The growth rate per day was calculated as the difference in patch size between consecutive samplings divided for the number of days. At the end of this period, that is 4 mo after the start of the experiment, *C. racemosa* was removed from all treated plots and its abundance in each plot was evaluated in laboratory as biomass, total length of stolons and number of fronds. Biomass was expressed as g dry weight obtained after drying the material for 48 h at 60°C.

Patch diameter and *Caulerpa racemosa* biomass, stolon length and the number of fronds were analysed by 2-way ANOVA with Nutrient (nutrient enriched vs. not enriched) as fixed factor and Area (4 levels) as random factor nested in Nutrient.

Macroalgal assemblages. At the end of the study period, experimental plots were sampled by scraping whole 400 cm^2 surface of the plots by means of a hammer and a chisel. Material collected for each plot was preserved in 4% formalin seawater. In the laboratory, all macroalgal species were identified and abundance of each species was estimated as percentage cover (percentage of plot surface occupied by the algal surface measured by spreading out the thalli; Ballesteros 1986). For each sample, total percentage cover was calculated by summing the percentage cover of all algae in the sample. Percentage cover of turf, encrusting and erect layers was also calculated as the sums of the percentage cover of all the algae belonging to each layer (Piazzi et al. 2001). Diversity was evaluated as the number of species per plot and by the Shannon-Weaver index ($H' = -\Sigma R_i/R_t \ln R_i/R_t$, where R_i is the percentage cover of the species i and R_t is the total percentage cover).

Multivariate analysis of variance based on permutations (PERMANOVA, Anderson 2001) was used to examine differences in patterns of composition and

Table 1. Nutrient concentrations in plots with and without added nutrients (mean ± SE, n = 12) and results of ANOVA analysis between the 2 experimental conditions. Significant p-values are in **bold** type

Nutrient	Plots without added nutrients (µmol l^{-1})	Nutrient-enriched plots (µmol l^{-1})	F	p
N-NH$_4^+$	1.7 ± 0.6	47.3 ± 7.1	11.5	**0.006**
N-NO$_3^-$	0.7 ± 0.3	48.8 ± 12.3	18.8	**0.001**
N-NO$_2^-$	0.3 ± 0.1	3.3 ± 0.9	1.4	0.286
P-PO$_4^{3-}$	<0.001	9.1 ± 2.5	4.1	**0.003**

abundance of species between treatments. The analysis consisted in a 3-way model with Nutrient (nutrient enriched vs. not enriched) and Invasion (*Caulerpa racemosa* added vs. not added) as fixed and orthogonal factors, Area (4 levels) as random factor nested in the interaction Nutrient × Invasion and 3 replicates for each Area. PERMANOVA was conducted on the Bray-Curtis dissimilarity matrix (Bray & Curtis 1957), calculated from untransformed data. SIMPER analysis (Clarke 1993) was used to identify the percentage contribution of each species to the Bray-Curtis dissimilarity between conditions. A 2-dimensional non-metric multidimensional scaling (nMDS) was used to provide a graphical representation of the data.

Values of total percentage cover, percentage cover of vegetation layers, species number and Shannon-Weaver index were analysed by 3-way ANOVA. Factors and levels considered in these analyses were the same described for the multivariate analysis. Cochran's *C*-test was used before each analysis to check for homogeneity of variance (Underwood 1997). Student Newman Keuls (SNK) test was used for *a posteriori* multiple comparison of means.

RESULTS

Caulerpa racemosa growth

In the plots where *Caulerpa racemosa* were added, patches of the alga reached their maximum size in September, when their diameter measured 42.2 ± 2.3 cm (means ± SE, n = 12) in the invaded plots and 55.5 ± 1.7 cm in the nutrient-enriched+invaded plots. At the end of the study period (October 2009), patch-diameter was 38.2 ± 3.6 cm and 51.1 ± 3.1 cm respectively in invaded plots and in nutrient-enriched+invaded plots (Fig. 1). The highest growth rate was
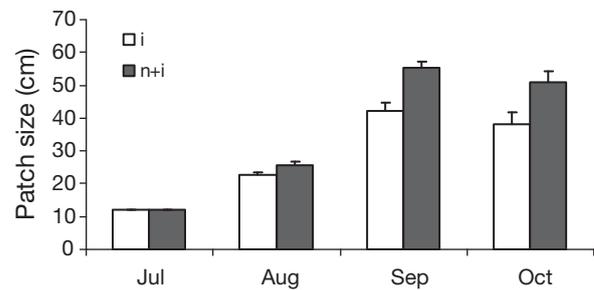

