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Could molecular effects of *Caulerpa racemosa* metabolites modulate the impact on fish populations of *Diplodus sargus*?

S. Gorbi [a,*], M.E. Giuliani [a], L. Pittura [a], G. d'Errico [a], A. Terlizzi [b], S. Felline [b], L. Grauso [c], E. Mollo [c], A. Cutignano [c], F. Regoli [a]

[a] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
[b] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Lecce, Italy
[c] Istituto di Chimica Biomolecolare, CNR, Naples, Italy

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ABSTRACT

The green alga *Caulerpa racemosa* is a non-native, invasive species in the Mediterranean, and an important stressor for several native organisms. The algal capacity to produce secondary metabolites has been suggested to modulate success of the *C. racemosa* invasion, although many of potentially involved biological pathways still remain unexplored.

In this respect, the aim of the present study was to investigate some molecular and cellular effects in the white seabream *Diplodus sargus*, an ecologically key species, which included the alien *C. racemosa* in its diet. Organisms were sampled in 2 seasonal periods from 3 locations of Southern Italy, each characterized by different levels of algal abundance.

The level of caulerpin, one of the main secondary algal metabolites, in fish tissues has been used as an indicator of the trophic exposure to the seaweed and related with molecular and cellular responses. Chemical analyses indicated that fish from invaded sites can accumulate caulerpin, with liver concentrations ranging from a few up to hundreds of μg/g. Biomarkers analyses revealed only limited alterations of the main antioxidant defences, such as glutathione reductase and levels of glutathione; on the other hand, increased enzymatic activities of cytochrome P450, glutathione S-transferases and acyl CoA oxidase, as well as enhanced gene transcription for peroxisome proliferator-activated receptor alpha, cytochrome P4501A and vitellogenin 1 were observed in fish more exposed to *C. racemosa* as indicated by liver concentrations of caulerpin higher than 50 μg/g.

Despite a direct molecular relationship with this algal metabolite could not be established, our results suggest that a *C. racemosa* enriched diet can modulate biotransformation and fatty acids metabolism of *D. sargus*. Assessing whether similar effects represent short- or long-term effects will be of crucial importance to understand consequences on the general health status and reproductive performance of exposed key fish species in the Mediterranean region.

1. Introduction

The introduction of non-indigenous species across the European seas is a dynamic and continuously growing process which has recently drawn the attention of scientists, politicians and media (Occhipinti-Ambrogi and Sheppard, 2007). Globalization and international trade, climate change, anthropogenic activities including aquaculture, shipping and transportation have been considered as the major driving forces behind the intensification of biological invasion phenomena (Streftaris et al., 2005; Schaffelke and Hewitt, 2007; Occhipinti-Ambrogi, 2007).

Although only a small percentage of introduced species can survive and invade a new habitat, their impact can be dramatic. The most harmful invaders completely displace native species, thus changing the original community structure and altering fundamental ecological processes, such as nutrient cycling, sedimentation, food resources and reproductive performance of many organisms (Boudouresque et al., 2005; Piazzi and Balata, 2008; Katsanevakis et al., 2010; Bulleri et al., 2011). Invasive aliens can also damage economical activities like fisheries and impact human health by causing various diseases (Perrings, 2002; Pimentel et al., 2005). In this respect the "Convention of Biological Diversity"

* Corresponding author. Tel.: +39 0712204142; fax: +39 0712204609.
E-mail address: s.gorbi@univpm.it (S. Gorbi).

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declared alien species as "One of the greatest threats to biodiversity, and to the ecological and economic well-being of society and planet" (Schaffelke and Hewitt, 2007).

The Mediterranean is one of the areas worldwide most severely affected by marine invasions, with about 1000 introduced species, representing more than 5% of the known flora and fauna (Boudouresque et al., 2005; Occhipinti-Ambrogi and Sheppard, 2007; Galil, 2008; Zenetos et al., 2012). Excluding the Lessepsian migrations, shipping and aquaculture are the major vectors for species of tropical origin, which have found favorable conditions for their spread due to the warming of Mediterranean (Streftaris et al., 2005; Occhipinti-Ambrogi, 2007).

The green alga *Caulerpa racemosa* is native from the south-western coast of Australia, and in the last decades it has been frequently reported as invasive species in Mediterranean soft bottoms, seagrass meadows and rocky shores, ranging from depths of 0 to up 70 m (Klein and Verlaque, 2008). Along to the Italian coasts, *C. racemosa* was firstly reported in 1993, and it has currently invaded also many Marine Protected Areas (Katsanevakis et al., 2010; Felline et al., 2012).

Beside the change in structure and composition of invaded assemblages, the extensive and uniform mats formed by *C. racemosa* have also direct effects on feeding habit of demersal species; sandy and rocky substrates are less accessible, and the alteration of predatory–prey interactions could potentially influence the decline of local fish populations (Galil, 2008; Bulleri et al., 2010; Vazquez-Luis et al., 2010).

Among the factors contributing to *C. racemosa* invasion, the production of secondary metabolites has a crucial role in interspecific competition for space (Mollo et al., 2008). Caulerpenyne, one of the major algal metabolites, has been suggested to act as chemical deterrent against herbivores, but several allelochemicals, neurotoxic and cytotoxic properties have been also reported for this compound (Brunelli et al., 2000; Erickson et al., 2006; Raniello et al., 2007). Biological effects have been described also for two other metabolites, caulerpin and caulerpicin which, however, do not seem to exert acute toxicity (Rocha et al., 2007).

The production of secondary metabolites varies according to the seasons, the highest level found in summer and autumn and the lowest in winter (Klein and Verlaque, 2008; Box et al., 2010), concurrently with the vegetative activity of the algae, which show the maximum of development from summer to autumn, a significant regression in winter, and a renewal of the growth in spring (Piazzi and Balata, 2008; Box et al., 2010).

Several studies have demonstrated that *C. racemosa* is becoming an important item within stomach contents of several species including *Boops boops*, *Pagellus acarne*, *Spondyliosoma cantharus*, *Sarpa salpa*, *Diplodus vulgaris* and *Diplodus sargus* (Klein and Verlaque, 2008; Box et al., 2009; Terlizzi et al., 2011). Recent studies with the white seabrem *D. sargus* firstly demonstrated a significant accumulation of caulerpin in organisms from invaded areas (Terlizzi et al., 2011; Felline et al., 2012). The presence of caulerpin in fish tissues was then used as indicator of trophic exposure to the seaweed and related to the appearance of some cellular and physiological alterations which included the slight modulation of antioxidant defences, cytochrome P450 (EROD), acetylcholinesterase and acyl CoA oxidase activities, as well as changes of hepatosomatic and gonadosomatic indices (Felline et al., 2012). These results allowed to hypothesize, for the first time, that biological large-scale effects of alien species on native fish assemblages might be modulated also by molecular mechanisms and interactions induced at cellular level.

