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## Bivalve trophic ecology in the Mediterranean: Spatio-temporal variations and feeding behavior

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### Abstract :

The trophic ecology of two bivalves, the clam *Callista chione* and the cockle *Glycymeris bimaculata* was studied using environmental and biochemical variables of the suspended particulate matter and the sediment. Samples were collected from two shallow sites, Pag and Cetina, in the coastal oligotrophic Mediterranean Sea, during a 17 month period.

The temporal variation of the particulate matter reflected a mixture between marine and terrestrial sources throughout the year, with a clear marine influence during summer and fall, and predominance of terrestrial inputs during spring and winter. The digestive gland was a useful rapid turnover tissue, where the carbon isotope signal was species-specific and the nitrogen isotope one was site-specific. FA markers in the digestive gland revealed a mixed diet where *Callista chione* fed more upon fresh material than *G. bimaculata* which relied largely on bacteria-derived detritus.

Overall, little feeding niche overlap was observed between the two species during the year, indicating resource partitioning, expected for a food-limited system. The present trophic ecology study in co-occurring species allowed identifying species-specific feeding adaptations to environmental variability.

### Highlights

► Particulate matter biochemical proxies show terrestrial inputs in the Adriatic Sea. ► POM quality in the shallow oligotrophic Adriatic Sea is best in spring and summer. ► Digestive gland is a useful rapid turnover tissue. ► Bivalve species show feeding adaptations to environmental variation. ► Bacterial diazotroph biomass has major importance in *G. bimaculata* diet. ► Feeding niche overlap between species is low, indicating resource partitioning.

**Keywords :** Feeding ecology, Bivalves, Suspension feeder, Biochemical variables, Stable isotopes, Fatty acids, Adriatic sea, Niche partitioning, Coastal ecosystems

29

## 30 **1. INTRODUCTION**

31 Coastal systems represent 8.5% of marine biomes and their related physical, chemical and  
32 biological processes are modulated by terrestrial influence (Costanza et al., 1997). In these  
33 systems, a mixture of organic particles from autochthonous and allochthonous origin contribute to  
34 the pool of particulate organic matter (POM) (Bode et al., 2006; Hedges et al., 1997; Savoye et al.,

2012). In temperate areas, benthic consumers are able to shift their diets along the year adapting to the available suspended material, e.g. following the magnitude and seasonality of primary production (Antonio et al., 2010). Bivalves, as suspension-feeding organisms, participate in the transfer of POM between the water column and the surface sediment layer (Schubert and Calvert, 2001). In the sediment-water interface, physical factors (e.g. currents, wind) and bioturbation enhance resuspension enabling a continuous supply of POM to suspension feeding organisms (Dubois et al., 2014; Orvain et al., 2012). Bivalves mainly feed upon phytoplankton but other sources such as detritus, bacteria, microphytobenthos and zooplankton can also constitute an important component of their diet (Davenport et al., 2000; Ezgeta-Balić et al., 2012; Grall et al., 2006; Kharlamenko et al., 2011; Peharda et al., 2012). The chemical characteristics of POM both in the pelagic and benthic compartments are tightly coupled to filter feeding activity, e.g. there is a positive feedback between phytoplankton abundance in the water column and the bivalve grazing activity (Charles et al., 1999; Page and Lastra, 2003; Prins et al., 1997; Ward and Shumway, 2004). Thus, investigating the origin and availability of food resources in POM in relation to their contribution to bivalve's diet allow to distinguish variations in the trophic activity, selective feeding, inter or intra-specific competition, and biogeochemical functioning of the ecosystem (Carlier et al., 2007; Dubois et al., 2014; Riera and Richard, 1996).