Fig. 1. *Caulerpa racemosa.* Diameter of patches (mean ± SE, n = 12) over the 4 mo of the experiment. i: invaded plots, n+i: nutrient enriched+invaded plots

registered between August and September, when it reached 0.49 ± 0.02 cm d^{-1} in invaded plots and 0.74 ± 0.02 cm d^{-1} in nutrient-enriched+invaded plots.

Caulerpa racemosa biomass (15.2 ± 2.4 and 30.2 ± 3.4 gr dry wt m^{-2} respectively in invaded and nutrient-enriched+invaded plots), stolon length (691.9 ± 113.4 cm and 991.7 ± 98.4 cm) and number of fronds (115.4 ± 24.2 and 226.7 ± 20.7), were all lower in invaded plots than in nutrient-enriched+invaded plots. ANOVA detected significant differences between treatments for all the above mentioned variables; moreover, a significant variability among areas was detected for biomass, stolon length and number of fronds (Table 2).

Macroalgal assemblages

A total of 65 macroalgal species were identified (7 Chlorophyta, 7 Fucophyceae and 51 Rhodophyta; Table 3). In control areas, algal assemblages showed a well defined stratified structure. Calcareous Corallinales completely covered the rocky bottom, forming a

Table 2. *Caulerpa racemosa.* Results of nested ANOVA analysis performed on variables patch diameter, stolon length, biomass and number of fronds. Significant p-values are in **bold** type; ns: not significant

Source	df	MS	*F*	p	Source	df	MS	*F*	p
Patch diameter					**Biomass**				
Nutrient = N	1	1568.16	42.70	**0.0006**	Nutrient = N	1	0.767	10.66	**0.017**
Area (N)	6	36.72	0.97	0.4756	Area (N)	6	0.072	5.15	**0.004**
Residual	16	37.83			Residual	16	0.014		
Total	23				Total	23			
Cochran's *C* = 0.453 (ns)					Cochran's *C* = 0.304 (ns)				
Stolon length					**No. of fronds**				
Nutrient = N	1	1315478	7.04	**0.037**	Nutrient = N	1	148444	23.91	**0.002**
Area (N)	6	186977.8	3.6	**0.018**	Area (N)	6	6207.41	3.85	**0.014**
Residual	16	51867.76			Residual	16	1614.28		
Total	23				Total	23			
Cochran's *C* = 0.396 (ns)					Cochran's *C* = 0.307 (ns)				

Table 3. Samples collected and mean percentage cover in experimental plots. c: control, n: nutrient-enriched, i: invaded, n+i: nutrient-enriched+invaded

