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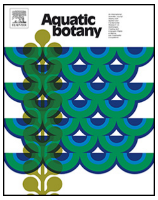

Differences in herbivory intensity between the seagrass *Cymodocea nodosa* and the green alga *Caulerpa prolifera* inhabiting the same habitat

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ABSTRACT

Seagrasses are frequently found mixed with other macrophytes, e.g., green macroalgae. We aimed to assess whether the magnitude of herbivory differed between two coexisting macrophytes, the seagrass *Cymodocea nodosa* (Ucria) Ascherson and the green seaweed *Caulerpa prolifera* (Forsskål) Lamouroux, at Gran Canaria Island (eastern Atlantic). Both in situ (field) and aquaria experimentation demonstrated a larger intensity of herbivory (between ca. 4–8 times) on *C. prolifera* than *C. nodosa*. At the scale of meadows, herbivorous fish abundance predicted the intensity of herbivory, in particular by the parrotfish *Sparisoma cretense*. A plant physical attribute ("force-to-fracture") negatively correlated with a larger consumption on *C. prolifera*, while differences in total phenolic compounds between both macrophytes were insignificant. Importantly, herbivory marks (bites) were significantly larger (ca. two times) on *C. nodosa* leaves than in *C. prolifera* fronds, so differences in the magnitude of herbivory between *C. nodosa* and C. *prolifera* were dependent on herbivorous size.

1. Introduction

Many organisms are associated with seagrasses, below the sediment linked to the rhizomes, upon the leaves and stems, and over the seagrass canopy (epi- and suprabenthic organisms, Herrera et al., 2014; Tuya et al., 2014b), which move throughout the meadow and constitute the main consumers of seagrass and associated vegetated material. Seagrasses are evolutionarily adapted to herbivory; this is indicated by the range of evolutionary adaptions to mitigate the consequences of herbivory (Karban and Myers, 1989), including mechanical and chemical elements (Lucas et al., 2000). Traditionally, it has been postulated that a small fraction of seagrass production is directly consumed by marine herbivores (Cebrián and Duarte, 1998; Valentine and Heck, 1999). The low consumption of seagrass by grazers has been explained by their poor nutritional quality (Prado and Heck, 2011), including a high content in cellulose that act as a structural deterrent. Recent studies; however, have pointed out that herbivory over seagrasses has a larger influence than previously considered (Tomas et al., 2005; Heck and Valentine, 2006; Doropoulos et al., 2009; Prado et al., 2007; Vergés et al., 2011). Seagrasses offer herbivores two main food sources: epiphytes on seagrass leaves and the seagrass itself (Wressnig and Booth, 2007), as well as flowers and seeds (Balestri and Cinelli, 2003). Epiphytes are a key element in the relationship between herbivores and seagrasses. Some studies suggest that epiphytic production may be elevated to exceed even that of seagrasses (Morgan and Kitting, 1984; Chiu et al., 2013). Temperate and sub-tropical fishes consuming seagrass material select seagrass leaves and parts of leaves with abundant epiphytic loads, whereas mesograzers usually feed on algae attached to seagrass leaves (Valentine and Heck, 1999; Goecker et al., 2005). It has been postulated that internal contents in N are a relevant factor mediating feeding preferences by marine herbivores (Vergés et al., 2007; Prado et al., 2010; Prado and Heck, 2011), despite in other occasions its influence is negligible (Lee et al., 2015).