In aquatic ecosystems, stable isotope analyses (SI), e.g.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , and fatty acid (FA) concentrations have been used to analyze the food web structure and infer trophic relationships (e.g. Antonio and Richoux, 2016; Giraldo et al., 2016; Kharlamenko et al., 2008, 2011; Pérez et al., 2013). SI analyses have been widely used to describe diet composition, trophic level and habitat characteristics in marine organisms (DeNiro and Epstein, 1978; Lopez et al., 2016; Lorrain et al., 2002; Peterson and Fry, 1987) due to a time-integrated signal of the food sources, assimilated in animal tissues (Ezgeta-Balić et al., 2014; Martínez del Rio et al., 2009; Peterson and Fry, 1987). The measurement of  $\delta^{13}\text{C}$  allows the identification of the main primary producers (Post, 2002) and also discriminates sources from both terrestrial and marine, and benthic and pelagic systems (e.g. Cloern et al., 2002; Savoye et al., 2003). On the other hand, the  $\delta^{15}\text{N}$  signature is useful to identify food chain structure, based on the principle that excretion and respiration of lighter N isotopes by consumers increase the  $\delta^{15}\text{N}$  content in their tissues (Peterson and Fry, 1987). FA are involved in the organism's energy storage (Parrish et al., 2000) and are effective tracers of food sources, since

they remain mostly invariable along trophic pathways, and can be associated to different groups of organisms, such as primary producers or heterotrophs (e.g. Dalsgaard et al., 2003; Napolitano et al., 1997). However, the higher the trophic level, the more complex is the interpretation of FA as dietary indicators (Budge et al., 2002) due to the incorporation of multiple diet sources and diverse metabolic pathways (Dalsgaard et al., 2003). Still, FA are good discriminators of food sources in suspended POM and sediment (Bergamino et al., 2014; Connelly et al., 2016; El-Karim et al., 2016; Ricardo et al., 2015) and also useful describing their seasonal and spatial variations (Boon and Duineveld, 1996). Lipids play an important role as metabolic energy reserves; thus, FA analyses in the digestive gland of bivalves have been employed to trace recent food ingestion (weeks-months) (Deudero et al., 2009; Ezgeta-Balić et al., 2012; Najdek et al., 2013).

The study of fine-scale spatio-temporal patterns in environmental biochemical variations (e.g. SI, FA) and in primary producers allow assessing the intra and interspecific variability in the diet of secondary consumers which is essential for comprehensive ecological studies (Antonio et al., 2012; Gonçalves et al., 2012; Underwood et al., 2000; Ventrella et al., 2008; Yurkowski et al., 2016). Likewise, other biomarkers characterizing the composition of suspended particulate matter (SPM) in coastal ecosystems such as C:N ratios and Chlorophyll *a* are often used as indicators of the quality of biogenic matter. However, few studies incorporate the analysis of biogenic silica, which is a major component in diatom biomass (Alomar et al., 2015; Leblanc et al., 2003; Ragueneau et al., 2002). Therefore, an extended particulate matter characterization including environmental variables (e.g., temperature, salinity) with the above mentioned biochemical parameters will lead to more accurate interpretations of benthic trophic ecology.

The burrowing bivalves *Glycymeris bimaculata* (Poli, 1975) and *Callista chione* (Linnaeus, 1758) are two common benthic suspension feeders inhabiting Atlantic and Mediterranean waters (Huber, 2010; Poppe and Goto, 1993). *Callista chione* is a commercially important species in many parts of the Mediterranean Sea (Galimany et al., 2015; Metaxatos, 2004; Tirado et al., 2002), which demands a thorough knowledge on its general ecology. Both of these species have a relative long life span of over 40 years (Bušelić et al., 2015; Ezgeta-Balić et al., 2011; Forster, 1981) making them interesting targets for sclerochronological research that includes use of shell as archives of environmental change (e.g. Butler and Schöne, 2017). Further on, they co-occur along the eastern

Adriatic coast (Peharda et al., 2010) and show spatial variations in growth (Ezgeta-Balić et al., 2011; Kovačev, 2016) and timing and duration of their reproductive periods (Purroy, 2017).

Seawater properties in the shallow eastern coastal Adriatic are exposed to high temporal and spatial variations mainly characterized by large temperature fluctuations and freshwater inputs (e.g. Ogrinc et al., 2005; Surić et al., 2015). The intrusion of freshwater is more evident in river mouth areas, where also the incorporation of nutrients and organic matter from terrestrial origin stimulates biological productivity and creates a characteristic fluctuating environment. Likewise, in this karst littoral the common groundwater discharge and coastal springs also influence the marine environment (Bonacci, 2015; Bonacci et al., 1997; Surić et al., 2015). These conditions have an ecological impact such as physiological stress, changes in biological responses and shifts in community structure (Hamer et al., 2010).