Taxon	c	n	i	n+i
Chlorophyta				
Cladophora echinus (Biasoletto) Kützing	0.3	0.5	0.3	0.0
Cladophora prolifera (Roth) Kützing	0.4	1.2	3.8	5.7
Flabellia petiolata (Turra) Nizamuddin	47.5	46.2	34.2	24.9
Halimeda tuna (J. Ellis e*t* Solander) J.V. Lamouroux	3.1	2.2	0.0	0.0
Palmophyllum crassum (Naccari) Rabenhorst	0.1	1.5	0.0	0.0
Pseudochlorodesmis furcellata (Zanardini) Børgesen	0.8	2.5	0.3	3.0
Valonia macrophysa Kützing	0.2	0.1	0.0	0.4
Ochrophyta				
Dictyopteris polypodioides (A.P. De Candolle) J.V. Lamouroux	0.4	0.1	0.0	0.0
Dictyota linearis (C. Agardh) Greville	0.7	0.8	0.5	0.5
Halopteris filicina (Grateloup) Kützing	0.8	1.0	3.2	4.4
Nereia filiformis (J. Agardh) Zanardini	0.2	0.1	0.1	0.0
Sphacelaria cirrosa (P.H. Roth) C. Agardh	0.0	0.1	0.0	0.0
Sphacelaria plumula Zanardini	0.1	0.1	0.1	0.0
Zanardinia typus (Nardo) G. Furnari	4.0	1.8	0.0	0.1
Rhodophyta				
Acrodiscus vidovichii (Meneghini) Zanardini	0.9	1.1	0.0	0.0
Acrosorium venulosum (Zanardini) Kylin	0.2	0.5	0.3	0.4
Acrothamnion preissii (Sonder) Wollaston	0.1	1.1	0.3	2.2
Aglaothamnion tenuissimum (Bonnemaison) Feldmann-Mazoyer	0.2	0.2	0.0	0.0
Amphiroa rubra (Philippi) Woelkerling	0.1	0.3	0.0	0.0
Anthithamnion piliferum Cormaci *et* Furnari	0.7	0.5	0.6	0.8
Apoglossum ruscifolium (Turner) J. Agardh	0.2	0.2	0.0	0.1
Botryocladia botryoides (Wulfen) Feldmann	1.1	0.4	0.5	0.3
Ceramium bertholdii Funk	0.0	0.0	0.0	0.0
Ceramium diaphanum (Lighfoot) Roth	0.1	0.1	0.0	0.0
Ceramium flaccidum (Kützing) Ardissone	0.0	0.0	0.0	0.0
Ceramium codii (H. Richards) Feldmann-Mazoyer	0.2	0.0	0.0	0.0
Champia parvula (C. Agardh) Harvey	0.4	0.1	0.2	0.1
Chondria capillaris (Hudson) M.J. Wynne	0.3	0.0	0.0	0.0
Contarinia squamariae (Meneghini) Denizot	0.7	0.4	0.0	0.0
Crouania attenuata (C. Agardh) J. Agardh	0.4	0.2	0.2	0.1
Dasya baillouviana (S.G. Gmelin) Montagne	0.1	0.5	0.2	0.6
Dasya ocellata (Grateloup) Harvey	0.0	0.0	0.0	0.0
Erythroglossum sandrianum (Kützing) Kylin	0.0	0.0	0.0	0.0
Eupogodon planus (C. Agardh) Kützing	1.9	1.1	0.7	0.6
Feldmannophycus rayssiae (Feldmann *et* Feldmann-Mazoyer) Augier *et* Boudouresque	0.7	1.4	1.4	0.8
Gelidium bipectinatum G. Furnari	0.0	0.0	0.0	0.2
Jania adhaerens J. V. Lamouroux	1.0	3.0	1.5	2.4
Halopithys incurva (Hudson) Batters	1.0	4.0	7.8	8.1
Halydyction mirabile Zanardini	0.0	0.4	0.0	0.0
Halymenia floresia (Clemente and Rubio) C. Agardh	0.0	0.0	0.1	0.0
Herposiphonia secunda (C. Agardh) Ambronn	0.0	0.0	0.0	0.0
Heterosiphonia crispella (C. Agardh) M.J. Wynne	1.1	1.3	0.8	1.1
Hypoglossum hypoglossoides (Stackhouse) Collins *et* Harvey	0.2	0.2	0.0	0.0
Laurencia chondrioides Børgesen	42.5	42.3	20.8	7.8
Lomentaria chylocladiella Funk	0.2	0.1	0.0	0.0
Meredithia microphylla (J. Agardh) J. Agardh	9.0	15.3	2.4	2.8
Monosporus pedicellatus (J.E. Smith) Solier	0.1	0.3	0.2	0.3
Osmundea pelagosae (Schiffner) F.W. Nam	2.4	0.6	0.0	0.0
Peyssonnelia rubra (Greville) J. Agardh	46.7	44.4	18.9	14.2
Peyssonnelia squamaria (S.G. Gmelin) Decaisne				
Phyllophora crispa (Hudson) P.S. Dixon	0.0	0.8	0.0	1.0
Plocamium cartilagineum (Linnaeus) P.S. Dixon	0.1	0.1	0.1	0.0
Polysiphonia elongata (Hudson) Sprengel	0.0	0.0	0.0	0.2
Polysiphonia furcellata (C. Agardh) Harvey	0.1	0.2	0.1	0.1
Polysiphonia perforans Cormaci, G. Furnari, Pizzuto *et* Serio	0.3	0.1	0.0	0.0
Pterothamnion plumula (Ellis) Nägeli	0.1	0.1	0.0	0.0
Rhodophyllis divaricata (Stackhouse) Papenfuss	0.2	0.0	0.0	0.0
Rodriguezella strafforelloi F. Schmitz	0.4	0.7	0.7	0.8
Sebdenia dicotoma Berthold	0.2	0.4	0.0	0.0
Spyridia filamentosa (Wulfen) Harvey	0.0	0.1	0.1	0.0
Tricleocarpa fragilis (linnaeus) Huisman *et* R.A. Towsend	13.2	9.6	7.0	3.7
Womersleyella setacea (Hollenberg) R.E. Norris	15.9	87.5	34.1	57.9
Wrangelia penicillata (C. Agardh) C. Agardh	0.2	0.0	0.0	0.0

