TITLE:

Size structure and dynamics of an invasive population of lineage 2 of *Asparagopsis taxiformis* (Florideophyceae) in the Alboran Sea.

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RUNNING TITLE: Population dynamics of *A. taxiformis*.

FOR CORRESPONDENCE[*]

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SUMMARY

In this study we present basic population data of the red macroalga *Asparagopsis taxiformis*, widely recognized as invasive in the Mediterranean Sea. A 13-month field study was carried out on a population located in southern Spain, addressing its phenology, population dynamics and demography. We further tested whether biomass variations were related to environmental variables at the study site. Gametophytes were present year-round while tetrasporophytes were only found in spring and summer. Recruitment capacity and vegetative growth of the gametophytes are discussed as important modulators for the population structure and enhancers of its persistence. Thallus size-time histograms revealed a high prevalence of small shoots that showed high mortality that was not related to self-thinning. Biomass of *A. taxiformis* was higher from March to July. Vegetative growth was the main way the gametophyte population was maintained, although the presence of tetrasporophytes and fertile gametophytes in the field confirms that sexual reproduction also occurs. Its continuous and high recruitment, in terms of the number of smallest shoots, makes this southern population of *A. taxiformis* a source of future invasive populations due to the intensive maritime traffic in the region.

KEY WORDS: Demography, Gini coefficient, phenology, self-thinning.

INTRODUCTION

The invasive macroalga *Asparagopsis taxiformis* (Delile) Trevisan de Saint Leon is considered to be a major threat to native coastal ecosystems in the Mediterranean region (Bordalba & Quesada 2010). Native to the Indo-Pacific region (Harvey 1855) and remarkably ubiquitous (see Figure 1B in Dijoux *et al.* 2014 for worldwide distribution), *A. taxiformis* is actually a species complex composed of six cryptic lineages, with different geographical distributions (Andreakis *et al.* 2007, 2016; Dijoux *et al.* 2014; Zanolla & Andreakis 2015). Broad physiological plasticity and high genetic variability (Zanolla *et al.*, 2015; Zanolla *et al.*, unpublished data) nominate IL2 to be the most invasive lineage within the species complex (in the sense of Boudouresque & Verlaque 2010). In the Mediterranean Sea the Indo Pacific lineage 2 (IL2) dominates the central and western basin (Andreakis *et al.* 2007; Dijoux *et al.* 2014) and lineage 3 has been only sampled once in Lebanon (Andreakis *et al.* 2007). Aside from its fast spread in the Mediterranean Sea, its presence is related to detrimental effects in native community. Hence, as a precautious strategy, it has been included in several Spanish and European management programs, yet most of the ecological characteristics of this noticeable invader remain unknown.

The species is characterized by the alternation of erect gametophytes (n), which can grow up to 26 cm (pers. obs.), with spring –summer filamentous tetrasporophytes (2n). They form pompom-like structures and live free floating and/or entangled in benthic algae. These filaments, in turn, are born from carpospores (2n) formed in the microscopic carposporophytes (2n) that remain parasitic to the gametophyte.

Effective monitoring strategies of invasive species should be based on the knowledge of their biology and ecology (Meinesz 2007). This information is also essential for understanding the invasive processes (Sakai *et al.* 2001), predicting their future impacts and for the implementation of preventive and management actions (Araujo *et al.* 2011). Despite many studies that have been conducted on *A. taxiformis* in the last decade (e.g. Andreakis *et al.* 2007, 2009; Altamirano *et al.* 2008; Sherwood 2008; Dijoux *et al.,* 2014; Zanolla *et al.,* 2014), no ecological studies providing information about its population structure or demographic dynamics are available. Many studies agree that population attributes of species invasions are essential in understanding how, when and where an invasive species will become dominant. Additionally, overall, little is known about the demographic characteristics and consequences of the complexities of triphasic life histories (Engel *et al.* 2001).

Given that impact of introduced species is directly linked to both final range of expansion and their local abundances in host communities (Parker *et al.* 1999), it is imperative to have detailed information on the ecology of the species in its range of distribution to increase our capabilities for prediction (Araujo *et al.* 2011). Therefore, the aim of this study was to describe the vegetative phenology, population structure and dynamics of an established population in southern Spain of lineage 2 of *A. taxiformis* (IL2). We further tested the relationship of biomass changes with several presumed control environmental variables. This study highlights the importance of vegetative traits in invasive population maintenance and therefore the risk of expansion of *A. taxiformis* in the Mediterranean region.