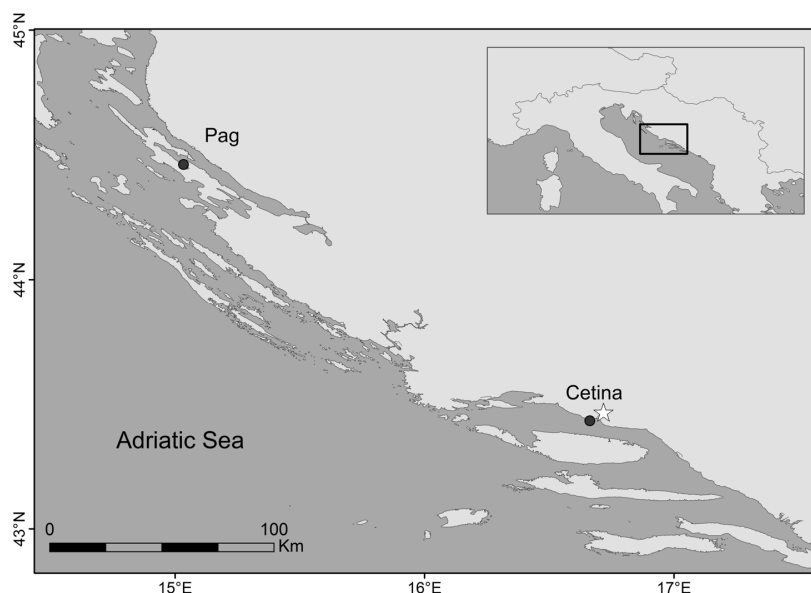
Within this context, the aim of this study was to evaluate the trophic ecology of two co-occurring suspension feeders in two different coastal settings influenced by freshwater inputs, based on a multiple tracer approach. To address this, we (1) characterized two coastal settings to identify relationships between environmental conditions with two main food sources: suspended particulate matter (SPM) and sediment (Sed); and investigated (2) spatio-temporal variations (hundreds of Km and months) in feeding patterns in bivalves using SPM and Sed and (3) trophic interactions to identify whether feeding niche overlap between species occurs. This study contributes to a better understanding of bivalve feeding mode adaptations to environmental variability in oligotrophic settings.

## 2. MATERIAL AND METHODS

### 2.1. Study area and sample collection

The study area is located on the Adriatic coast off central Croatia (Figure 1). Two sites were selected based on earlier studies, which showed differences in environmental settings and in local bivalve growth (Ezgeta-Balić et al., 2011). One study site was located in Pag Bay off the island of Pag (44° 27'42" N, 15°01'36"E) whereas the other site, Cetina (43°26'13" N, 16°41'14"E), was located close to the mouth of the Cetina River, situated on the main direction of the river plume. Both study sites have karstic origin and are under the influence of submarine freshwater springs (Surić, 2005). In these shallow coastal sites, samples were collected by SCUBA and skin diving

within a range of 2 to 5 m water depth. Previous studies on the distribution of benthic species (Peharda et al., 2010) identified these sites with sufficient population abundances for conducting a temporal collection design based on monthly samplings, ensuring collection of specimens throughout the study period.



**Fig 1.** Sampling sites in the Croatian Adriatic Sea: Pag and Cetina. A star indicates the location of the Cetina river. The black square in the inlet represents the study area.

Study was conducted between May 2014 and October 2015. Seawater temperature was recorded hourly with a Tinytag data logger deployed *in situ* and salinity was monthly measured *in situ* with a YSI-Pro probe. Twenty adult specimens of both *Glycymeris bimaculata* and *Callista chione* (hereafter also referred as Gly and Cal, respectively), were collected monthly at each site, measured for biometric analysis and processed within 3 hours by sectioning the muscle and dissecting the digestive gland (DG) from each individual. The DG is a rapid turnover tissue enabling the detection of isotopic signatures of recent ingestions (days to weeks) (e.g. Ezgeta-Balić et al., 2012). Analyses were carried out on three replicates, each of them constituted by a pool of tissues from three specimens. Each sample was stored at -20°C for subsequent analysis.