secondary substrate colonized by encrusting, turf and erect algae. Species of the genus *Peyssonnelia* were the most common encrusting forms, while *Womersleyella setacea*, *Heterosiphonia crispella*, *Eupogodon planus* and *Feldmannophycus raissiae* were widely present in the turf. The erect layer was mostly composed of *Flabellia petiolata*, *Laurencia chondroides* and *Tricleocarpa fragilis*.

PERMANOVA detected a significant interaction Nutrient × Invasion (Table 4); pair-wise test comparisons showed significant differences between treatments, which were clearly separated in the nMDS ordinations (Fig. 2).

The SIMPER test showed that an increase of nutrient load caused the rise in percentage cover of turf species, especially *Womersleyella setacea*, and of some erect layer species such as *Meredithia microphylla* and *Laurencia chondroides*. In invaded plots, the percentage cover of encrusting taxa *Peyssonnelia* spp., *Zanardinia typus* and calcareous Corallinales and the erect species *Flabellia petiolata*, *Halimeda tuna*, *Laurencia chondrioides* and *Meredithia microphylla* decreased, while *Halopithys incurva*, *Womersleyella setacea* and *Cladophora prolifera* showed a high tolerance to *C. racemosa* colonization and increased percentage cover. Responses of macroalgal assemblages in the nutrient-enriched+invaded plots were similar to those in the invaded plots; however, in nutrient-enriched+invaded plots, there was a more pronounced decrease of sensitive taxa such as *Peyssonnelia* spp., *Zanardinia typus*, encrusting Corallinales, *Laurencia chondrioides* and *Flabellia petiolata*, while cover of *Womersleyella setacea* increased (Table 5).

Table 4. Results of PERMANOVA analysis on species composition and abundance of macroalgal assemblages in experimental plots and pairwise tests for interaction factor N × I (Nutrient × Invasion). N–: not nutrient-enriched (c, i: control vs. invaded); N+: nutrient-enriched (n, i: nutrient vs. nutrient+ invaded); I–: not invaded (c, n: control vs. nutrient-enriched); I+: invaded (i, n + i: invaded vs. nutrient-enriched+invaded). Significant $p_{(perm)}$ values are in **bold** type

Source	df	MS	pseudo-F	$p_{(perm)}$
N	1	3135.6	72.02	**0.001**
I	1	6811.7	156.46	**0.001**
N × I	1	609.8	14.00	**0.001**
Area (N × I)	4	43.5	0.57	0.835
Residual	40	75.9		
Total	47			