The aim of the present work was to further confirm and provide new insights on the potential impact of a *C. racemosa* enriched diet on cellular responses of *D. sargus*. In relation to seasonal fluctuations of *C. racemosa*, organisms were collected in two seasonal periods, June and October 2012, along three areas of the Southern Apulian coast, Brindisi (BR), Porto Cesareo (PC) and Torre Guaceto (TG), differently characterized in terms of *C. racemosa* abundance; also *C. racemosa* is expected to differ between June and October in terms of vegetative activity, production and content of secondary metabolites (Box et al., 2010). In the present ecotoxicological study, the measurement of caulerpin bioaccumulation in fish liver was considered as a marker of trophic exposure to *C. racemosa* and it was integrated with a battery of biochemical and molecular alterations which could represent early warning signals of metabolite-induced disturbance in white seabream (Terlizzi et al., 2011; Felline et al., 2012).

Biotransformation and conjugation reactions were analyzed as transcriptional response of CYP1A and catalytic activities of both 7-ethoxyresorufin *O*-deethylase (EROD) and glutathione S-transferases, to evaluate if these pathways are involved in metabolism and possible excretion of algal metabolites. Acyl-CoA oxidase activity and gene transcription of peroxisome proliferator-activated receptor alpha gene (PPARα) have been selected as markers of peroxisomal proliferation which is known to respond to a large class of natural products, with important effects on lipid metabolism and beta-oxidation of fatty acids. Specific biological effects potentially induced by algal metabolites on *D. sargus* were also evaluated on acetylcholinesterase activity, a typical biomarker of neurotoxic damages, and on vitellogenin gene (VTG1) expression, to elucidate possible endocrine effects and implications on reproductive performance.

Since accumulation of bioactive molecules is frequently associated to the intracellular enhancement of reactive oxygen species (ROS), variations of antioxidant defences typically represent sensitive biomarkers for the onset of an oxidative challenge (Esterhuizen-Londt et al., 2011; Gorbi et al., 2012; Regoli and Giuliani, in press); such data, however, may be difficult to summarize in a synthetic index reflecting a varied susceptibility to oxidative stress or general health condition of exposed organisms. In this respect, the analyses of individual antioxidants (catalase, glutathione reductase, Se-dependent and Se-independent glutathione peroxidases, level of total glutathione) were complemented in this study with the measurement of total oxyradical scavenging capacity (TOSC) which quantifies the overall resistance toward ROS like peroxyl and hydroxyl radicals (Regoli and Winston, 1999; Gorbi and Regoli, 2003).

The overall results of this study were expected to add further evidence on molecular effects of a *C. racemosa* enriched diet on the Mediterranean *D. sargus*, thus supporting the role of cellular mechanisms by which invasive species can alter the structure and the functioning of natural assemblages.

2. Materials and methods

2.1. Fish sampling

Specimens of *D. sargus* were sampled in June and October 2012 in 3 sites along to the Apulian coast (Southern Italy), the area between Brindisi and Lecce (BR), Porto Cesareo (PC) and Torre Guaceto (TG). All these locations are considered as pristine sites, unaffected by chemical pollution (Lionetto et al., 2003; Spada et al., 2013), and both PC and TG are included among Marine Protected Areas. These sites are characterized by different levels of *C. racemosa* proliferation, which is very abundant in TG and BR and only occasionally found in PC. From each site, 10 individuals of *D. sargus* were sampled in June and October 2012 (mean weight 374 ± 71 g and 410 ± 87 g respectively). Liver and gill tissues were

immediately excised, frozen in liquid nitrogen and maintained at −80 °C till processed for analyses.

2.2. Chemicals

The following chemicals were supplied by Sigma–Aldrich (St. Louis, MO, USA): 7-ethoxyresorufin, β-nicotinamide adenine dinucleotide (NADPH), dichlorofluorescein-diacetate (DCF-DA), horseradish peroxidase (HRP), indole-3-acrylic acid, palmitoyl-CoA, H_2O_2, cumene hydroperoxide (CHP), α-keto-γ-methiolbutyric acid (KMBA), 2-2′-azo-bis-(2methylpropionamidine)-dihydrochloride (ABAP), 1-metyl-2-phenylindole), and Trizol reagent. For molecular analyses, iScript cDNA Synthesis Kit and iQ™ SYBR® Green Supermix were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.3. Chemical analyses

Lyophilized samples of fish liver were extracted with ethyl acetate by homogenization with a pestle and ultrasound. Indole-3-acrylic acid was converted into its methyl ester by reaction with diazomethane and added as internal standard (80 ng/mg lyophilized tissue). After removal of the organic solvent, extracts were reconstituted in MeOH at a final concentration of 0.5 mg/ml. For each individual liver, organic extracts were analyzed by ultra-performance liquid chromatography/mass spectrometry (UPLC–MS/MS) to quantify the algal metabolite.

UPLC–MS/MS analyses were carried out on Acquity UPLC system (Waters, Milford, MA, USA) on line with API 3200 triple quadrupole mass spectrometer (ABSciex, Foster City, CA, USA) using multiple reaction monitoring (MRM) analysis. A Turbo VTM interface equipped with a turbo ion spray probe in negative ionization mode was used. Data processing was performed on Analyst and Multiquant software packages (ABSciex). UPLC analyses were carried out on Acquity CSH Fluoro Phenyl column (Waters, 1.7 μm, 2.1 × 100 mm) maintained at 45 °C. The mobile phase consisted of a MeOH:H_2O gradient from 40 to 95% of MeOH in 3.5 min at 0.45 ml/min. Injection volume was 2 μl. Three MRM transitions were monitored (397.0→365.0 *m/z*; 397.0→337.0 *m/z* and 397.0→278.0 *m/z*) for the algal metabolite identification, and the MRM transition (200.2→168.2 *m/z*) was used for the internal standard. The quantitation of caulerpin was achieved selecting the most intense transition 397.0→365.0 *m/z*. The calibration curve was constructed by adding known amount of caulerpin in MeOH and consisted of 2 blank samples and 6 calibration points (in triplicate) at concentrations in the range 1–3000 ng/ml. The resulting peak areas under MRM trace were measured and plotted against concentration.