Cymodocea nodosa is a seagrass distributed across the entire Mediterranean and the adjacent Atlantic coasts, from the southern Iberian Peninsula to Senegal, including Madeira and the Canary Islands (Cunha and Araujo, 2009). Meadows constituted by *C. nodosa* are found on shallow soft substrates of Gran Canaria Island (Tuya et al., 2014a), where it may form mixed meadows with green rhizophytic seaweeds of the genera *Caulerpa*, particularly *Caulerpa prolifera* (Fig. A1). As a result of environmental deterioration, frondose *C. nodosa* meadows can turn into bottoms dominated by *C. prolifera*; this has been reported from the Mediterranean and the southern Iberian Peninsula (Ceccherelli and Cinelli, 1997;

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Lloret et al., 2005), as well as from the Canary Islands (Tuya et al., 2013b). *C. nodosa* may be an important food source for macro-herbivores (Cebrián et al., 1996a). In addition, leaves of this seagrass are colonized by epiphytic assemblages that may provide food for associated invertebrates (Vizzini et al., 2002; Tuya et al., 2013a). Accompanying macrophytes, e.g., green seaweeds, can also represent an additional food source for herbivores. However, certain macrophytes have developed several mechanisms to minimize herbivory (Duffy and Hay, 1990). The primary deterrent substances in seagrasses and seaweeds are phenolic compounds (Arnold et al., 2012), which have been linked to a variety of functions, preventing bacterial infections (Harrison and Chan, 1980), protecting algae from high PAR and UV damage (Pavia et al., 1997) and deterring grazers (Van Alstyne and Paul, 1990). Yet, there is some controversy in the real effectiveness of phenols as grazer deterrents (Close and McArthur, 2002; Vergés et al., 2007). For algae within the genera *Caulerpa*, it has been largely hypothesized that the presence of repulsive (toxic) secondary metabolites, e.g., caulerpenyne, may also deter herbivores (Box et al., 2010). Preference for vegetated material among herbivores is; however, not exclusively related to chemical attributes (Hay and Kappel, 1994), but also to the physical structure and configuration of macrophytes, e.g., their resistance to breakage (Duffy and Hay, 1990; Lucas et al., 2000; Prado and Heck, 2011).

In mixed meadows (i.e., those constituted by seagrasses and green seaweeds), macro-herbivores have several choices of food, what may generate different patterns of vegetation consumption. The aim of this work was to compare the magnitude of herbivory between the seagrass *C. nodosa* and the green alga *C. prolifera*; these two macrophytes inhabit the same habitat (mixed meadows on shallow subtidal waters) at Gran Canaria Island (eastern Atlantic). Differences in the intensity of herbivory were compared by combining in situ assays, that assessed indirect (bite marks) and direct (rates of consumption of fresh material) measures of herbivory, and an aquaria experiment that quantified rates of consumption on fresh material under controlled laboratory conditions. We set out these procedures to specifically test whether the intensity of herbivory differed between *C. nodosa* and *C. prolifera*. We additionally hypothesized that spatial and temporal variation in herbivory intensity on these two macrophytes is connected with differences in the abundances of herbivorous fish. Finally, we analyzed differences in phenolic compounds concentration (a chemical attribute) and leaf/frond resistance to breakage (a physical attribute) between both macrophytes to help to explain differences in herbivory patters.

