Chemoecological study of the invasive alga *Caulerpa taxifolia* var. *distichophylla* from the Sicilian coast

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Abstract

Marine invasive species and their bioactive metabolites have become critical ecological issues in the Mediterranean Sea. In particular, the highly invasive green algae *Caulerpa taxifolia* and *Caulerpa cylindracea* are known to contain the bioactive sesquiterpene caulerpenyne (**1**) and the bisindolic alkaloid caulerpin (**2**), potentially acting as chemical stressors for native species. The recent spread of a variety of *C. taxifolia*, *Caulerpa taxifolia* var. *distichophylla* (Sonder) Verlaque, Huismane & Procaccini, also raises urgent questions about its chemical composition. Indeed, the only chemical data available for this alga are limited to the seasonal variations of caulerpenyne (**1**) in samples collected in the Eastern Mediterranean. In this study, we confirmed the presence of **1** also in *C. taxifolia* var. *distichophylla* collected along the Sicilian coast, while **2** was not detected in the alga. However, caulerpin was found both in a Mediterranean specimen of *C. taxifolia*, and at a much higher level in the congeneric *C. cylindracea*. This suggests that *C. taxifolia* var. *distichophylla* differs from *C. taxifolia* in its secondary metabolism, potentially exerting dissimilar chemically-mediated impacts on native biota. Further chemical investigations on the terpenoidic content of *C. taxifolia* var. *distichophylla* led to isolate and identify squalene 2,3 oxide (**3**), phytol (**4**), and plastoquinone (**5**), along with the two unreported sesquiterpene lactones **6** and **7**. Finally, chemoecological assays clarified that caulerpenyne (**1**) at its natural

concentration in *C. taxifolia* var. *distichophylla* elicits avoidance responses in native shrimp, although sensitization was a prerequisite to significantly induce food rejection.

Keywords
Biological invasions, invasive macroalgae, secondary metabolites, chemical defense, *Caulerpa taxifolia*

Introduction

A better understanding of the chemical composition of marine invasive species has become a research priority (Mollo et al. 2008, 2015), especially in the case of exotic macrophytes that are capable of replacing keystone native species, causing environmental and economic damages (Boudouresque and Verlaque 2002). Among them, both the feather-like green alga *Caulerpa taxifolia* (Vahl) C. Agardh, 1817, and the grape-like *Caulerpa cylindracea* Sonder 1845, are known to contain a variety of secondary metabolites, among which the toxic sesquiterpene caulerpenyne (**1**) and the bisindolic alkaloid caulerpin (**2**) (Figure 1) have been suggested to act as chemical stressors for Mediterranean species (Raniello et al. 2007; Vitale et al. 2018; Defranoux and Mollo 2020). In addition, caulerpenyne (**1**) has been indicated as the most active metabolite involved either in the chemical defense of *C. taxifolia*, and in interspecific competition (antifeedant and antifouling effects) (Lemée et al. 1993, 1997). Beyond their ecological role, both compounds displayed a range of biological activities of interest for possible pharmacological and biotechnological applications (Sfecci et al. 2017; Vitale et al. 2018; Defranoux and Mollo 2020, and references therein).

Compound **1** was first isolated and characterized from *Caulerpa prolifera* (Amico et al. 1978), a native species that is distributed throughout the Mediterranean Sea. It has subsequently been found as a major metabolite in most *Caulerpa* species (Meyer and Paul 1992), including *C. taxifolia*. This compound has been shown to exhibit cytotoxic activity on sea urchin eggs and fish hepatocytes (Lemée et al. 1993; Galgani et al. 1996), and phytotoxic effects on leaf tissue of the native seagrass *Cymodocea nodosa* (Raniello et al. 2007). However, measurements of caulerpenyne (**1**) levels required to determine cell toxicity, skin toxicity or food poisoning, suggested that the toxicological risk to humans is minimal (Parent-Massin 1996).

The alkaloid caulerpin (**2**), first isolated from *Caulerpa* species collected in the Philippines (Aguilar-Santos 1970), was also later reported for *C. taxifolia* (Maiti and Thomson 1977; Mao et al. 2006), but its presence in

C. taxifolia living in the Mediterranean still remains to be clarified. It is worth mentioning that, contrarily to a statement in the literature (Máximo et al. 2018), caulerpin (**2)** has also never been detected in *C. prolifera*, which is endemic to the Mediterranean. The compound is not toxic at relatively high levels when tested on mice (Vidal et al. 1984). However, several studies indicate that **2** accumulate in the tissue of fish feeding on the invasive *C. cylindracea* (Terlizzi et al. 2011; Felline et al. 2012, 2014, 2017; Gorbi et al. 2014), and suggest its possible role in fish metabolic and behavioral alterations (Magliozzi et al. 2017, 2019; Vitale et al. 2018). In addition, it has been proposed that **2** acts as a chemosensitizer by inhibiting the multixenobiotic resistance pump (MXR) in the sponge *Geodia cydonium* (Schröder et al. 1998).