2.4. Biochemical analyses

Activity of 7-ethoxyresorufin *O*-deethylase (EROD) was measured according to ICES (1998) in the S9 fraction of individual livers homogenized (1:5, w/v) in 100 mM K-phosphate buffer pH 7.5, 0.15 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 12,000 *g* for 15 min, the resulting supernatants (S9) were immediately incubated at 30 °C in a final volume of 1 ml containing 100 mM K-phosphate buffer pH 7.5, 4 μM 7-ethoxyresorufin, and 0.25 mM β-nicotinamide adenine dinucleotide (NADPH); 2 ml acetone were added after 5 min to stop the reaction. Incubation mixtures stopped at time zero were used as blank values and were subtracted from the sample fluorescence. Spectrofluorimetric analyses (535/585 nm) were quantified by reference to resorufin standards (0.02–1 μM).

The peroxisomal enzyme acyl CoA oxidase (AOX) was analyzed in liver samples homogenized in 1 mM sodium bicarbonate buffer (pH 7.6), containing 1 mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500 *g* for 15 min at 4 °C. The H_2O_2 production was spectrophotometrically measured in a coupled assay by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 μM sodium azide, 0.01% Triton X-100, 1.2 U/ml HRP in a final volume of 1 ml. After a pre-incubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrate palmitoyl-CoA at a final concentration of 30 μM. Readings were carried out against a blank without substrate at 502 nm.

Acetylcholinesterase (AChE) activity was analyzed in the gills homogenized in 0.1 M Tris–HCl buffer pH 7.2, 0.25 M sucrose and centrifuged at 10,000 *g* for 10 min. Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18 ± 1 °C, $\lambda = 412$ nm, $\varepsilon = 13.6$ mM/cm.

Enzymatic activities of glutathione S-transferases (GST), catalase (CAT), glutathione reductase (GR) and glutathione peroxidases ($GPxH_2O_2$ and GPxCHP) were measured in the liver cytosolic fractions obtained by the homogenization (1:5, w/v) in 100 mM Tris–HCl buffer pH 7.5, 0.1 mM phenylmetylsulfonilfluoride (PMSF), NaCl 1.8%, and centrifugation at 100000 *g* for 1 h at 4 °C (Regoli et al., 2005). CAT was analyzed by the consumption of H_2O_2 at 240 nm ($\varepsilon = -0.04$ mM/cm); the assay concentration was 12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0. GR was quantified by the loss of NADPH during the reduction of oxidized glutathione GSSG ($\lambda = 340$ nm, $\varepsilon = -6.22$ mM/cm); the final assay conditions were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 0.12 mM NADPH. GPx were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to reconvert the formed GSSG to its reduced form. The decrease of absorbance was monitored at 340 nm ($\varepsilon = -6.22$ mM/cm) with 0.5 mM hydrogen peroxide (H_2O_2) or 0.8 mM cumene hydroperoxide (CHP) as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms, in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM NaN_3 (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH. The rate of the blank reaction was subtracted from the total rate. Total glutathione was analyzed in samples homogenized (1:5 w:v ratio) in 5% sulfosalicilic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37,000 *g* for 15 min. The resulting supernatants were enzymatically assayed. (Regoli et al., 2005).

The total oxyradical scavenging capacity (TOSC) was determined in liver cytosolic fractions obtained as previously reported without PMSF in the homogenization buffer (Regoli et al., 2005). The TOSC assay measures the overall capability of cellular antioxidants to absorb different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Regoli and Winston, 1999). Peroxyl radicals (ROO•) were generated by the thermal homolysis of 20 mM 2-2′-azo-bis-(2methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals (•OH) were produced by the Fenton reaction of iron-EDTA (1.8 μM Fe^{3+}, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM K-phosphate buffer. Ethylene formation in control and sample reactions was analyzed by gas-chromatographic analyses according to Regoli and Winston (1999). The TOSC values were quantified from the equation: $TOSC = 100 - (\int SA / \int CA \times 100)$, where $\int SA$ and $\int CA$ are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay.

Table 1
Primer pair sequences, amplicons size, annealing temperatures and accession numbers of genes investigated in quantitative PCR.

Gene	Primer pairs sequences	Amplicon size	Annealing *T*	Accession *n.*
CYP1A	Fwd: ATGACCTGTTTGGAGCTGGTTTTG Rev: TTCCCTGTATCTCTGGGTATGAGAC	90 bp	62 °C	HG003580
PPARα	Fwd: TGAGGGAGATCCACGGAGCCT Rev: TGAACGGCTGCTTGCTGGTCT	96 bp	62 °C	HG003581
VTG1	Fwd: ACCCAAAGAACGTGATGTGAT Rev: TGTAGGATGCTGGTAAGGCAGG	80 bp	62 °C	HG003582

The content of malondialdehyde (MDA) was measured in liver samples homogenized (1:3 w/v ratio) in 20 mM Tris–HCl pH 7.4, centrifuged at 3000 *g* for 20 min and then derivatized in 1 ml reaction mixture containing 10.3 mM 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1), HCl 32%, 100 μl water and an equal volume of sample or standard (standard range 0–6 μM 1,1,3,3-tetramethoxypropane, in 20 mM Tris–HCl, pH 7.4). The tubes were vortexed and incubated at 45 °C for 40 min. Samples were cooled on ice, centrifuged at 15,000 *g* for 10 min and spectrophotometrically analyzed at 586 nm; levels of MDA were calibrated against a malondialdehyde standard curve and expressed as nmol/g wet weight (Shaw et al., 2004).

Protein concentrations were measured by the spectrophotometric method of Lowry with bovine serum albumin (BSA) as standard.

2.5. Molecular analyses

2.5.1. RNA isolation and cDNA synthesis

Total RNA was purified from *D. sargus* liver, homogenized in Trizol reagent (Sigma–Aldrich) according to the manufacturer's protocol. Total RNA concentrations were measured using NanoDrop® ND-1000 UV–Visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total cDNA was generated by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) from 1 μg of total RNA for each sample using combined oligo(dT) and random hexamer primers for the iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad).

2.5.2. Cloning and sequence identification

Since nucleotide sequences for the genes of interest were unknown, partial fragments of cytochrome P450 (CYP1A), peroxisome proliferator-activated receptor alpha (PPARα) and vitellogenin 1 (VTG1) genes of *D. sargus* were amplified, cloned and sequenced. PCR primers for the amplification of CYP1A (Fwd: GGACAACATTCGGGACATCACAGACTC; Rev: CATTGTGAGACCGTATTCTGGGGTCA), PPARα; (Fwd: AAGTGCCTCTCTGTGGGCATG; Rev: CTTGGCGAACTCCGTCAGCTC), and VTG1 (Fwd: AAATTCATGGTTCTGCTGAAGAAGGA; Rev: ACAGGTCTGTCCCTTCATCCAGTC) were designed on conserved regions of homolog genes (with accession number DQ898145, AY590299 and HQ846509, respectively).