Pairwise tests (N × I)	$p_{(perm)}$			$p_{(perm)}$
N–: c, i	**0.001**		I–: c, n	**0.001**
N+: n, n+i	**0.001**		I+: i, n + i	**0.001**
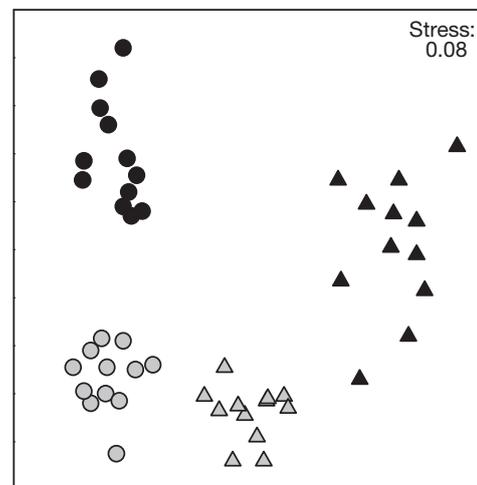
Fig. 2. nMDS ordination of species composition and abundance of macroalgal assemblages of all replicate plots: (◯) control, (●) nutrient-enriched, (△) invaded, (▲) nutrient-enriched+invaded
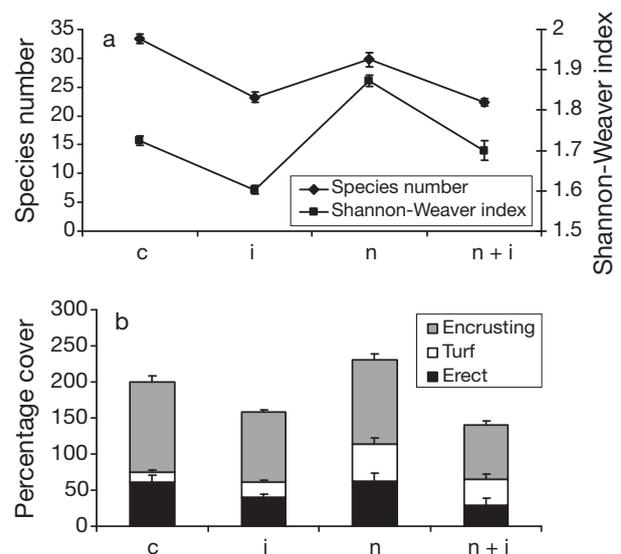

Fig. 3. Vegetation cover of experimental plots (mean ± SE, n = 12). c: control, i: invaded, n: nutrient-enriched, n+i: nutrient-enriched+invaded. (a) Number of species and Shannon-Weaver index; (b) percentage cover of vegetation layers (encrusting, turf and erect)

In control plots, species number and total percentage cover were 33.3 ± 1.7 and 195.8 ± 8.9 respectively; lowest values for both these variables were found in the nutrient-enriched+invaded plots. Invaded plots showed intermediate values between control plots and nutrient-enriched+invaded ones. Total percentage cover and percentage cover of turf layer showed the highest values in nutrient-enriched plots (238.9 ± 8.4) while percentage cover of encrusting and erect

layers showed minimum values in nutrient-enriched+ invaded plots (Fig. 3). ANOVA detected significant interaction between nutrient and invasion for total percentage cover, number of species, and percentage cover of all 3 algal layers. The SNK test showed that values of species number, total percentage cover and cover of erect and encrusting layers in invaded plots (both treatments) were significantly lower than in non-invaded plots, while the lowest values were detected in nutrient-enriched+invaded plots. The SNK test also showed that differences between nutrient-enriched plots (both treatments) and not enriched plots were not significant for species number or for cover of erect and encrusting layers, while total percentage cover and cover of turf layer were significantly higher in nutrient-enriched than in not enriched plots; turf cover was also significantly higher in nutrient-enriched plots than in nutrient-enriched+invaded plots. The Shannon-Weaver index was higher in non-invaded plots than in invaded ones and in nutrient-enriched plots compared to not enriched ones (Table 6).

Table 5. Results of SIMPER test showing taxa contributing most to differences between experimental treatments. c: control, n: nutrient-enriched, i: invaded, n+i: nutrient-enriched+ invaded. Values in bold in the right-hand column show the average dissimilarity between the 2 treatments in the columns to the left