2. Materials and methods

2.1. Field observational approach

Four study sites were selected in mixed meadows constituted by the seagrass *C. nodosa* and the rhizophytic seaweed *C. prolifera* at the east coast of Gran Canaria Island (Fig. 1); depth ranged between 8 and 12 m, all bottoms were sandy and proximity from the adjacent coast varied between 150 and 250 m. These mixed meadows are permanent all year round (Tuya et al., 2013b, 2014b); the biomass of *C. nodosa* varies between 120 and 170 g DW m^{-2} and the biomass of *C. prolifera* between 0 and 70 g DW m^{-2} across sites 10 s of meters apart (Tuya et al., 2013b). Alternative vegetation is sparse and mainly restricted to epiphytes growing on seagrass leaves. The seagrass shows a clear seasonal pattern, including a maximum in shoot density and biomass in summer and a minimum in winter (Tuya et al., 2006). No information is available on seasonal patterns of *C. prolifera*. We developed different types of assays to evaluate the magnitude of herbivory on *C. nodosa* leaves and fronds of *C. prolifera*. Firstly, we conducted an indirect approach by estimating herbivory pressure as the number of bite marks left by herbivores on both *C. nodosa* leaves and *C. prolifera* fronds; since the majority of fishes inhabiting these seagrass systems are small-sized (Espino et al., 2011), complete removal of seagrass leaves is an unlikely process. The study was carried out at two times: October 2013 and May 2014 to test for the effect of seasonality on responses. At each of the 4 meadows, 12 leaves of *C. nodosa* and 12 fronds of *C. prolifera* were haphazardly collected by SCUBA divers; adjacent leaves/fronds were >2 m apart. Samples were quickly transported to the laboratory and preserved in ice until analysis. At the same time of collection, fish assemblages were counted at daylight hours (between 9:00 and 12:00 a.m.) through underwater visual censuses, following ($n = 4$) 25 × 4 m transects per meadow (100 m^2 of observation per census); the abundance and size of each fish species was annotated according to standard procedures implemented in the study region for seagrass meadows (Tuya et al., 2006). No major herbivorous invertebrates (e.g., sea-urchins) were found in the study area. Once in laboratory, we measured the length of *C. nodosa* leaves (from the ligule to the upper tip of each leaf) and *C. prolifera* fronds (from the base of the stipe to the upper tip of the frond). Fronds with proliferations were not considered to avoid confusion. Bite marks were recorded for each leaf/frond through image analysis (imageJ freeware); all material was then preserved in silica gel. Some bite marks were clearly crescent-shaped (Fig. A2), a clear indication of consumption by herbivorous fishes (Hay, 1984; Kirsch et al., 2002; White et al., 2011; Lee et al., 2015). In these cases, we recorded each bite size, as the maximum diameter of the mark (cm). The cover of epiphytic material was also annotated by using a qualitative, visual, scale: 0 (cover: <1%), 1 (cover: 1–10%), 2 (cover: 10–20%), 3 (cover: 20–40%), 4 (cover: 40–60%) and 5 (cover: >60%). When the apical part of either seagrass leaves or *C. prolifera* fronds was damaged, we omitted to record these as bite marks, due to the difficult of ascertaining if these marks resulted from herbivory or other type of damage (e.g., currents and/or swells).

We took measurements of the "force-to-fracture" (FTF), as a way to assess the physical resistance to breakage of both macrophytes; these measurements were calculated with a dynamometer. The tip of each of $n = 30$ leaves and fronds of both *C. nodosa* and *C. prolifera* was attached to the pin of the dynamometer; the force (Newtons) necessary to tear each leaf/frond was then annotated. All leaves and fronds were collected at Gando meadow (May 2014), encompassing the entire range of available sizes; measurements were taken from fresh material immediately after collection. On the 17th December 2013, we randomly collected leaves of *C. nodosa* and thalli of *C. prolifera* (ca. 0.25 g FW each thalli, $n = 9$) from Gando meadow (8–10 m) to analyze differences in total phenolic compounds. All material was stored at −80 °C until analysis. Once in the laboratory, all thalli were initially cleaned and epiphytes removed. In all cases, we selected the central parts of the thalli, with no evidence of grazing activity. All samples were grounded with a mortar and a pestle in sand at 4 °C, and extracted overnight in centrifuge tubes with 2.5 ml of 80% (v/v) methanol (Betancor et al., 2014). The mixture was centrifuged at 4000 rpm for 30 min and the supernatants were collected (Sigma 2-16PK, Göttingen, Germany). Total phenolic compounds, expressed as mg GAE g^{-1} DW (Gallic Acid Equivalent), were determined using gallic acid as a standard (Folin and Ciocalteu, 1927) after 120 min in darkness at 4 °C. The absorbance was then measured at 760 nm in a spectrophotometer (Thermo Scientific Evolution 201, UV-visible, China).

2.2. Field experimentation

Through an in situ experiment, we offered herbivores fresh *C. nodosa* leaves and *C. prolifera* fronds alternatively attached with clothespins to plastic mesh frames (Fig. A3). This was a way to