The PCRs were performed on 1 μl total cDNA from *D. sargus* liver for 30 cycles at the annealing temperature of 60 °C for CYP1A, 55 °C for PPARα, and 50 °C for VTG1. All primer pairs gave a single band pattern for the expected amplicon size (673 bp, 459 bp and 294 bp, respectively). The PCR products were cloned into pGEM-T Easy Vector System in JM109 *Escherichia coli* strain (Promega). Each plasmid was sequenced using the ABI 3730xl 96-capillary DNA Analyzer (Eurofins MWG Operon). Sequences were confirmed as CYP1A, PPARα and VTG1 through the NCBI nucleotide BLAST alignment tool (http://www.ncbi.nlm.nih.gov/BLAST), and registered at the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) with the accession number HG003580, HG003581 and HG003582.

2.5.3. Quantitative real-time PCR

Absolute quantitative real-time PCR with gene-specific primer pairs (Table 1) was performed for evaluating the mRNA levels of individual target genes, using SYBR green method in an iQ5 iCycler thermal cycler (Biorad, CA, USA). Total cDNA, synthesized as described above, was diluted 1:5 and quantified by NanoDrop® ND-1000 UV–Visible Spectrophotometer. Each 25 μl DNA amplification reaction contained 12.5 μl of iQ™ SYBR® Green Supermix (Bio-Rad), 1 μg of cDNA and 200 nM of each forward and reverse primers. The three-step real-time PCR program included an enzyme activation step at 95 °C (3 min), 40 cycles each characterized by 95 °C (10 s), 62 °C (20 s) and 72 °C (20 s), followed by a melting analysis at 95 °C for 1 min, 62 °C for 10 s and thereafter decreasing fluorescence detection with increasing temperature between 62 and 95 °C. Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. Obtained cycle threshold (*C*t) values were converted into mRNA copy number using standard plots of *C*t versus log copy number. The standard plots were generated for each target sequence using serial dilution of known amounts of plasmid containing the amplicon of interest. Obtained data from triplicate runs for individual target cDNA amplification were averaged and expressed as initial mRNA copy number. This absolute quantification method is a well-validated procedure, as the so-called housekeeping genes have been shown to be modulated by experimental exposure (Arukwe, 2006).

2.6. Statistical analyses

Statistical analyses were performed using the R statistical software (R Development Core Team, 2011). Analysis of variance (ANOVA) was applied for all parameters to test differences between sites and groups characterized by different tissue levels of caulerpin. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Bartlett test and mathematical transformation applied if necessary; post-hoc comparison (Newman–Keuls) was used to discriminate between means of values ($n = 5$).

Table 2
Concentration of caulerpin in liver of white seabrem *D. sargus* sampled from Torre Guaceto (TG), Brindisi (BR) and Porto Cesareo (PC), in June and October 2012. Values of caulerpin concentrations are expressed in μg per gram of dry weight (μg/g).

	Caulerpin μg/g			Caulerpin μg/g			Caulerpin μg/g	
	June	October		June	October		June	October
TG1	4.44	114.08	BR1	9.38	28.35	PC1	9.96	6.34
TG2	3.83	0.08	BR2	28.21	235.75	PC2	41.89	0.00
TG3	0.00	93.60	BR3	11.34	10.31	PC3	14.77	0.00
TG4	0.00	27.94	BR4	30.80	10.68	PC4	22.09	0.00
TG5	6.91	5.67	BR5	12.65	29.09	PC5	5.86	34.05
TG6	6.51	136.95	BR6	25.76	5.50	PC6	0.00	0.00
TG7	0.00	40.98	BR7	13.09	16.61	PC7	3.72	0.00
TG8	13.71	16.64	BR8	26.93	61.29	PC8	17.93	0.00
TG9	0.00	39.02	BR9	13.49	114.42	PC9	31.43	131.41
TG10	0.00	10.07	BR10	7.41	3.20	PC10	24.43	0.00
mean	3.54	48.50	*mean*	17.91	51.52	*mean*	17.21	17.18
st.dev.	4.56	48.76	*st.dev.*	8.89	73.08	*st.dev.*	13.14	41.52

Table 3
Biomarker responses in white seabream *Diplodus sargus* sampled in June 2012. Mean values ± standard deviations ($n = 5$). The p values are reported for significant effects and different letters indicate significant differences between groups of means (Newman Keuls post hoc); n.s. not statistically significant.

Caulerpin accumulation	0 μg/g (Absent)	<10 μg/g (Low)	10–50 μg/g (Medium)	
Parameter				
EROD (pmol/min/mg prot)	68.54 ± 36.15	36.93 ± 13.18	54.64 ± 22.88	n.s.
GST (nmol/min/mg prot)	1424 ± 197 (a)	1110 ± 81 (b)	1146 ± 164 (b)	$p < 0.05$
AOX (nmol/min/mg prot)	1.489 ± 0.408 (a)	2.582 ± 0.415 (b)	2.319 ± 0.341 (b)	$p < 0.05$
AChE gills (nmol/min/mg prot)	10.31 ± 3.25	7.95 ± 2.29	7.27 ± 1.41	n.s.
CAT (μmol/min/mg prot)	223 ± 20	222 ± 30	221 ± 29	n.s.
GSH (μmol/g tiss)	2.357 ± 0.467 (a)	1.658 ± 0.276 (ab)	1.544 ± 0.217 (b)	$p < 0.05$
GR (nmol/min/mg prot)	16.25 ± 1.26 (a)	11.87 ± 2.41 (b)	11.45 ± 1.07 (b)	$p < 0.05$
$GPxH_2O_2$ (nmol/min/mg prot)	175.0 ± 94.5	337.1 ± 170.2	242.4 ± 68.7	n.s.
GPxCHP (nmol/min/mg prot)	375.1 ± 71.7	329.7 ± 184.3	328.7 ± 63.6	n.s.
TOSC ROO• (UTOSC/mg prot)	469.0 ± 10.3	547.0 ± 76.1	457.1 ± 35.0	n.s.
TOSC HO• (UTOSC/mg prot)	799.6 ± 126.0	830.9 ± 26.5	732.7 ± 99.9	n.s.
MDA (nmol/g tiss)	56.06 ± 10.86	61.46 ± 16.85	50.56 ± 10.64	n.s.

EROD: 7-ethoxyresorufin O-deethylase; GST: glutathione S-transferases; AOX: acyl CoA oxidase; AchE: acetylcholinesterase; CAT: catalase; GSH: levels of total glutathione; $GPxH_2O_2$ and GPxCHP: Se-dependent and sum of Se-dependent and Se-independent glutathione peroxidases; TOSC: total oxyradical scavenging capacity toward peroxyl (ROO•) and hydroxyl (•OH) radicals; MDA: content of malondialdehyde.