## 2.2. Environmental variables and food sources

Seawater samples were collected ~0.5 m above the seafloor using a Niskin bottle (between 10-20 L) and vacuum filtered in the laboratory. Prior to filtration water containers were gently agitated

to homogenize seawater and filters were rinsed with distilled water. Pre-weighed nitrocellulose filters (Whatman<sup>TM</sup>, 47 mm diameter, 0.45  $\mu$ m mesh size) were used to measure bulk suspended particulate matter (SPM) and biogenic silica content (BSi) while pre-combusted (450°C, 6 h) and pre-weighted glass fiber filters (Whatman<sup>TM</sup> GF/F, 25 mm diameter, 0.7  $\mu$ m mesh size) were used to measure elemental composition and ratios of C (weight %) and N (weight %), and their isotopic composition ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ; see 2.3), fatty acids (FA) and lipid proportions (see 2.4). The concentration of SPM was determined on the dry mass of the total suspended particles retained per unit of volume after drying (60°C, 24 h) (expressed as mg/L). SPM filters were also used for BSi content analysis following a sequential alkaline digestion (2 to 5 h) with  $\text{NaCO}_3$  to distinguish between biogenic and lithogenic silica sources (DeMaster, 1991; Mortlock and Froelich, 1989). Around 1 L of seawater per replicate was filtered for Chlorophyll *a* (Chl *a*) analyses through glass fiber filters which were folded, wrapped in aluminum foil, frozen and stored at -20°C. Chl *a* was extracted in 90 % acetone (Strickland and Parsons, 1972) and measured in a Turner Systems (Sunnyvale, CA) fluorometer (Trilogy). Triplicate samples were conducted for all chemical analysis. Additionally, satellite-derived Chl *a* data was extracted from the MODIS-Aqua Sensor provided by the EU Copernicus Marine Service (CMEMS).

Surface sediment samples (ca. upper 2 cm) (Sed) were collected monthly by SCUBA diving using a plastic core and preserved at -20°C. Homogenized and ground samples were used for %C, %N and Si analyses (see 2.3), Chl *a* samples were extracted in 90% acetone (4°C, 12 h) (Lorenzen and Jeffrey, 1978), BSi content was measured following the procedure for SPM, and total carbon (TC) was measured in a LECO Truspec CN-2000 analyzer. Inorganic carbon (IC) (expressed in dry weight %) was calculated by the difference between TC and OC content (see 2.3). To identify the effect of grain size on the OC content, grain size was determined in a HORIBA LA950V2 laser scattering particle size distribution analyzer after removal of organic matter in a 20% hydrogen peroxide solution (Table 1).

### 2.3. Stable isotope analysis (SIA)

Particulate organic carbon and nitrogen elemental and isotopic compositions were measured in filters dried at 50°C for 24 h, 50 mg of Sed and 1 mg of DG. Sed and DG were freeze-dried and individually homogenized using an agate mortar and pestle. Aiming to remove carbonates, SPM