MATERIALS AND METHODS

Study area and sampling procedure- In the Alboran Sea, stable populations of *Asparagopsis taxiformis* can be found from Almería to Cádiz (Altamirano *et al.* 2008) almost uninterruptedly. This study was specifically conducted in Marina del Este, Granada (Spain, 36° 43'N, 3° 43'W), on a 400 m long semi-exposed coast where *A. taxiformis* inhabits rocky substrate at 1-12 m depth. Sampling was carried out monthly by SCUBA diving at 9.5 m depth, between July 2010 and November 2011. For genetic analysis at least eight specimens (four tetrasporophyte and four gametophyte stages) were collected randomly several times during the study period. A fragment of each specimen, cleansed of visible epiphytes and dried between paper tissues, was desiccated immediately in silica gel and stored until DNA extraction. Their genetic lineage corroboration was performed using the *cox*2-3 intergenic spacer following Andreakis *et al.* (2004). In a first sampling, spatial distribution pattern of the species in the study location was assessed using three 40 x 40 cm quadrats subdivided in 10 x 10 cm squares. Algal material was carefully scraped from each 10 x 10 square, placed in individual plastic bags and taken to the laboratory where it was weighed. Using these data, we estimated the aggregation index by means of the ratio between variance and mean of the biomass (Blackmann 1942) and assessed the minimal sampling area. The significance of the aggregation index was determined by the X^2 test, according to Blackmann (1942). Therefore, in the subsequent monthly samplings, three 25 x 25 cm quadrats were randomly placed and all algal material was thoroughly removed. Thanks to its morphology and presence of rhizoids, *A. taxiformis* gametophytes were easy to pull up from the rocky substrate without losing material during the removal. Care was taken not to sample the same area twice. The presence of gametophytes and tetrasporophytes was visually monitored during each sampling to establish the phenological calendar of *A. taxiformis*.

Structure and dynamics of the population - The size distribution of all shoots of *A. taxiformis* gametophytes per area was achieved by separating them into 13 size classes, each one of 2 cm (considering the main vertical axis). They were counted, freshly weighed (FW), and then oven dried at 60°C for 48 h to obtain the dry weight (DW). Rhizoids were separated from the shoots and weighed in the same way. Total biomass of the sample was computed by the addition of these two weights. The shoots of 0-2 cm were considered as new recruits for the population.

Size hierarchy (inequality) within the studied population was analyzed by means of Gini coefficient (G) (Weiner & Solbrig 1984) that measures the mean size differences among individuals in a population and thus is a commonly used indicator of the heterogeneity of size though time. It ranges from a value of 0 (when all individuals are the same size), to a maximum of 1 (when all individuals except one have a size of zero).

Given the particular size structure of the shoots, the self-thinning effect was tested as a mechanism of growth interference (Yoda 1963) by means of the biomass-density relationship using the total biomass and number of shoots collected between February and June 2011, when active growth occurs.

Environmental variables- Monthly data of average temperature, salinity and mean daylight hours were obtained from online servers for the study area (www.aemet.es and www.fomento.gob.es). Photosynthetic active radiation (PAR) was measured every minute in the air using a radiometer UV-PAR multifilter NILU-6 (Geminali, AS, Oslo, Norway) located near the study site. These data were provided by the Photobiology Service of the Central Services from the University of Málaga (Spain). Turbidity of the water was measured in every occasion using a Secchi disk. Seawater was taken monthly at 9.5 m depth, and analyzed in an automated nutrient analyzer QuAAtro AQ2 AACE (Seal Analytical Ltd, Fareham, UK), using the standard methods to measure ammonium (Slawyk & MacIsaac 1972), nitrate and nitrite (Shinn 1941; Wood *et al.* 1967) and phosphate (Murphy & Riley 1962).

Statistical analysis- Linear regression analysis was applied to the biomass of rhizoids and to the biomass of the smaller size class.

As previously recommended (Flores-Moya *et al.* 1997; Arenas & Fernández 2000), we applied the reduced major axis (RMA), also known as Model II regression, to obtain the functional relationship between biomass and density for self-thinning. The Pearson correlation coefficient was used to calculate 95% confidence intervals to test the significance of the correlation between biomass and density. RMA regression was calculated using the computer program RMA (Bohanak & van der Linde 2004).We further applied a Principal Component Analysis (PCA) to seek for significant correlations among independent or linearly combined environmental variables and total biomass of *A. taxiformis* IL2 during the study. Statistical tests were performed using the SIGMAPLOT software (Systat Software Inc., version 11.0), except for the PCA, which was carried out using MVSP software (Kovach Computing Services, version 3.1).