Although **1** and **2** have been often invoked as a primary cause of the ecotoxicological impact of invasive *Caulerpa* algae, it is still unclear whether *Caulerpa taxifolia* var. *distichophylla*, which is considered a variety of *C. taxifolia* based on genetic data (Jongma et al. 2013), contains both compounds. Indeed, the spread of this alga along Mediterranean coasts (Musco et al. 2014; Schembri et al. 2015; Aplikioti et al. 2016; Cevik et al. 2016; Mannino and Balistreri 2017; Shakman et al. 2017; Bitar et al. 2017; Di Martino et al. 2018; Chartosia et al. 2018; Mannino et al. 2019) raises urgent questions about its chemical composition (Musco et al. 2014). Currently, our knowledge on this issue is limited to the seasonal variation in the level of **1** in *C. taxifolia* var. *distichophylla* from the Gulf of Iskenderun in Turkey (Cevik et al. 2016). Here we examine the distribution of **1** and **2** in Mediterranean specimens of *C. taxifolia, C. taxifolia* var. *distichophylla*, and *C. cylindracea*, and report a more in-depth chemical analysis of *C. taxifolia* var. *distichophylla*. The study also aims at clarifying whether the compounds can actually play a role in chemical defense against native species.

Material and Methods

General Procedures. NMR experiments were recorded at the ICB-NMR Service Centre on a Bruker DRX 600 MHz spectrometer equipped with a TXI CryoProbe, a Bruker Avance-400 spectrometer using an inverse probe fitted with a gradient along the z-axis, and on a Bruker Avance III HD spectrometer equipped with a CryoProbe Prodigy. The NMR spectra were acquired in $CDCl_3$, and reported the chemical shifts in parts per million relative to the solvent ($CHCl_3\delta_H$ 7.26 and δ_C 77.0). ESIMS was performed on a Micromass Q-TOF MicroTM coupled with a HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW (resolution specification 5000 FWHM, deviation < 5 ppm RMS in the presence of a

known lock mass). HRESIMS spectra were acquired on a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific).

Collection of *Caulerpa taxifolia* var. *distichophylla* and extraction procedure. *Caulerpa taxifolia* var. *distichophylla* was sampled in Donnalucata along the southeastern Sicilian coast (36°45′ 17.82″ N, 14°38′ 35.13″ E) during July 2016 and stored at -20°C until the extraction. The alga clearly differed from *Caulerpa taxifolia* in its slender thallus and the lack of large rhizoidal pillars. The biological material (wet weight: 240.3 g) was cut into small pieces and then extracted with acetone both by grinding and ultrasonic treatment to increase extraction efficiency. The aqueous residue was diluted with water and extracted with diethyl ether (Et_2O) to give 950 mg of crude extract.

Extract fractionation and purification of compounds 1 and 3-7. The Et_2O extract of *C. taxifolia* var. *distichophylla* was preliminary analyzed by thin-layer chromatography (TLC) on normal phase TLC plates (Merck Kieselgel 60 F254, 0.2 mm), by using different ratios of petroleum ether/diethyl as eluent. Cerium Sulfate reagent and a UV254nm beam were employed to detect the spots. The crude extract was then first fractionated on a Sephadex LH-20 column (Amersham Pharmacia Biotech; Uppsala, Sweden) by using an isocratic solvent system of $CHCl_3$/MeOH (1:1) as mobile phase. The collected fractions were analyzed by TLC and then combined into eleven main fractions (A-K), which were analyzed by ^{1}H NMR. Fractions B (300 mg), C (84 mg), and F (20 mg) were separately subjected to further purification on columns packed with Silica gel Merck Kieselgel 60 (70-230 mesh ASTM 0.063-0.200 mm) and eluted with a petroleum ether/diethyl ether gradient, to give:

- pure squalene 2,3 epoxide (**3**; 7mg) and plastoquinone A (**5**; 9.5mg) from fraction B;
- caulerpenyne (**1**; 10 mg), and phytol (**4**; 7.0 mg) from fraction C;
- compounds **6** (0.5 mg) and **7** (0.5 mg) from fraction F, whose NMR and MS data are given below.

Compound 6: ^{1}H NMR signals ($CDCl_3$) δ: 6.83(1H, s, H-2); 6.09 (1H, bd, H-1), 5.34 (1H, bs, H-8), 2.30 (2H, m, H_2-4), 1.99 (2H, m, H_2-9), 1.68 (3H, s, H_3-13), 1.40 (1H, m, H10a), 1.25 (1H, m, H-10b), 0.92 (3H, s, H_3-15), 0.87 (3H, s, H_3-14). ^{13}C NMR signals ($CDCl_3$) δ 170.6 (s, C-12, indirect detection from HMBC *J*=7Hz), 141.2 (d, C-2), 139.3 (s, C-3, indirect detection from HMBC *J*=7Hz), 135.4 (s, C-7, indirect detection from HMBC *J*=7Hz),121.8 (d, C-8),96.3 (d, C-1), 52.8 (d, C-6), 32.8 (t, C-5 or C-10), 32.3 (t, C-10 or C-5), 29.2

(q, C-15), 28.6 (t, C-4), 26.8 (q, C-14), 25.5 (t, C-9). ESIMS *m/z* 273 $[M + Na]^+$. HRESIMS *m/z* 273.1467 (calc. for $C_{15}H_{22}O_3$ 273.1461)