Taxon	Mean % cover	Mean % cover	Contribution (%)
	c	**i**	**19.4**
Peyssonnelia spp.	45.42	18.92	19.31
Encrusting Corallinales	200	174.17	18.97
Laurencia chondrioides	38.75	20.75	13.11
Flabellia petiolata	42.5	35.83	6.28
Meredithia microphylla	9.1	3.08	5.69
Halopithys incurva	1.83	7.82	5.18
Womersleyella setacea	18.42	24.08	4.36
Zanardinia typus	4.02	1.33	3.01
Cladophora prolifera	0.38	3.77	2.48
Halimeda tuna	3.08	0	2.24
	c	**n**	**18.48**
Womersleyella setacea	18.42	87.5	42.81
Laurencia chondrioides	38.75	42.33	7.04
Meredithia microphylla	9.1	15.33	6.07
Halopithys incurva	1.83	4	2.66
Jania adhaerens	0.97	3.03	1.47
Pseudochlorodesmis furcellata	0.83	2.53	1.1
	c	**n+i**	**35.19**
Encrusting Corallinales	200	135.42	27.62
Peyssonnelia spp.	45.42	14.17	13.29
Laurencia chondrioides	38.75	8.18	13.01
Womersleyella setacea	18.42	48.75	12.95
Flabellia petiolata	42.5	25.75	7.17
Meredithia microphylla	9.1	2.77	3.36
Halopithys incurva	1.83	8.08	3.06
Tricheocarpa fragilis	10.25	4.93	2.7
Cladophora prolifera	0.38	5.67	2.26
Zanardinia typus	4.02	0	1.72
Halimeda tuna	3.08	0	1.31
	i	**n+i**	**22.84**
Encrusting Corallinales	174.17	135.42	28.88
Womersleyella setacea	24.08	48.75	18.37
Laurencia chondrioides	20.75	8.18	9.38
Flabellia petiolata	35.83	25.75	7.6
Tricheocarpa fragilis	11.17	4.93	4.86
Peyssonnelia spp.	18.92	14.17	3.82
Meredithia microphylla	3.08	2.77	2.41
Pseudochlorodesmis furcellata	0.33	2.95	1.95

DISCUSSION

The results of the present study show that *Caulerpa racemosa* growth was enhanced by nutrient enrichment. Moreover, the effects of invasion on native macroalgal assemblages were stronger where nutrients were added.

The growth rates of *Caulerpa racemosa* observed in the present study, the values of biomass and the percentage cover in not nutrient-enriched plots were lower than those reported for shallower habitats in the same area (Piazzi & Cinelli 1999, Piazzi et al. 2001) and more similar to values reported for deeper populations along the French coasts (Ruitton et al. 2005), indicating the influence of depth in spread dynamics of the alga. Nutrient enrichment caused an increase in *C. racemosa* growth rate, resulting in higher values of biomass, stolon length, number of frond and patch size, which were clearly detected in spite of high variability among selected areas. These findings are in accordance with previous observations of the ecological characteristics of other *Caulerpa* species. The expansion of *C. prolifera* Forsskål (Lamouroux) along French coasts has been suggested to be related to the increase of domestic sewage pollution (Ollivier 1929) and thick mats of *C. verticillata* J. Agardh have been observed in sewage canals in Florida (Lapointe et al. 1994). The spread of introduced species, such as *C. taxifolia* in the Mediterranean Sea, *C. brachypus* f. *parvifolia* (Harvey) Cribb in Florida and *C. ollivierii* Dostál in the Bahamas is also considered to be enhanced by eutrophication (Chisholm et al. 1995, Lapointe et al. 2005, Lapointe & Beldford 2010). Results of the present study showed that invasion by *C. racemosa* may also be faster in eutrophic areas, suggesting that it could be favored by an increase of pollution in the coastal water of the Mediterranean Sea.

Effects of nutrient enrichment on the structure of macroalgal assemblages were lower than those described in other studies (Karez et al. 2004, Kraufvelin 2007) since no species disappeared as a consequence

Table 6. Results of ANOVA analysis on total percentage cover, percentage cover of vegetation layers (erect, encrusting, turf), species number per sample, and values of Shannon-Weaver index (H'), and results of SNK test for interaction factor N × I (Nutrient × Invasion). N(I): N × I comparisons for pairs of levels of factor 'Nutrient'; I(N): N × I comparisons for pairs of levels of factor 'Invasion'; c: control; n: nutrient-enriched; i: invaded; n+i: nutrient-enriched+ invaded; N–: not nutrient-enriched (c and i); N+: nutrient-enriched (n and n+i); I–: not invaded (c and n); I+: invaded (i and n+i). Significant p-values are in **bold** type; ns: not significant