RESULTS

The genetic survey of the samples indicated that the population located at Marina del Este belonged to *Asparagopsis taxiformis* invasive lineage 2. While gametophytes could be found at every sampling, tetrasporophytes were only present in August and September 2010, and in April, June, July and from September to November 2011. Quantitative estimations of tetrasporophytes could not be achieved due to its sparse spatial and temporal distribution and, in general, low density. Therefore, our results correspond to the gametophyte stage. In relation to its spatial distribution, a variance: mean ratio of 2.6 ± 0.7 (significantly >1, $X^2_{0.05} = 36.96, P = 6.57$) indicated that *A. taxiformis* presents an aggregated distribution pattern in this study area.

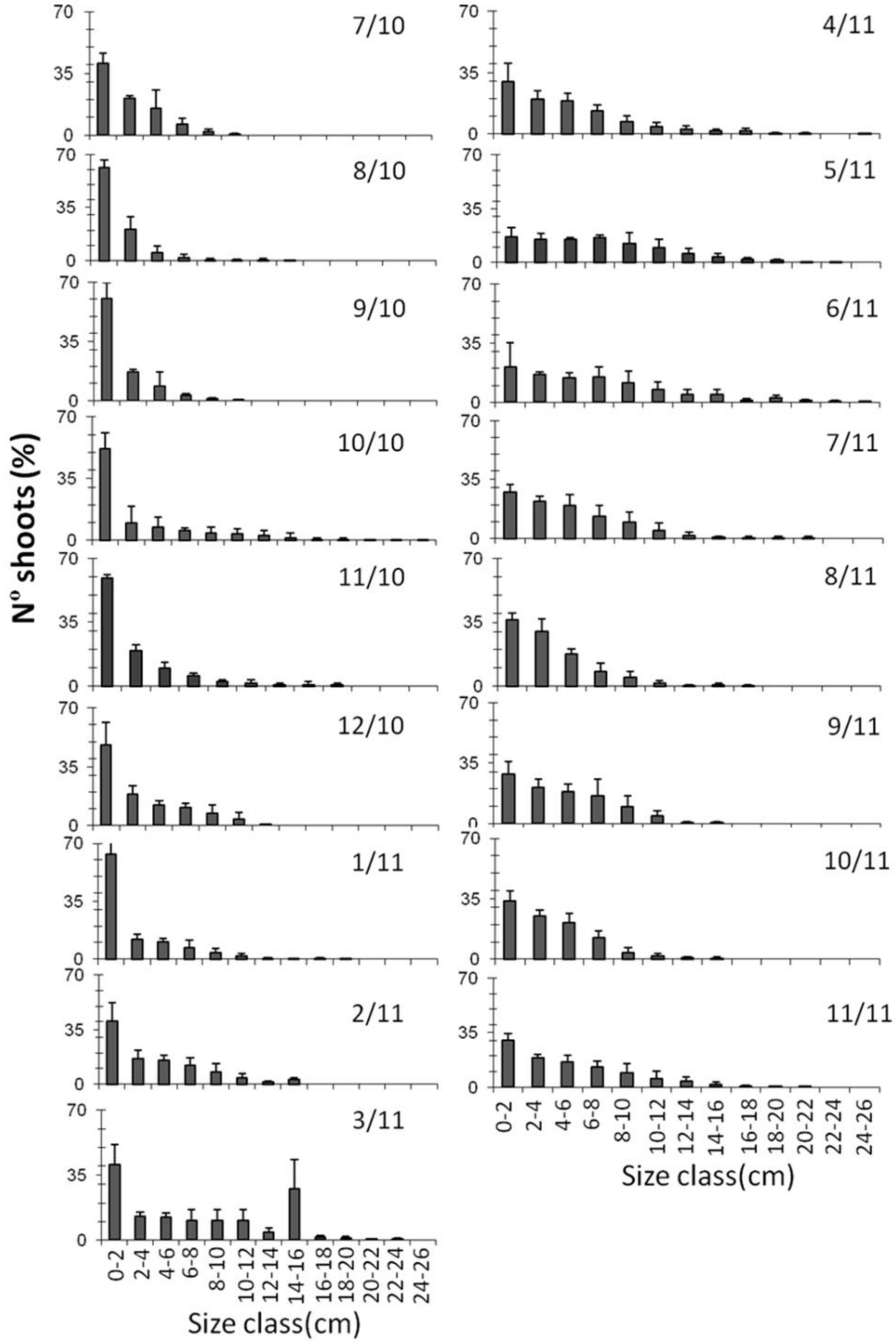

Fig. 1 Size frequency distributions of shoots of *A. taxiformis* during the study period. Data are expressed as mean ± SD. Date of sampling (month/year) is shown in the upper right corner of every plot.

Structure and population dynamics

Size distribution analysis of the *A. taxiformis* population highlighted an absolute dominance of the smaller shoot size classes (0-4 cm) throughout the whole study period (an average of 2271.2 ± 1234.3 shoots m^{-2}; Fig. 1). For instance, from August to November 2010, and in January 2011 they represented up to 60% of the total number of shoots.

Biomass of *A. taxiformis* IL2 varied in time without a marked seasonality (Fig. 2a). It showed five peaks during the study, recorded in August, October and December 2010 and from March to April 2011 and in June 2011. Mean length of shoots reached a peak in May and June 2011 (Fig. 2b) while a decrease was observed in August and September 2010. Maximum values of the Gini coefficient were recorded in October 2010 and March 2011 (Fig. 2c). Lower estimates were recorded from May to July 2011 and September and October of the same year.

The resulting RMA parameters described a non-significant relationship between density and biomass pooling across sampling times (R = -0.234; P = 0.324, n=15), indicating that self-thinning did not occur in the studied population of *A. taxiformis*.

Environmental variables

Winter temperatures dropped to 14ºC in November, while in the spring and fall, mean temperature was 18ºC, followed by an increase of 4ºC in the summer (Fig. 3a). Salinity was relatively homogeneous during the sampling period, ranging between 36.6 and 37.2 psu. The amount of sun hours and PAR showed similar variations (Fig. 3b) and no turbidity was detected at -9.5m. Thus, we assumed that light attenuation was proportional to the incident light. As for the water nutrient concentrations, ammonium was negligible in some months, while a maximum value of 9.29 µM was