Compound 7: ^{1}H NMR signals ($CDCl_3$) δ: 5.98 (1H, bs, H-12); 5.87 (1H, s, H-2), 5.38 (1H, bs, H-8), 2.52 (1H, m, H-4a), 2.35 (1H, m, H-4b), 2.00 (2H, m, H_2-9), 1.68 (3H, s, H_3-13), 1.42 (1H, m, H10a), 1.27 (1H, m, H-10b), 0.92 (3H, s, H_3-15), 0.85 (3H, s, H_3-14). ^{13}C NMR signals ($CDCl_3$) δ 171.9 (s, C-1, indirect detection from HMBC *J*=7Hz),139.1 (s, C-3, indirect detection from HMBC *J*=7Hz), 135.4 (s, C-7, indirect detection from HMBC *J*=7Hz), 122.2 (d, C-8), 118.2 (d, C-2), 98.2 (d, C-12) 52.1 (d, C-6), 32.5 (t, C-5 or C-10), 31.9 (t, C-10 or C-5), 30.8 (t, C-4), 28.2 (q, C-15), 27.2 (q, C-14), 25.2 (t, C-9). ESIMS *m/z* 273 $[M + Na]^+$. HRESIMS *m/z* 273.1465 (calc. for $C_{15}H_{22}O_3$ 273.1461).

Distribution and relative abundance of caulerpenyne (1) and caulerpin (2) in *C. taxifolia, C. taxifolia* var. *distichophylla, and C. cylindracea*. Specimens of *C. taxifolia* and *C. cylindracea* were sampled along the coast of the Campania region (southern Italy) in the archipelago of Li Galli (40°34′ 57″ N, 14°26′ 05″ E), with the permission of the Marine Protected Area of Punta Campanella. Each algal sample was extracted with acetone as described above for *C. taxifolia* var. *distichophylla*. The diethyl ether soluble portion of acetone extracts from *C. taxifolia, C. cylindracea* and *C. taxifolia* var. *distichophylla* were dissolved in MeOH to obtain a final concentration of 5.0 μg/μL for LC-MS analysis. Solutions of 50 μM of pure caulerpenyne (**1**) and caulerpin (**2**) were also prepared. Chromatographic separations were carried out on an Accela LC Systems (Thermo Scientific) by using a Jupiter 5μ C18 column (Phenomenex 300 A, 150 x 2 mm) and a MeOH/water/ FA 0.1% linear gradient from 50% to 100% MeOH in 10', holding for 5' at 100% MeOH/FA 0.1 %, (flow 200 μL/min). The solvent gradient returned to starting conditions in 5' and a re-equilibration step of 5' was included before the successive run. MS analysis was performed on a LTQ XL linear ion trap mass spectrometer (Thermo Scientific) equipped with an ESI source operating in positive ion mode.

Quantification of caulerpenyne (1) in *Caulerpa taxifolia* var. *distichophylla*. Three samples of *Caulerpa taxifolia* var. *distichophylla* were collected in Donnalucata and individually extracted with acetone by adding acetyl farnesol as internal standard (IS, 2.0 mg). After acetone evaporation, the aqueous residues were subsequently partitioned with diethyl ether. The ether extracts were then dissolved in MeOH (5 mL) and diluted to obtain a final concentration of 3.0 μg/mL of IS for LC-MS analysis.

Chromatographic separation was carried out on Alliance HPLC system (Waters) by using an Ascentis C-18 column (Sigma Aldrich, 250 x 4.6 mm) and a MeOH /water linear gradient from 50% to 100% MeOH in 30', holding for 15' at 100% MeOH (flow 1 mL/min); the HPLC eluate was split and channeled 9/10 to PDA detector 210-400 nm) and 1/10 to mass spectrometer. Mass analysis was performed on a QT of mass spectrometer (Waters) equipped with an ESI source operating in positive ion mode. The calibration curve of **1** was prepared by using five calibration points of the pure standard in MeOH (0.3, 1, 3, 10 and 30 µg/mL) Area of extracted ion at 397.2 m/z ($M+Na^+$) was used as an analytical response for linearity curve calculation (R^2=0.9998).

Study of phytol (4) distribution by GC-MS analysis. GC-MS analyses were performed on an ion trap mass spectrometer equipped with EI source (70 eV) (Polaris Q; ThermoScientific) coupled with a GC system (GCQ; ThermoScientific) with a 5% phenyl column (Trace TR-5, 30 m × 0.25 mm × 0.25 µm; ThermoScientific) and using helium as a gas carrier. Elution of phytol required a temperature program starting at 120 °C for 3 min, followed by a 5 °C min−1 gradient up to 220 °C, then 20 °C min−1 up to 310 °C, holding for 5 min. Algal samples (60 µg/µL) and reference compound **4** were directly injected in split (1:10) mode, with a blink window of 3 min, inlet temperature of 210 °C, transfer line set at 250 °C, and ion source temperature of 230 °C.