Descriptive multivariate statistical analysis (PCA, principal component analysis) was performed using the FactoMineR R-package (Lê et al., 2008) and was applied to discriminate between different groups of caulerpin bioaccumulation.

3. Results

The analysis of caulerpin bioaccumulation in the liver indicated some differences between organisms sampled in the two periods and from various sites (Table 2). It is worth noting that the present quantitative analyses yielded an improvement in terms of accuracy, specificity, and sensitivity in comparison with our previous works (Terlizzi et al., 2011; Felline et al., 2012). Except for PC organisms, fish generally showed higher mean levels of caulerpin bioaccumulation in October than in June (Table 2). Tissue concentrations varied also considering the sampling site, although these differences are not significant due to the large inter-individual variability; greater mean values were generally observed in organisms sampled in BR compared to those from TG and PC (Table 2).

According to individual caulerpin tissue concentrations, organisms sampled in June were divided, irrespective of the sampling site, in three groups corresponding to a content “absent” (0 μg/g d.w.), “low” (<10 μg/g d.w.), “medium” (levels of caulerpin between 10 and 50 μg/g d.w.); for fish sampled in October, also a group with “high” content of caulerpin concentration (>50 μg/g d.w.) could be considered. Five specimens from each of those groups were randomly chosen for the biochemical and molecular measurements: individuals from at least 2 sites were always present in each bioaccumulation group, with the only exception of “absent” in October, containing fish only from PC. All the analyzed specimens resulted to be females and histological analyses demonstrated the presence of different gametogenic stages without revealing whether it was the first reproductive season as females (unpublished results).

Biochemical results in white seabreams sampled in June are shown in Table 3. Catalytic activity of cytochrome P450 (EROD) did not exhibit significant variation between the 3 groups of organisms accumulating different levels of caulerpin; on the contrary both the glutathione S-transferases (GST) and the acyl CoA oxidase significantly differ in fish with both low (<10 μg/g) and medium (10–50 μg/g) levels of caulerpin compared to fish without this algal metabolite (Table 3). In the same two groups of fish, a limited and not statistically significant decrease of AChE activity was also observed (Table 3). Among antioxidant responses, glutathione (GSH) and glutathione reductase activity (GR) were decreased in organisms with low or medium levels of caulerpin; this limited prooxidant challenge induced by caulerpin accumulation in June 2012 was also supported by the lack of variations for other sensitive antioxidant enzymes, including catalase and glutathione peroxidases, and for total oxyradical scavenging capacity and level of malondialdehyde (Table 3).

Table 4 presents results obtained in the 4 groups of fish sampled in October. Significant induction of cytochrome P450 activity was observed in organisms with medium (10–50 μg/g) and high (>50 μg/g) content of caulerpin, suggesting the involvement of biotransformation pathway in metabolism of this algal metabolite. No significant effects were observed for GST, while acyl CoA oxidase showed a significant enzymatic induction in organisms with high levels of caulerpin; the high-content group exhibited also a significant induction of acetylcholinesterase. Oxidative stress biomarkers did not exhibit variations in terms of individual antioxidants, total oxyradical scavenging capacity and lipid peroxidation products in fish sampled in October (Table 4).

Molecular analyses required the preliminary identification of specific sequences for CYP1A, PPARα and VTG1 genes. Obtained results showed a significant transcriptional induction of PPARα, in fish with medium and high content of caulerpin both in June and October, and of CYP1A in fish with high levels of caulerpin in October (Fig. 1); these molecular data almost supported the results on enzymatic induction of acyl CoA oxidase and EROD observed for the same groups of fish (Table 4, Fig. 1). Levels of VTG1 mRNA significantly increased in organisms with high level of caulerpin in October, while a certain induction, although not statistically significant, was also observed in both June and October for fish exhibiting a medium content (10–50 μg/g) of the algal metabolite.

A principal component analysis (PCA) was performed on those biomarkers showing significant responsiveness toward caulerpin content, including EROD, AOX, CYP1A, PPARα, VTG1: the 2 dimensional plot explained more than 80% of total variance, clearly separating groups of fish with medium or high content of caulerpin (irrespective of the sampling period) from those with low or absent levels (Fig. 2A). On the other hand, when all analyzed biomarkers were considered, separation of groups appeared more influenced by the sampling period (June vs October) than the content of caulerpin (Fig. 2B).

4. Discussions

In the recent decades, biological invasions by alien species have been recognized among the major threats to biodiversity and

Table 4
Biomarker responses in white seabream *Diplodus sargus* sampled in October 2012. Mean values ± standard deviations ($n = 5$). The p values are reported for significant effects and different letters indicate significant differences between groups of means (Newman Keuls post hoc); n.s. not statistically significant.

Caulerpin accumulation	0 μg/g (Absent)	<10 μg/g (Low)	10–50 μg/g (Medium)	>50 μg/g (High)	
Parameter					
EROD (pmol/min/mg prot)	17.89 ± 1.49 (a)	30.66 ± 13.12 (ab)	49.73 ± 7.54 (b)	39.29 ± 9.24 (b)	$p < 0.05$
GST (nmol/min/mg prot)	1316 ± 194	1189 ± 311	1592 ± 185	1258 ± 175	n.s.
AOX (nmol/min/mg prot)	1.959 ± 0.083 (a)	1.765 ± 0.026 (a)	1.839 ± 0.450 (a)	2.584 ± 0.159 (b)	$p < 0.05$
AChE gills (nmol/min/mg prot)	5.25 ± 0.28 (a)	3.91 ± 0.27 (a)	5.22 ± 1.13 (a)	10.25 ± 0.12 (b)	$p < 0.05$
CAT (μmol/min/mg prot)	199 ± 29	209 ± 21	259 ± 18	225 ± 30	n.s.
GSH (μmol/g tiss)	1.384 ± 0.178	1.233 ± 0.404	1.574 ± 0.473	1.532 ± 0.138	n.s.
GR (nmol/min/mg prot)	9.46 ± 1.84	9.97 ± 1.94	11.51 ± 2.24	10.41 ± 2.08	n.s.
$GPxH_2O_2$ (nmol/min/mg prot)	203.0 ± 15.8	196.4 ± 34.9	188.7 ± 12.1	198.6 ± 52.2	n.s.
GPxCHP (nmol/min/mg prot)	284.8 ± 66.2	269.5 ± 36.5	263.4 ± 45.8	294.2 ± 17.1	n.s.
TOSC ROO• (UTOSC/mg prot)	454.3 ± 97.2	406.2 ± 0.2	453.1 ± 29.9	428.5 ± 21.8	n.s.
TOSC HO• (UTOSC/mg prot)	1013.1 ± 58.3	665.2 ± 171.8	924.0 ± 195.1	902.1 ± 178.5	n.s.
MDA (nmol/g tiss)	41.07 ± 14.17	35.30 ± 2.53	41.48 ± 12.62	47.47 ± 8.32	n.s.