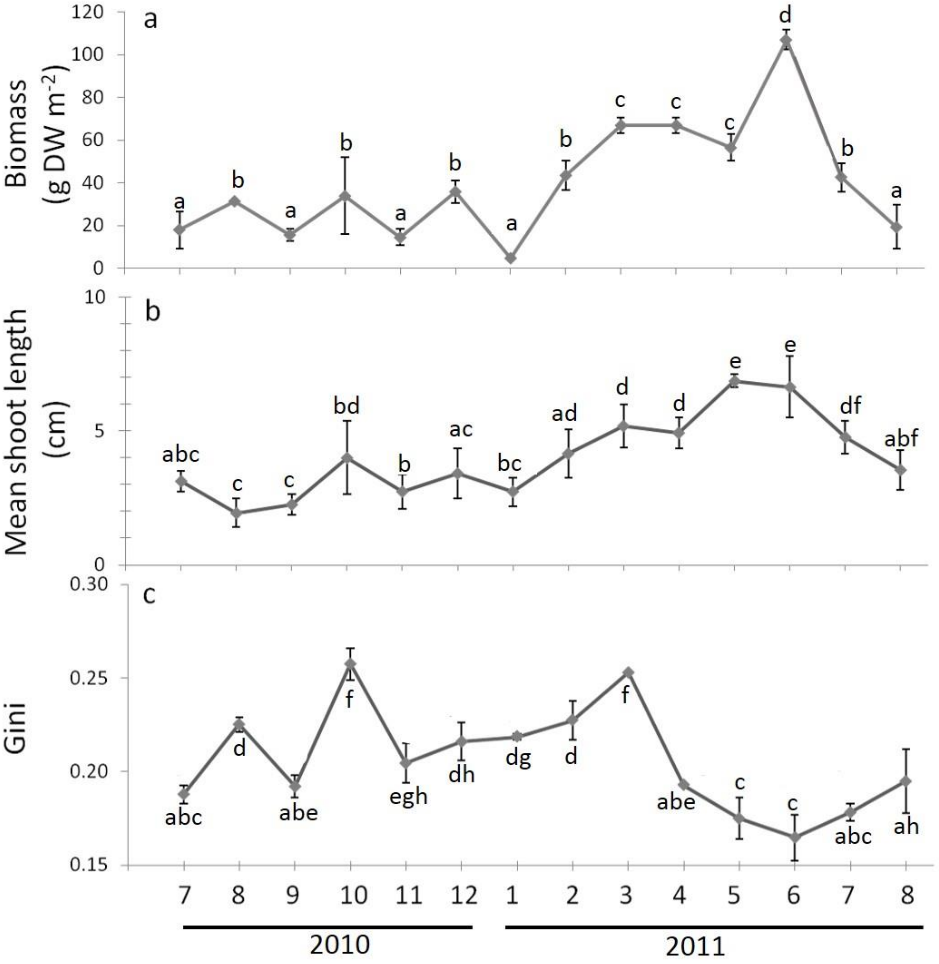

Fig. 2. Total biomass (a), mean shoot length (b) and Gini coefficient (c) in *A. taxiformis* along the study period (July 2010-November 2011). Data are expressed as mean ± SD.

recorded in November 2010 (Fig. 3c). Phosphate, on the other hand, was only detected in October 2010, which coincided with high concentrations of nitrate and nitrite. In general, nitrate values averaged 0.6 μM during the study and nitrite concentrations ranged between 0 to 0.1 μM (Fig. 3c).