Feeding deterrence activity. Compounds **1** and **2** were tested for their activity as feeding deterrents against the native generalist shrimp *Palaemon elegans* (Rathke, 1837), which is not an endangered species, following a procedure described in the literature (Giordano et al. 2017). The shrimps were collected along the coast of Pozzuoli, Italy, in not protected areas, and habituated to the control food in captivity for a week before experiments. After three days of fasting, ten randomly picked shrimps were assayed as a series of individual replicates for each tested compound and the control (n =10 for each series). Shrimps were placed individually into 500 ml plastic beakers filled with 300 ml of seawater. Treated and control food were proposed in parallel to shrimp as red-colored pellets. The presence of a red spot visible by transparency in the stomach of the shrimps after 30 min was considered as acceptance of food, while the absence of the spot gave a rejection response. To evaluate possible sensitization or satiety effects, both treated and control food were further administered to shrimp after a 30-min interval. Statistical analysis between treatments and controls was performed using the two-tailed Fisher-Exact test, with a= 0.05 as significant level.

Results

Chemical investigation. The Et_2O extract from *C. taxifolia* var. *distichophylla* was preliminary analyzed by *thin-layer chromatography* (TLC) showing, as expected, a complex metabolic pattern. Within the major lipophilic components of the alga, comprising chlorophylls, carotenoids, fatty acid and sterols, the TLC analysis also suggested the presence of the sesquiterpenoid **1** [UV positive spot at Rf 0.75 (petroleum ether/ Et_2O 1:1)] but did not allow the detection of the alkaloid **2** [red color reaction with Cerium Sulphate reagent at Rf 0.35 (petroleum ether/ Et_2O 1:1)].

The subsequent fractionation of the Et_2O extract, performed with chromatographic methods including Sephadex LH-20 and SiO_2 chromatography, led to the isolation of the sesquiterpenoid caulerpenyne (**1**) along with the other main components of the terpenoidic fraction of the extract. These latter include the triterpenoid squalene-2,3-epoxide (**3**), the diterpenoid phytol (**4**), and the isoprenoid quinone plastoquinone (**5**) which were easily identified by comparison of their spectroscopic data with the literature (Allen et al. 1967; De Napoli et al. 1982; Arigoni et al. 1999).

An in-depth investigation of the minor components of the lipophilic extract led also to the isolation of two unreported sesquiterpene lactones, compounds **6** and **7**. Their structures were characterized by NMR and mass spectrometry (see Methods section). The two compounds share the same molecular formula C15H22O3 as inferred by the sodiated peaks at *m/z* 273.1467 (for compound **6**) and 273.1465 (for compound **7**) in the HR-ESIMS spectra. Analysis of mono and bi-dimensional NMR experiments revealed the presence in the structure of both compounds of a 6-ethyl-1,5,5-trimethyl-cyclohex-1-ene unit linked to a γ-hydroxyl-α,β-unsaturated-γ-lactone moiety. The two metabolites differ only in the connectivity between these two substructures, being lactone ring linked to aliphatic chain through the α-carbon in compound **6**, whereas in compound **7** the linkage involved the β position. According to this, the main differences between the NMR data of compounds **6** and **7** were related to the resonances of methine on the γ-lactone ring (=CH- 2) which shift from δ_H 6.83/ δ_C141.2 in compound **6** to δ_H 5.87/ δ_C 118.2 in compound **7**.

Distribution and relative abundance of caulerpenyne (1) and caulerpin (2) in *C. taxifolia, C. taxifolia* var. *distichophylla, and C. cylindracea*. The LC-MS analysis of the diethyl ether soluble portion of acetone extracts of *C. taxifolia*, *C. cylindracea* and *C. taxifolia* var. *distichophylla* showed that caulerpenyne (**1**) is present in all the investigated algae, though it is much less abundant in *C. cylindracea*. Conversely, caulerpin

(**2**) was not detected in *C. taxifolia* var. *distichophylla*, while it was found in *C. taxifolia* and, at a significantly higher level, in *C. cylindracea* (Figure 4; Figure S1)

LC-MS quantifications of caulerpenyne. Levels of caulerpenyne (**1**) were evaluated in the acetone extracts from three samples of *C. taxifolia* var. *distichophylla* by using a sensitive LC-MS method by adding acetyl farnesol as the internal standard (IS). Extracted ion chromatograms (EICs) were obtained from each LC-MS run for molecular ions sodium adducts at 397.2 m/z (**1**). The area of extracted ion peak of caulerpenyne (**1**) was used to quantify the metabolite in the algal extracts by interpolation of a calibration curve (Table 1). Caulerpenyne concentration showed high variability, ranging over one order of magnitude, i.e. from 80 to 1020 μg/g of wet alga. No trace of **2** (M+Na+ 421.1 *m/z*) was detectable in the three extracts under the same experimental conditions (Figure 5).

Study of phytol (4) distribution by GC-MS. GC-MS analyses showed the presence of phytol (**4**) in both *C. taxifolia* var. *distichophylla* and *C. taxifolia*, while it was not detected in *C. cylindracea* (Figure S2).

Feeding deterrence activity. Artificial food treated with caulerpenyne (**1**) did not show significant activity as feeding deterrent against *P. elegans*, even when assayed at the concentration of 0.9 mg per gram of wet food, which is almost equivalent to the higher concentration detected in the fronds of *C. taxifolia* var. *distichophylla* from Turkey (Cevik et al. 2016), and is slightly below the maximum level observed in the present study in the alga from Sicily (1.0 mg of **1** per gram of wet alga). However, as summarized in Figure 6, when the caulerpenyne-treated food was administered again after 30' to the same group of shrimps, it was rejected by all individuals, who evidently became sensitized after the first administration. Conversely, caulerpin (**2**) did not show any feeding deterrent property even in a second administration, up to the relatively high concentration of 3.0 mg per gram of wet food.