Source	df	MS	F	p	Source	df	MS	F	p
Percentage cover					**Turf**				
N	1	6627	31.7	**0.001**	N	1	32489.6	445.9	**0**
I	1	238854.1	1145.8	**0**	I	1	863.6	11.8	**0.004**
Area (N × I)	12	208.4	0.3	0.959	Area (N × I)	12	72.8	0.4	0.906
N × I	1	47125.3	226.0	**0**	N × I	1	6320.4	86.7	**0**
Residual	32	542.5			Residual	32	148.9		
Total	47				Total	47			
Cochran's C = 0.243 (ns)					Cochran's C = 0.241 (ns)				
SNK test N × I	N(I)	c < n	I(N)	c > i	SNK test N × I	N(I)	c < n	I(N)	c = i
		i > n+i		n > n+i			i < n+i		n > n+i
Erect					**Species number**				
N	1	283.2	0.9	0.344	N	1	58.5	12.8	**0.003**
I	1	24201.1	82.8	**0**	I	1	910.0	199.4	**0**
Area (N × I)	12	291.9	1.1	0.382	Area (N × I)	12	4.5	1.6	0.141
N × I	1	4700.5	16.1	**0.001**	N × I	1	22.6	4.9	**0.045**
Residual	32	262.0			Residual	32	2.8		
Total	47				Total	47			
Cochran's C = 0.191 (ns)					Cochran's C = 0.204 (ns)				
SNK test N × I	N(I)	c = n	I(N)	c > i	SNK test N × I	N(I)	c = n	I(N)	c > i
		i > n+i		n > n+i			i > n+i		n > n+i
Encrusting					H'				
N	1	11535.1	48.9	**0**	N	1	0.157	18.1	**0.001**
I	1	58569.2	248.29	**0**	I	1	0.124	14.3	**0.002**
Area (N × I)	12	235.8	1.44	0.198	Area (N × I)	12	0.008	0.7	0.693
N × I	1	1743.6	7.39	**0.018**	N × I	1	0.001	0.1	0.707
Residual	32	163.5			Residual	32	0.011		
Total	47				Total	47			
Cochran's C = 0.174 (ns)					Cochran's C = 0.229 (ns)				
SNK test N × I	N(I)	c = n	I(N)	c > i	SNK test	N	N– < N+		
		i > n+i		n > n+i		I	I– > I+		

of nutrient enrichment, nor was there an increase in cover of typical species of polluted habitats. Probably the length of the experiment (about 4 mo) allowed observation of only the early phases of the eutrophication process in deep subtidal Mediterranean rocky assemblages. Nutrient enrichment caused an increase of macroalgal cover, mostly due to turf species, a pattern that has been widely described elsewhere (Gorostiaga & Diez 1996, Diaz et al. 2002, Gorgula & Connell 2004, Russell et al. 2005). Increasing nutrient levels in coastal waters are considered to be the principal cause of the dominance of turf-forming species, which can spread more quickly under nutrient overload conditions than other seaweeds (Rosenberg & Ramus 1984, Hein et al. 1995, Pedersen & Borum 1996, 1997).

The effects of addition of *Caulerpa racemosa* were similar to those described for naturally invaded deep subtidal Mediterranean assemblages (Piazzi et al. 2007a, Piazzi & Balata 2008, 2009), i.e. modification of the assemblage structure and reduced macroalgal cover and biodiversity.

The addition of *Caulerpa racemosa* in combination with nutrient enrichment resulted in more extensive modification of the assemblage structure, compared to the other treatments, and a reduction in percent cover of sensitive species was observed. *C. racemosa* competes with benthic organisms by overgrowth of stolons (Piazzi et al. 2001, Kružić et al. 2008, Baldacconi & Corriero 2009) and the enhanced abundance of *C. racemosa* caused by nutrient enrichment can amplify the effect of stolon cover on benthic assemblages. This effect can lead to severe consequences particularly in coralligenous habitats, which are highly sensitive to environmental alterations (Balata et al. 2005). The maintenance of this habitat is related to the balance between growth and death of calcareous organisms and a long recovery period is required following any damage, owing to the low growth rate of Corallinales,

which are the main builders of this habitat in the Mediterranean region (Garrabou & Ballesteros 2000). Many sessile organisms can completely or partially recover during the vegetative rest period of *C. racemosa* (Piazzi & Ceccherelli 2006, Klein & Verlaque 2009), but this period in not sufficient to allow the recovery of encrusting Corallinales. The death of the latter due to *C. racemosa* cover could have serious consequences for the conservation of Mediterranean coastal habitats.