All environmental variables fluctuated during the study, but no significant correlations were found between each of them and *A. taxiformis* biomass (data not

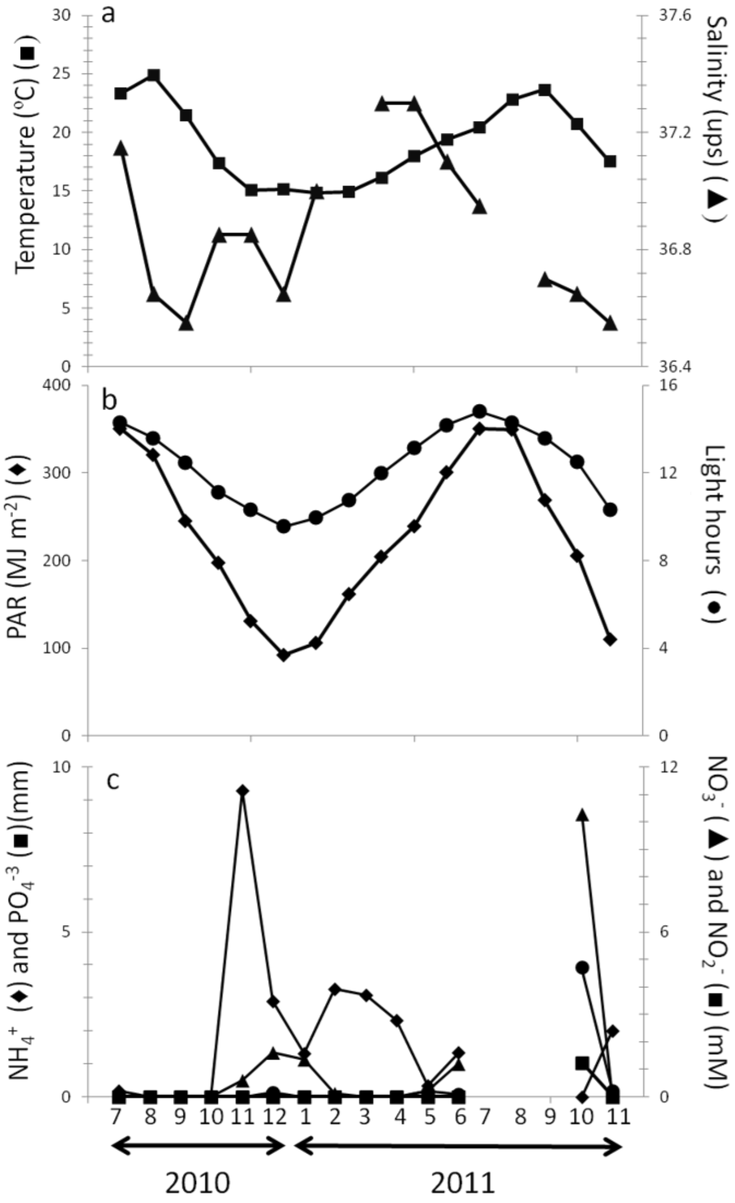

Fig. 3. Environmental variables during the study period (July 2010-Novemeber 2011): a) temperature (■) and salinity (▲); b) monthly doses of PAR (♦) and monthly light hours (●); c) ammonium (♦), phosphate (■), nitrate (▲) and nitrite (●) concentration in the seawater column. No nutrient data are available for July, August and September 2011.