Discussion

The present work confirms and quantify the presence of the sesquiterpene caulerpenyne (**1**) in *Caulerpa taxifolia* var. *distichophylla* from the Sicilian coast by means of liquid chromatography/mass spectrometry (LC-MS). The same procedure did not allow detection of the alkaloid caulerpin (**2**) in the alga, although it was detected both in a sample of *Caulerpa taxifolia* collected in the Mediterranean, and in greater abundance in *Caulerpa cylindracea* (Figure 4, Figure S1). These differences in the chemical composition of the three

invasive algae suggest that they could exert different chemically-mediated effects on native species. Initially, palatability assays carried out on the native generalist shrimp *Palaemon elegans* did not show significant feeding deterrent activity of caulerpenyne (**1**) at its highest natural concentration in *C. taxifolia* var. *distichophylla* (Figure 6, time 0). However, the treated food was rejected by all individuals in a subsequent administration at a 30 min interval (Figure 6, time 30'), suggesting that sensitization is mandatory to significantly elicit avoidance behavior. Control food was instead completely consumed by all shrimp during both feeding sessions, excluding any satiety effects. Conversely, caulerpin (**2**) did not show any feeding deterrent property even in a second administration, up to the relatively high concentration of 3.0 mg per gram of food. Accordingly, the palatability of **2** together with the lower level of **1** detected in *C. cylindracea*, may explain why the native herbivore *Paracentrotus lividus* avoided *C. taxifolia* var. *distichophylla* and preferred the more palatable *C. cylindracea* (Noè et al. 2018). On the other hand, *C. cylindracea* is also consumed as food by both humans and native fish (Vitale et al. 2018, and references therein). Conversely, higher levels of **1** could explain the substantial physiological stress (reduced motility and coordination) observed in sea urchins after feeding on *C. taxifolia* var. *distichophylla* (Vega Fernández et al. 2019). In addition, the chemical study of the terpenoidic content of *C. taxifolia* var. *distichophylla* revealed the presence of the acyclic diterpene alcohol phytol (**4**), which is a constituent of chlorophyll (Van Den Brink and Wanders 2006). Compound **4** was also present in *C. taxifolia* but not in *C. cylindracea* (Figure S2). It is worth mentioning here that it was also isolated from both the leaves of the smooth sumac (*Rhusglabra*), and the feces of the sumac flea beetle *Blepharidarhois*, and has been shown to act as a feeding deterrent against the generalist predatory ant, *Formica subsericea* (Vencl and Morton 1998). Squalene 2,3 oxide (**3**), which is an intermediate product in the synthesis of phytosterols (Abe 2007), was also found in *C. taxifolia* var. *distichophylla*, along with plastoquinone A (**5**), an isoprenoid quinone molecule involved in the light-dependent reactions of photosynthesis but also functioning as an antioxidant by reducing reactive oxygen species (Mubarakshina and Ivanov 2010). If confirmed, the possible action of **3** and **5** against abiotic stresses, such as UV radiation and heat, could confer a competitive advantage to *C. taxifolia* var. *distichophylla* in photophilic environments. Finally, within the minor terpenoidic components of the alga, two unreported sesquiterpenoids, compounds **6** and **7**, were also isolated. Their structures are characterized by an α,β-unsaturated-γ-lactone linked to a 6-ethyl-1,5,5-trimethyl-cyclohex-1-ene moiety. They differ from each other in the carbon connectivity between the aliphatic ethyl

chain and the γ-lactone ring, being the carbon involved in linkage the α-carbon in compound **6** and the β-carbon in compounds **7**. Biosynthetically, they could be derived from enzymatic cyclization of the 1,4 dialdehyde **8**, in free or enolacetate forms. These putative precursors were not detected in the extract of *C. taxifolia* var. *distichophylla* but they have been previously reported in *Caulerpa ashmeadii* (Paul et al.,1987). Compounds **6** and **7** are the endo double bond isomers of the two monocyclofarnesol derived sesquiterpenoids **9** and **10**, respectively reported from *Caulerpa bikinensis* (Paul and Fenical 1982), and from a sponge belonging to the genus *Ircinia* (Hahn et al. 2014). Compound **9** showed toxic and feeding deterrence properties, while it has been reported that the closely related compound **10** activates peroxisome proliferator-activated receptor delta (PPARδ) that is a nuclear receptor playing a pivotal role in lipid metabolism of animals, mitochondrial function, and insulin secretion, with an EC50 value of 18 μg/mL (Hahn et al. 2014). The lack of activity observed in the same assay for the compound **9** seems to suggest that the carbon connectivity from the γ-lactone to the linear alkyl chains is crucial for the bioactivity. Future studies on the new compounds **6** and **7** isolated from *C. taxifolia* var. *distichophylla* might help to further elucidate their ecological role and the possible interaction between this class of metabolites and the PPARδ receptor.