EROD: 7-ethoxyresorufin O-deethylase; GST: glutathione S-transferases; AOX: acyl CoA oxidase; AchE: acetylcholinesterase; CAT: catalase; GSH: levels of total glutathione; $GPxH_2O_2$ and GPxCHP: Se-dependent and sum of Se-dependent and Se-independent glutathione peroxidases; TOSC: total oxyradical scavenging capacity toward peroxyl (ROO•) and hydroxyl (•OH) radicals; MDA: content of malondialdehyde.

ecosystem functioning (Occhipinti-Ambrogi, 2007; Tamburello et al., 2013).

The green alga *C. racemosa* was introduced in the Mediterranean Sea from south-western coast of Australia, rapidly demonstrating its invasive capacity with a variety of negative effects on benthic communities (Klein and Verlaque, 2008). The production of secondary metabolites confers successful advantages for interactions with competing native macrophytes, and as anti-predatory defence against grazers. However, a limited knowledge still exists on the biological effects induced by these molecules; recent studies on human cell lines suggested a bioactive potential for caulerpin, with antiviral and anti-inflammatory properties, capability to inhibit mitochondrial respiration, alter multixenobiotics resistance and exert cytotoxic activity in melanoma cells (Rocha et al., 2007; Pinto et al., 2012).

Very recently, the occurrence of *C. racemosa* has been demonstrated in the stomach content of the *D. sargus,* typically feeding on shellfish and benthic invertebrates, which exhibited a significant accumulation of caulerpin in the liver, at concentrations up to nearly 26 μg/g d.w. (Terlizzi et al., 2011). The presence of caulerpin in the white seabream has been further confirmed in organisms collected from *C. racemosa* invaded areas (Felline et al., 2012); hepatic levels ranging from 0 to nearly 187 μg/g were paralleled by the alteration of some enzymatic pathways (including antioxidants, cytocrome P450 and acyl CoA oxidase activities) in fish with the highest content of caulerpin. In this respect, a potential role of algal

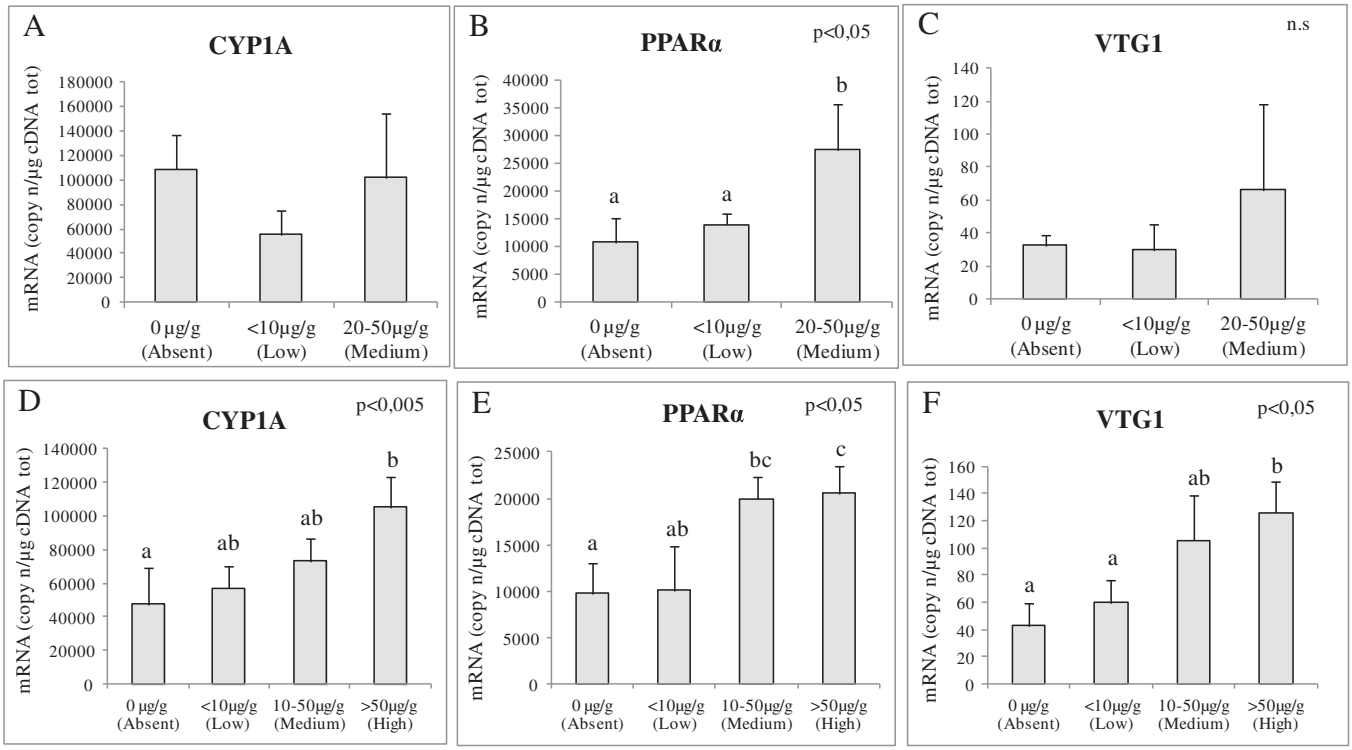

Fig. 1. Transcriptional responses of CYP1A, PPARα and VTG1 genes in the liver of *D. sargus* sampled in June 2012 (A–C) and October 2012 (D–F). Data, expressed as copy number per μg of total cDNA, are given as mean values ± standard deviations ($n = 5$). Different letters indicate significant differences between groups of means (Newman Keuls post hoc); n.s. not statistically significant.