shown). Thus, data were recombined around new axes named “components” in order to maximize the explanation of the whole data variability. PAR and nitrogen were the major components of Axis I (loadings values > 0.34), whereas those more relevant in Axis II were temperature and the amount of sun hours (loadings values > 0.45). Nevertheless, no significant correlation was found between biomass of *A. taxiformis* and Axis I (R = 0.058; P = 0.829; n = 16) or II (R = 0.059; P = 0.828; n = 16).

DISCUSSION

The year-round presence of *Asparagopsis taxiformis* IL2 in Marina del Este highlights the importance of this species as an invader in the studied area, given that permanent invasive populations are likely to have more impact on native communities. Vegetative propagation was accounted for in this study as the major form of population maintenance, though sexual reproduction, evidenced by the formation of the tetrasporophytic generation, could play a pivotal role in contributing to the genetic variability this lineage has in the Mediterranean area (Andreakis *et al.* 2009).

In the present study, the environmental conditions inherent to southern Spain are not restrictive for *A. taxiformis*. We suggest that the year round presence of *A. taxiformis* IL2 is related to the ability to maintain its photosynthetic performance in the thermal range of 12-26ºC (Zanolla *et al.* 2015). Similarly, biomass, percentage cover and production in *Womersleyella setacea* (Hollenberg) R. E. Norris, another invasive tropical taxa, did not present a clear seasonal pattern in the Mediterranean Sea (Cebrián & Rodríguez-Prieto 2012). This lack of seasonality was related to a continuous asexual vegetative spread throughout the year, as it happens with *A.*

taxiformis, and to the stability that temperature and nutrient availability gain with depth.

For seaweeds, temperature, photoperiod and nutrients are the most relevant bottom-up factors in modulating their distribution and abundance (Breeman 1988). Abiotic dependence on many biological mechanisms may therefore be used to exploit temporal windows of increased risk of invasion. In the case of the studied population of *A. taxiformis*, total biomass was not directly related to the combination of environmental variables tested here. However, we cannot discard entirely their influence, given that the species responds to the environment by producing several cohorts that contribute differently to the whole year production (unpublished data). This year-round biomass production supported by favorable climate conditions at the study site probably prevents finding these significant correlations.

The biomass increase observed in the spring, followed by a strong decay in the summer, indicates that this might be an important growing time for this species, though it should be verified in other *A. taxiformis* populations.

Population dynamics

The active recruitment period for *A. taxiformis* was restricted from late summer to the end of winter, not being so noticeable in the rest of the study. Our methodology did not allow us to discern between those recruits generated by vegetative propagation from those generated by tetraspore germination, but the sporadic presence of tetrasporophytes leads to think that vegetative recruitment plays a relevant role in this population. The analytical approach used to uncover *A. taxiformis* demography was taken from its sibling species, *A. armata*, subject of similar studies (Aranda *et al.* 1984; Flores-Moya *et al.* 1997). *Asparagopsis taxiformis* size distribution, dominated by the smallest thalli, has also been reported for other clonal seaweeds, such as *Mazzaella laminaroides* (Bory) Fredericq (Martínez & Santelices 1992) and *Gelidium corneum* (Hudson) J.V. Lamouroux, in which frond recruitment is a consequence of vegetative growth from a prostrate axis rather than from the development of new fronds by spores (Santos 1995; Carmona & Santos 2006). This is further supported by the fact that tetrasporophytes were not observed continuously throughout the year, which produces the tetraspores that give rise to young gametophytes.

The importance of vegetative multiplication of gametophytes has already been shown in populations of *A. taxiformis* from India, since sexual cycle does not seem to complete on those coasts (Kumar *et al.* 2000). In the present study, multiplication of gametophytes is supported by the significant positive correlation ($R = 0.32$; $n=38$; $P < 0.05$, Suppl. Fig 1) between the biomass of rhizoids and the biomass of the smallest shoots (the 0-2 cm size class). This positive correlation suggests that greater rhizoid development leads to the formation of new shoots. Another form of vegetative propagation was observed in the studied population, consisting in the proliferation of apical cells of the gametophytes in a hook-like structure that detach from the main thallus (pers. obs.)These propagules can reach up to 5,855 per shoot (Mairh 1977), thus representing an important way of vegetative proliferation.