Conclusions

Biological invasions by non-indigenous macrophytes have been widely recognized as an important threat to the Mediterranean ecosystem, and may result in significant economic and social impacts. Unfortunately, little information is available on the factors determining the success of invasive algae in new environments. In this study, we focused on the invasive green alga *C. taxifolia* var. *distichophylla*, which turned out to differ from *C. taxifolia*, and from the congeneric *C. cylindracea*, in its chemical composition. Chemoecological evaluations also led us to propose that the sesquiterpene caulerpenyne (**1**), at its natural concentration in *C. taxifolia* var. *distichophylla*, can elicit sensitization and food avoidance in native species. Furthermore, we provided preliminary knowledge for future investigations aimed at clarifying the natural functions of the other substances isolated from *C. taxifolia* var. *distichophylla* and their role in the chemically-mediated interactions of the alga with native species.

Acknowledgments

We thank the administration of the Marine Protected Areas of Punta Campanella (Italy) for allowing access, fieldwork, and sampling in the protected area. We acknowledge Carlo D'Angiolella for his help in performing the assays with shrimp. FD thanks INCIPIT CONFUND project (grant agreement n. 665403) co-funded by HORIZON 2020/Marie Sklodowska Curie Action.

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418
419

Table 1 Quantification of **1** in three samples of *C. taxifolia* var. d*istichophylla*

	wet weight (g)	volume (mL)	ether extract (mg)	internal standard recovery (%)	amount of **1** in the extract (μg)	concentration of **1** in the extract (μg/mg)	concentration of **1** per wet weight (μg/g)	concentration of **1** per volume (μg/mL)
sample 1	6.10	5.50	37.60	59.60	485.85	12.92	79.65	88.34
sample 2	8.70	8.00	61.10	52.80	3298.40	53.98	379.13	412.30
sample 3	6.90	7.00	45.50	64.80	7040.59	154.74	1020.37	1005.80