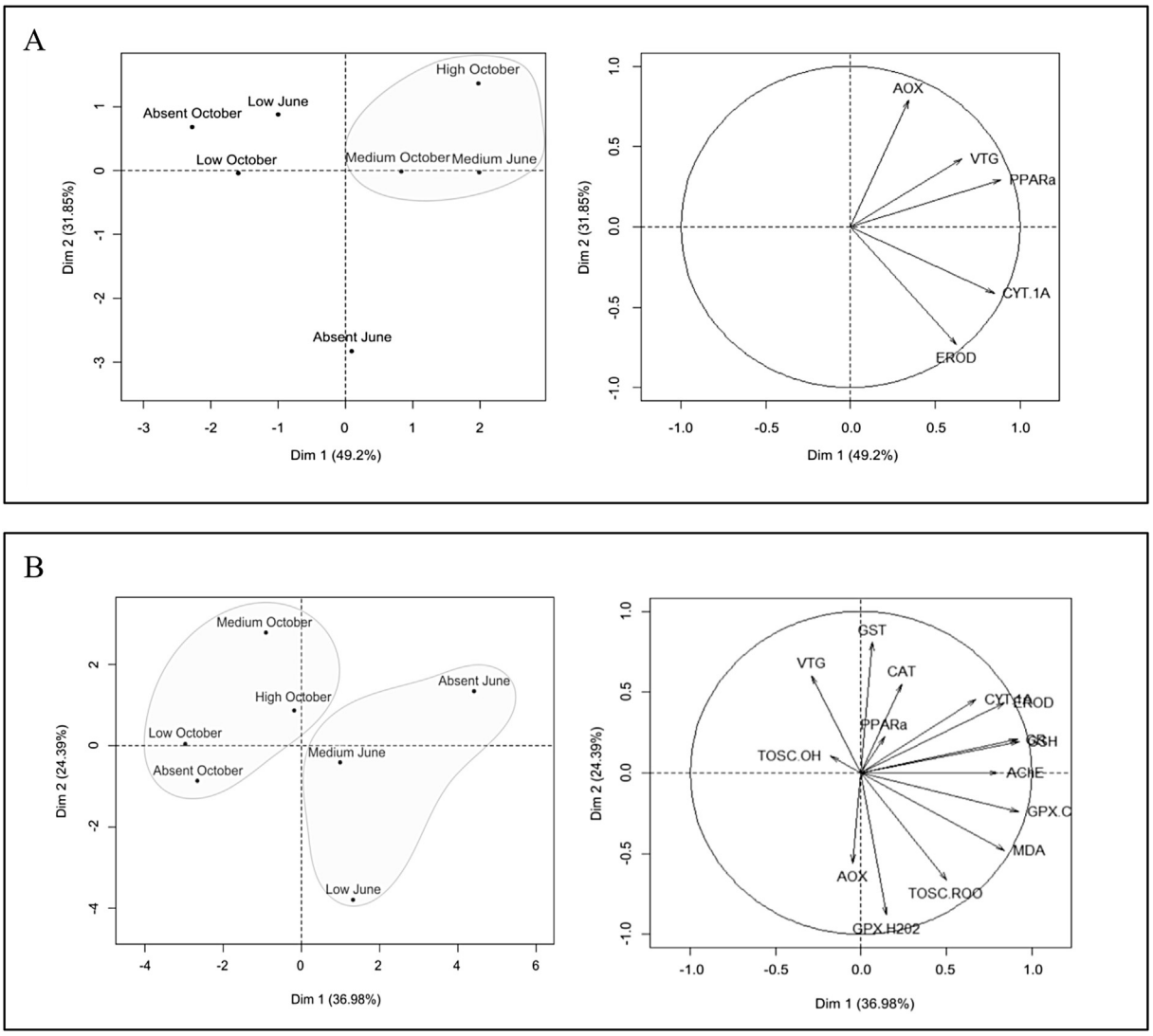

Fig. 2. A. PCA analysis performed on biomarkers data including EROD (7-ethoxyresorufin O-deethylase); AOX (acyl CoA oxidase); CYT1A (cytochrome P4501A); PPARα (peroxisome proliferator-activated receptor alpha) and VTG1 (vitellogenin 1). B. PCA analysis performed on all biomarkers data including EROD, AOX, CYT1A, PPARα, VTG1, GST (glutathione S-transferases), AChE (acetylcholinesterase), CAT (catalase), GSH (glutathione), GPxH_2O_2 and GPxCHP (Se-dependent and sum of Se-dependent and Se-independent glutathione peroxidases) TOSC ROO• and TOSC •OH (total oxyradical scavenging capacity toward peroxyl and hydroxyl radicals), MDA (malondialdehyde).

metabolites was firstly hypothesized in modulating possible adverse effects on the health status of this fish population (Felline et al., 2012).

In the present work, white seabreams were collected in the same already investigated areas along the south Apulian coast (Terlizzi et al., 2011; Felline et al., 2012), but in two different seasonal periods, June and October 2012, to address possible seasonal fluctuations in both caulerpin bioaccumulation and biological responsiveness. The measured biochemical endpoints were selected on the basis of our previous results (Felline et al., 2012) and further integrated with the study of transcriptional levels of cytochrome P4501A (CYP1A), peroxisome proliferator-activated receptor-alpha (PPARα) and vitellogenin 1 (VTG1): the overall aim was to get additional insights on the involvement of these specific pathways in an ecological link between molecular and physiological responses.

Chemical results on caulerpin accumulation indicated generally higher levels in white seabreams collected in October compared to those from June. This difference can be related to the biological cycle of *C. racemosa* characterized by a high vegetative activity from June to November, with a subsequent growth arrest until May; the invasive potential of the seaweeds is not affected during this period because the stolons remain adherent to the substrate allowing a resumption of rapid growth in summer (Ruitton et al., 2005). Production of secondary metabolites is positively correlated with seawater temperature and their content in the algal thallus exhibits a clear seasonal pattern, with minimum values in winter to early spring, an increase during the summer, reaching the maximum at the end of October (Box et al., 2010). According to such algal physiological characteristics, fish sampled in October from the invaded areas showed levels of caulerpin in the liver, often exceeding 50 μg/g (with a maximum of 235 μg/g), while in June the single highest value was 42 μg/g.

From this and previous works on the range of caulerpin concentrations in liver of the white seabream (Terlizzi et al., 2011; Felline et al., 2012), four bioaccumulation groups were defined in this study (absent, 0 μg/g d.w.; low, <10 μg/g d.w.; medium, 10–50 μg/g d.w.; high >50 μg/g d.w.) to evaluate differences of biomarker responses in each sampling period. A significant induction of cytochrome P450 was measured as both catalytic activity

(EROD) and mRNA CYP1A levels, in organisms collected in October and showing medium or high levels of caulerpin in the liver. The same result was not observed in June when a higher basal EROD activity in fish with absent levels of caulerpin (68.54 ± 36.15 vs 17.89 ± 1.49 pmol/min/mg prot in October) might have limited the responsiveness of biotransformation pathway. Despite there is no molecular evidence that caulerpin or other metabolites can act as AhR agonists, laboratory experiments with the Mediterranean scorpion fish *Scorpaena porcus* exposed to *Caulerpa taxifolia* demonstrated that the toxic effects of algal metabolites (mainly caulerpenyne) on the activities of cytochrome P450 monooxygenases were dependent on the contact time between the fish and the algae (Uchimura et al., 1999). In this respect, considering the seasonality of the vegetative activity and of metabolites production by *C. racemosa* (Box et al., 2010), we can reasonably hypothesize that in October the algal enriched diet could more strongly exert its influence on the cytochrome P450 pathway.