Since the correlation between density and mean size was not significant, self-thinning was not the cause of the high mortality of younger shoots. Therefore, this mortality is probably caused by other factors. In other clonal Rhodophyta, e.g. *Gelidium corneum*, *Mazzaella parksii* (Setchell and N. L. Gardner) Hughey, P.C. Silva and Hommersand, *Pterocladiella capillacea* (S.G. Gmelin) Santelices and Hommersand and *Chondrus crispus* Stackhouse, the lack of this intraspecific regulating mechanism was related to the persistence of fixation structures and shoot regrowth (Scrosati 2005), density-dependent production of shoots (Scrosati & De Wreede 1997) and physical factors (Fernández & Menéndez 1990). In *A. taxiformis* the absence of self-thinning might be related to density-dependent production of shoots, abiotic factors (nutrient availability) or growth from pre-existing rhizoids, which might prevent them from reaching the minimal size for self-thinning to occur. Thus, more research is needed to reveal the cause of the high mortality of these young shoots.

The results of this study suggest that size inequality is a major characteristic in the population of *A. taxiformis* studied. Variation in biomass and Gini coefficient showed different patterns during the study period. High values of G can be achieved by the presence of most size classes in different proportions, which happened in October 2010 and March 2011. Both peaks of G match with a period of active growth and intermediate mean frond sizes. G reduction in March 2011, on the other hand, might be attributed to a peak in the formation of reproductive structures in the gametophytes that starts in this month (pers. obs.). Another explanation for the G reduction might rely on the fact that, maturation not being simultaneous, tallest reproductive fronds would stop growing whereas juvenile fronds could still keep on growing, which would translate into a reduction of G (Arenas & Fernández 2000). In general, Gini coefficients remained high all through the cooler season and were lower in the hot season when recruitment is reduced and all size classes equal their abundance.

Following the results given in the present study, *Asparagopsis taxiformis* IL2 is expected to expand or at least maintain its populations in the area. The dynamics of this population, by overlapping two macroscopic stages of its life cycle show how two different life stages can combine and thus promote the success of this species as an invader. Gametophytes would be in charge of population maintenance, as well as being the main culprit of the negative impact of this species in native communities, which is aggravated by its year round presence. On the other hand, tetrasporophytes would be in charge of remote dispersal and therefore the species expansion, apart from increasing genetic variability through meiosis. This hypothesis is supported by 1) higher survival rates in ballast waters conditions, associated with higher thermal photosynthetic performance of the tetrasporophytes compared to gametophytes (M. Zanolla pers. obs., Zanolla *et al.* 2009); and 2) their own morphology: tetrasporophytes are mostly found free floating, hence being able to be easily transported by currents.

Once established, *A. taxiformis* populations are difficult to eradicate by manual means, given their high biomass and coverage. Therefore, facing management recommendations, we propose in those areas where this species has been recently introduced, to carry out a specific experiment of gametophytes removal to assess its viability. This would mitigate the impact of this stage on the host macroalgal communities. If effective, it should be done from August to January, when biomass is lower. Rhizoids should be carefully removed from the substrate. *A. taxiformis* shoots, once detached from substrate lose their re-fixation capacity (R. Carmona pers.com.), which, in this case, might be advantageous to avoid its expansion.

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Figure legends

Fig. 1. Size frequency distributions of shoots of *A. taxiformis* during the study period. Data are expressed as mean ± SD. Date of sampling (month/year) is shown in the upper right corner of every plot.

Fig. 2. Total biomass (a), mean shoot length (b) and Gini coefficient (c) in *A. taxiformis* along the study period (July 2010-November 2011). Data are expressed as mean ± SD.

Fig. 3. Environmental variables during the study period (July 2010-Novemeber 2011): a) temperature (■) and salinity (▲); b) monthly doses of PAR (♦) and monthly light hours (●); c) ammonium (♦), phosphate (■), nitrate (▲) and nitrite (●) concentration in the seawater column. No nutrient data are available for July, August and September 2011.

Supp. Fig.1: Linear regression analysis applied to biomass values from new recruits and rhizoids in *A. taxiformis*.