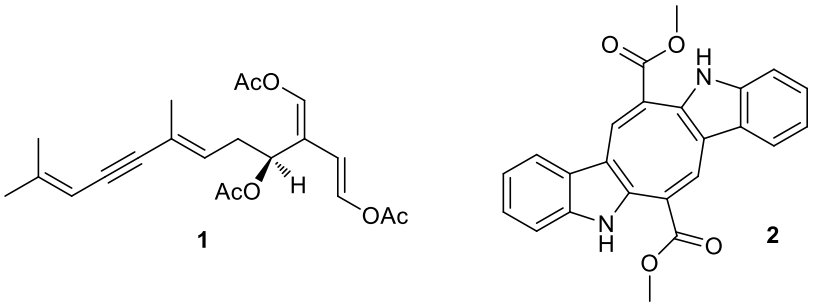

Fig. 1

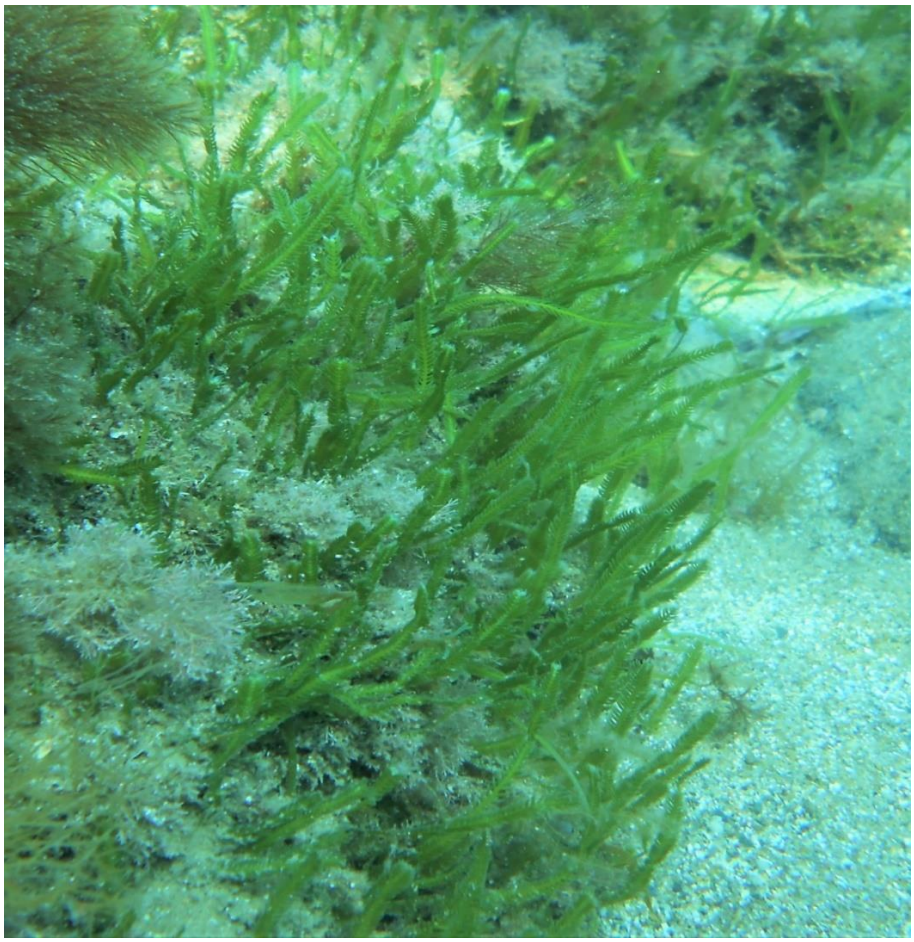

Fig. 2

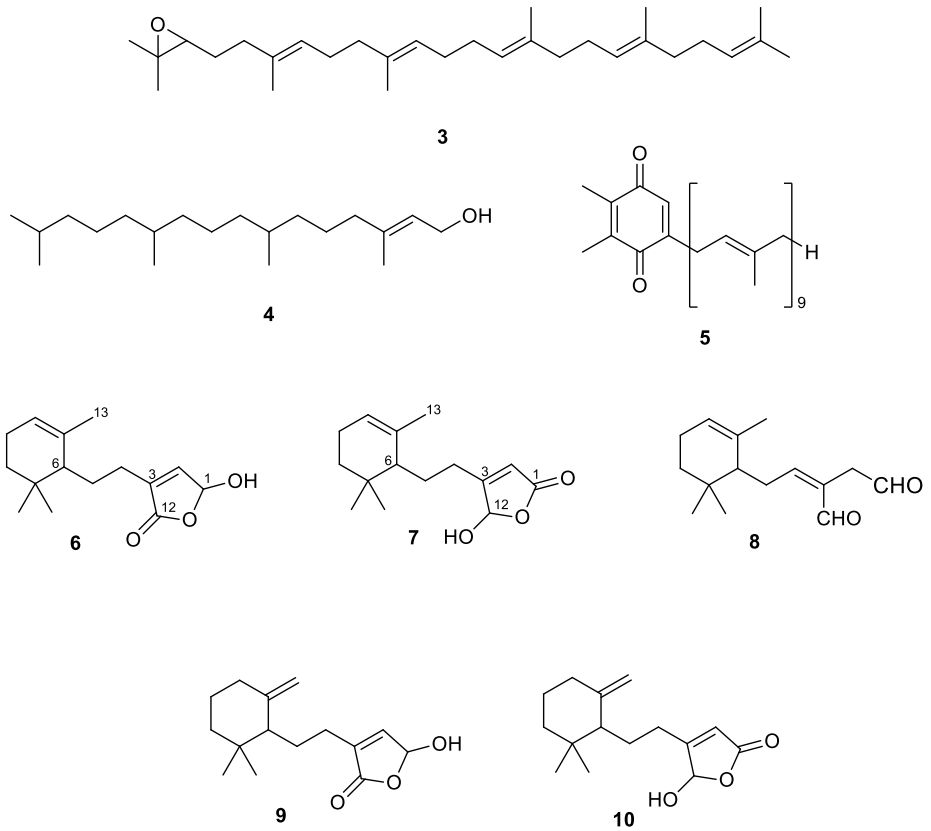
O
3
OH
4
O
O
H
9
5
13
6
3
1
OH
12
O
O
6
13
6
3
1
O
12
O
HO
7
CHO
CHO
8
OH
O
O
9
O
O
HO
10

Fig. 3

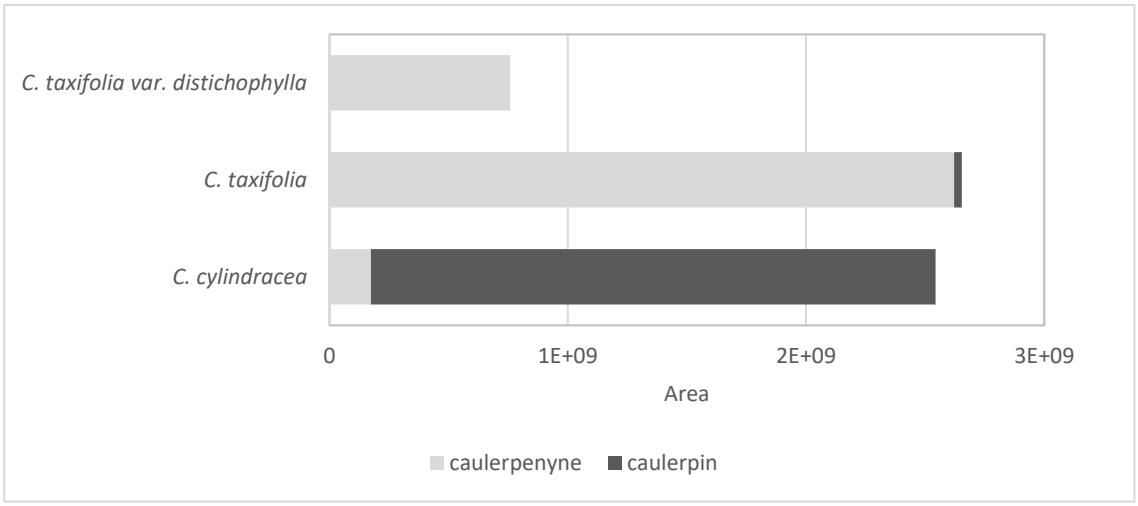
C. taxifolia var. distichophylla
C. taxifolia
C. cylindracea
0
1E+09
2E+09
3E+09
Area
caulerpenyne
caulerpin