The onset of peroxisomal proliferation had already been suggested in white seabreams including *C. racemosa* in the diet (Felline et al., 2012), and the involvement of this pathway was further confirmed in this work. The activity of acyl CoA-oxidase (AOX) was significantly enhanced in *D. sargus* accumulating caulerpin both at low and medium levels in June and at high levels in October. The transcriptional induction of peroxisomal proliferator activated receptor α in fish with medium and high content of caulerpin, further supported the activation of peroxisome proliferation events at the molecular level. Peroxisomes have a key role in lipid metabolism and beta-oxidation of fatty acids, being modulated by a variety of natural molecules, synthetic products and anthropogenic pollutants (Cajaraville et al., 2003; Bocchetti et al., 2008; Gorbi et al., 2012). The effects reported in this study for fish accumulating caulerpin do not necessarily reflect a direct relationship of this algal metabolite with PPARα. Nonetheless, considering caulerpin content as a marker of trophic exposure to *C. racemosa*, the observed peroxisomal proliferation in this study could mechanistically explain recent findings on the content and profile of polyunsaturated fatty acids in *D. sargus* (Felline et al., submitted for publication): fish naturally experiencing a *C. racemosa* enriched diet were characterized by a progressive reduction of high unsaturated fatty acids (including ω3 and ω6 fatty acid) and consequent decrease of nutritional quality of fish filet.

Potential neurotoxic effects of *C. racemosa* were investigated in this study because other alkaloid molecules, structurally similar to caulerpin and extracted from terrestrial plants, were shown potent inhibitors of acetylcholinesterase (Andrade et al., 2005; Zhan et al., 2010); lower levels of AChE were also previously measured in the muscle of fish containing caulerpin (Felline et al., 2012). Contrasting to such results, in this study a significant induction of AChE was observed in gills of white seabreams accumulating high levels of caulerpin in October, while no difference was recorded in June. The different response in muscle and gills could be consistent with the greater sensitivity of muscle tissues than gills in teleosts (Sharbidre et al., 2011; Anandhan et al., 2012).

Enhanced intracellular generation of reactive oxygen species (ROS) and oxidative stress conditions are important pathways of toxicity mediated by several classes of chemicals and biologically active compounds (Regoli and Giuliani, in press). Oxidative mechanisms have been proposed also in the metabolism of Caulerpales metabolites by various marine organisms, and in their adaptation to the environmental presence of the algae (Box et al., 2009; Sureda et al., 2006; Terlizzi et al., 2011). Higher levels of glutathione peroxidases and glutathione reductase have been measured in the labrid *Coris julis* from areas with *C. taxifolia* or *Caulerpa prolifera*, compared to specimens sampled from *Posidonia oceanica* meadows (Sureda et al., 2006); further, the sea urchin *Paracentrotus lividus* fed in laboratory conditions with *C. racemosa*, revealed significantly increased activities of glutathione reductase and of glutathione levels (Tejada et al., 2013). Prooxidant effects of a *C. racemosa* enriched-diet have already been suggested in white seabreams by the modulation of some antioxidant enzymes, such as catalase and glutathione peroxidases (Terlizzi et al., 2011), and of the overall capability to counteract reactive oxygen species (Felline et al., 2012). This study confirmed some variations of antioxidant defenses, with the inhibition of glutathione reductase and lower levels of glutathione in fish accumulating caulerpin at both low and medium content in June. Overall these effects reflected a slight prooxidant challenge as indicated by the lack of variations for the total oxyradical scavenging capacity and malondialdehyde levels, which remained almost constant in different groups and sampling periods. In general different trends of the antioxidant responses in marine organisms exposed to *C. racemosa*, could be modulated by several biological and abiotic factors, including seasonality of reproductive cycle and feeding habit (Gorbi et al., 2005), experimental conditions (field or laboratory exposures), changes in algal abundance and secondary metabolites production, all factors which could overall influence the antioxidant responses in a unpredictable way. In addition, especially when organisms are exposed to a limited oxidative pressure, it is quite common to obtain discrepancies in the responses of antioxidants and, due to different time-courses of activation both at the transcriptional and catalytic levels, the same antioxidants can be induced, depleted or do not vary at all (Regoli et al., 2011; Giuliani et al., 2013; Benedetti et al., 2014).

This study also demonstrated a significant increase of VTG1 mRNA levels in organisms with high hepatic content of caulerpin (>50 μg/g) in October and, to a lower extent, in those with medium accumulation (10–50 μg/g) from both the sampling periods. All the analyzed specimens were females which is not surprising, considering digynic hermaphroditism of the white seabream *D. sargus* and the predominance of females in larger size classes, i.e. >20 cm length (Morato et al., 2003; Mouine et al., 2007). In addition, organisms without any caulerpin accumulation showed similarly low mRNA basal levels in both the sampling periods: in fact, June and October correspond to a resting stage status for the white seabreams of Southern Italy which exhibit gonad maturations from February to April, and a post-spawning period from April to May (Morato et al., 2003; Mouine et al., 2007). Transcriptional induction of VTG in aquatic vertebrates is widely indicated as a typical biomarker of exposure to estrogenic compounds in male and juvenile fish, while a similar effect is more difficult to envisage in adult females (Canapa et al., 2007). Despite an estrogenic potential of Caulerpales metabolites has never been hypothesized, our results may suggest similar effects for a *C. racemosa* enriched diet, with possible consequences on the reproductive success of the white seabreams, as already postulated by the evidence of a significantly lower gonado-somatic index in fish sampled from invaded areas (Felline et al., 2012).

The overall results of this study indicated that exposure to *C. racemosa* through diet causes caulerpin accumulation and effects on important cellular processes, primarily those related to the CYP450 biotransformation pathway, fatty acid metabolism, and vitellogenin modulation. Descriptive PCA analyses performed only with these biological responses (measured at both biochemical and molecular levels) confirmed this trend, separating fish on the basis of caulerpin content and clustering together those with a medium or high accumulation of this algal metabolite. However, when the whole set of biological responses was considered, including those reflecting the general oxidative status, organisms were grouped according to the sampling period, thus allowing to exclude a deleterious impairment of the health condition of white seabreams accumulating caulerpin.

In conclusion the present study provided additional evidences to support the hypothesis of a molecular modulation of invasive algae impact on local fish populations. At this stage precise mechanisms triggered by various metabolites remain to be elucidated and actually ongoing researches include specific laboratory exposures and in silico models validation studies. Finally field investigations on reproductive potential, feeding preferences and fatty acid profile of natural seabreams should further clarify the link between sublethal, molecular effects of *C. racemosa* and/or specific metabolites, with long term, acclimatization responses in *D. sargus* populations.

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