Turf species showed lower values in plots where both stressors were present than in nutrient treated plots without the addition of *Caulerpa racemosa.* This finding confirms previous observations showing that turf species are more affected by *C. racemosa* than erect layer species, probably because the former are more susceptible to the alga's competitive mechanisms (Piazzi et al. 2001, Balata et al. 2005). However, despite the occurrence of antagonism between *C. racemosa* and turfs, the introduced turf-forming species *Womersleyella setacea* showed higher abundance in plots where both *C. racemosa* and nutrients were added than in control plots. In fact, *W. setacea* was one of the species that most increased their abundance following nutrient enrichment and was less affected by *C. racemosa* colonization than other turf-species. Thus, the final result of the interaction between nutrient enrichment and *C. racemosa* invasion was the shift from a well structured macroalgal assemblage to one strongly dominated by a small number of invasive species. This replacement process implies a simplification of the architectural complexity of macroalgal assemblages, due to a decrease in species richness and the reduction or disappearance of engineering species (such as encrusting Corallinales, Peyssonneliaceae, and erect Udoteaceae).

Although the aim of the present study was not to evaluate the role played by pollution in modifying vulnerability of macroalgal assemblages to invasion, biotic and environmental changes observed in the nutrient-enriched plots suggest that these may have lead to a further reduction in these assemblages' resistance to *Caulerpa racemosa*. In fact, the spread of invasive seaweeds is considered to be favored not only by the high ecological fitness of the invaders but also by the characteristics of the receiving environment (Dunstan & Johnson 2007) and anthropogenic disturbance, causing changes in the resource availability, often leads to a higher incidence and abundance of invaders (Schaffelke and Hewitt 2007, Valentine et al. 2007). The amount of resource availability across the resource spectrum is another factor that determines the success or failure of a seaweed invasion (Dunstan & Johnson 2007). Thus, tolerance by *Caulerpa* species to changes in resource availability and the presence of

degraded receiving habitats are both considered to promote colonization by these species (Occhipinti-Ambrogi & Savini 2003). For example, sediment traits of urban wastewater impacted seabed are described to enhance the competitiveness of *C. taxifolia* when it is in contact with Mediterranean seagrasses (Chisolm et al. 1995, Ceccherelli & Cinelli 1997). Changes in the structure of assemblages following nutrient enrichment and in particular the increase of *Womersleyella setacea*, suggest further positive feedback between impacts, as the presence of algal turfs can favor colonization by *C. racemosa* (Ceccherelli et al. 2002, Piazzi et al. 2003, Bulleri & Benedetti-Cecchi 2008). Different roles, as passenger or driver of ecosystem modifications (MacDougall & Turkington 2005), have been attributed to *C. racemosa* (Bulleri et al. 2010) and the importance of nutrient enrichment in this context could be an interesting topic for further investigation.

Nutrient concentrations tested in the present study were higher than those usually measured in the Mediterranean coastal area affected by diffuse sources of pollution. Nevertheless, values of nutrient concentration comparable to those in our study were measured in some marine coastal areas subjected to major point sources of nutrient pollution, such as sewage effluents from intensive fish farming installations (Gennaro et al. 2006) and outfalls from domestic waste treatment plants (Arevalo et al. 2007). Given the long time response of macroalgal assemblages to diffuse eutrophication sources and the short vegetative period of *Caulerpa racemosa* (July to October), we had to stress the ecosystem by means of an experimental set-up that could induce a response in assemblages over a short time period that was comparable to that caused by chronic exposure to nutrient pollution. Further investigations could test the effects of changes in amount and temporal variability of nutrient levels; in fact, impacts are known to be dependent on both the intensity of stresses and their patterns of variations (Benedetti-Cecchi et al. 2006, Bertocci et al. 2007, Vaselli et al. 2008).

Non-native macroalgal invasions are expected to increase in coming years (William & Smith 2007), as is land-based nutrient pollution in coastal waters (Howarth & Marino 2006). Our results agree with other findings (Ceccherelli & Cinelli 1997, Steen 2003, Sánchez & Fernández 2006, Lapointe & Beldford 2010), indicating that the spread of some species of non-native macroalgae in the oligotrophic regions can be favored by nutrient enrichment. The role of eutrophication in aiding invasions of non-native macroalgae must be recognized and more experimental studies on this topic are needed in order to predict the consequences of possible synergisms between these aspects of global change and to identify appropriate strategies to effectively manage the phenomenon in the future.

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