Fig. 4

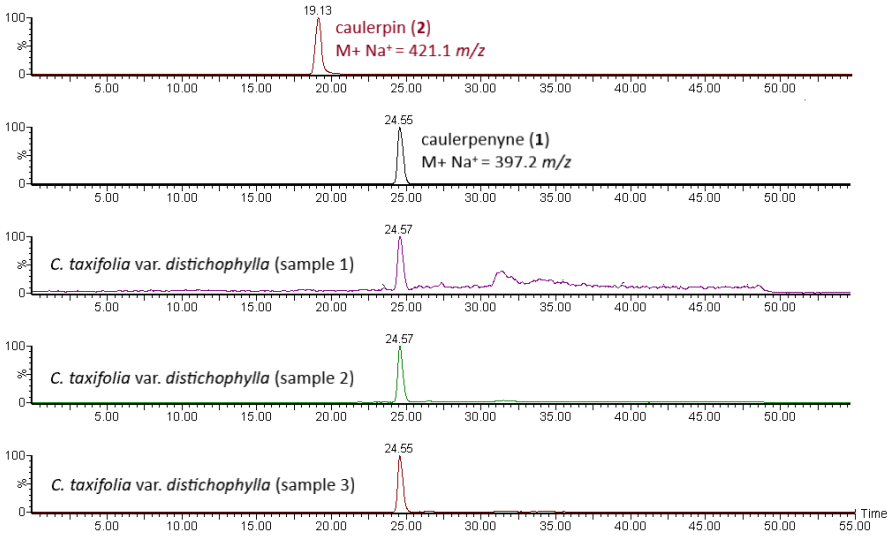
19.13
caulerpin (2)
M+ Na+ = 421.1 m/z
24.55
caulerpenyne (1)
M+ Na+ = 397.2 m/z
24.57
C. taxifolia var. distichophylla (sample 1)
24.57
C. taxifolia var. distichophylla (sample 2)
24.55
C. taxifolia var. distichophylla (sample 3)
100
%
0
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
Time

Fig. 5

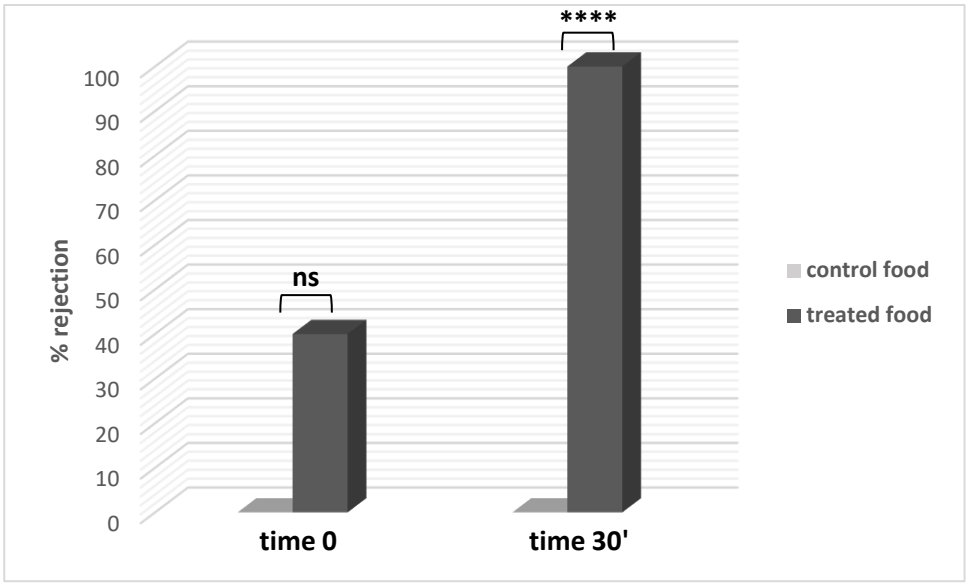
****
100
90
80
70
60
50
40
30
20
10
0
% rejection
ns
control food
treated food
time 0
time 30'

Fig. 6

Captions to figures 1-4

Fig. 1 Structures of caulerpenyne (**1**) and caulerpin (**2**)

Fig. 2 *C. taxifolia* var. *distichophylla*

Fig. 3 Structures of squalene-2,3-epoxide (**3**), phytol (**4**), plastoquinone (**5**), and sesquiterpene lactones **6** and **7** from *C. taxifolia* var. *distichophylla*, along with the structures of related compounds **8**-**10** isolated from other *Caulerpa* species.

Fig. 4 Relative abundance of caulerpenyne (**1**) and caulerpin (**2**) in the extracts from *C. taxifolia*, *C. taxifolia* var. *distichophylla*, and *C. cylindracea.*

Fig. 5 LC-MS profiles (EICs) of pure standard caulerpin (**2**) and caulerpenyne (**1**) and crude extracts from *C. taxifolia* var. *distichophylla* (samples 1-3).

Fig. 6 *P. elegans* alimentary response to food pellets treated with caulerpenyne (**1**) at the concentration of 0.9 mg per gram of food in comparison with control food. The artificial food was offered to groups of shrimps in two subsequent administrations at a time interval of 30 minutes. The significant differences were evaluated using the two-tailed Fisher's exact test ($n = 10$ for each bar, $\alpha = 0.05$). ns: not significant; ****: extremely significant, $P<0.0001$.