filters and DG samples had a 6 h exposure to HCl fumes (Lorrain et al., 2003) while Sed samples were exposed to 24 h (due to the high content of carbonates) prior to isotopic analyses. Further, to avoid effects of acidification in Sed (e.g. Walthert et al., 2010), non-acidified subsamples were used for the analysis of  $\delta^{15}\text{N}$ . To eliminate residual HCl and water, all samples were ventilated overnight at room temperature and dried in a hotplate (50°C, 6 h). Filters were folded and placed into tin cups (12.5 mm x 5 mm) whereas Sed and DG samples were placed into tin cups wrapped by silver cups to avoid losses due to tin corrosion (8 mm x 5 mm). Stable isotope ratios of carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ), and elemental %C and %N analyses were conducted on a Carlo Erba Elemental Analyzer EA1108 coupled to an isotope-ratio mass spectrometer ThermoFinnigan MAT253 in the Unidade de Técnicas Instrumentais de Investigación, University of A Coruña (Spain). Results are expressed in standard unit notation (VPDB for the carbon and atmospheric air for the nitrogen):  $\delta X = ([R \text{ sample} / R \text{ standard}] - 1) \times 1000$ , where X represents  $^{13}\text{C}$  or  $^{15}\text{N}$ , and R is the ratio of  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ , respectively. An acetanilide standard was used for the quantification of the amounts of %N and %C. Isotopic data are given in the conventional delta notation in units of parts per thousands (‰) with a resolution of  $\pm 0.15\text{‰}$ . Lipid content in tissues may affect  $\delta^{13}\text{C}$  values, where high lipid concentrations result in depleted  $^{13}\text{C}$  biasing the isotopic signal (DeNiro and Epstein, 1978; Focken and Becker, 1998). However, previous studies in bivalves did not find this relationship (Ezgeta-Balić et al., 2014; Lorrain et al., 2002) therefore, lipid extraction was not conducted in the present study. A two-source isotopic mixing model was used in MixSIAR package, a graphical user interface (GUI) built on R software (Parnell et al., 2010), that uses an algorithm based on Bayesian statistics to determine the probability distributions for proportional food source contribution to the mix diet of consumers (Semmens et al., 2013). This model accounts for uncertainty in isotopic values when estimating the contribution of sources in the diet due to the incorporation of diet-tissue discrimination factors. Discrimination factors of  $0.5 \pm 0.13\text{‰}$  for  $\delta^{13}\text{C}$  and  $2.3 \pm 0.18\text{‰}$  for  $\delta^{15}\text{N}$  considered for benthic consumers (Alomar et al., 2015; McCutchan et al., 2003) were incorporated into the model. Despite the increasing number of studies for prey-predator fractionation values, there are no species-specific values for our target species; therefore, conventional fractionation values for benthic consumers can be applied, as supported by Yokoyama et al., (2005).

#### 2.4. Fatty acid analysis (FA)

SPM, Sed and DG samples were collected monthly during one year period, kept frozen and freeze-dried prior to biochemical analyses. For total lipid analysis all samples were weighted (DG samples were blended) and after, a dichloromethane-methanol (DCM:MeOH) mixture (2:1) sonicated in a water bath at 30°C was added. After separation DCM phases were pooled and evaporated to dryness and weighed. Total lipids of digestive glands were re-dissolved in DCM and neutral lipids were separated according to Pernet et al., (2012). All extracts (total lipids of SPM and Sed and neutral lipids of DG) were saponified (1.2 M NaOH), acidified (6M HCl) and methylated (14% BF<sub>3</sub> in methanol) then extracted in DCM. Fatty acid methyl esters (FAMES) were analyzed by Agilent gas-liquid chromatography (GLC) 6890 N GC System equipped with a 5973 Network Mass Selective Detector, capillary column (25 m x 0.3 mm x 0.25 µm; cross-linked 5% phenylmethylsiloxane) and ultra-high purity helium as the carrier gas. The GLC setting was programmed column temperature rise from 145°C by 4°C min<sup>-1</sup> up to 270°C at a constant column pressure of 2.17 kPa. Retention times, peak areas and mass spectra were recorded with Chemstation software. Bacterial FAME standard mix, FAMES mix C18-C20, polyunsaturated fatty acids standards (PUFA1 and PUFA3), cod liver oil and various individual pure standards of FAME were used (Supelco). FAs were reported as percentages of the total fatty acids (% TFA, mean ± SD) and grouped as saturated (SFA), monounsaturated (MUFA), PUFA, and bacterial fatty acids (BACTERIAL; 15:0+15iso+15anteiso+17:0+17iso+17anteiso+18:1(n-7); (Mayzaud et al., 1989; Najdek et al., 2002). Unsaturation degree (UND) was calculated according to Pirini et al. (2007). The ratio of C16:1/C16:0 (>1.0) and the relative dominance of C20:5 (n-3) (EPA) and C14:0 are considered as diatom indicators (Ackman et al., 1968; Kharlamenko et al., 1995; L  veill   et al., 1997) while the ratio of C22:6 (n-3)/C20:5 (n-3) (DHA/EPA) (>1.0), DHA, 16:0 and C16:1/C16:0 (<1.0) are predominant in dinoflagellates (Budge and Parrish, 1998; Dalsgaard et al., 2003). DHA, EPA and ARA (20:4 (n-6)) are considered Essential FA (EFA; Alkanani et al., 2007) and are transferred to microzooplankton (Ventrella et al., 2008). Herbivorous calanoid copepod markers are C20:1 and C22:1 (Falk-Petersen et al., 1987). A marker indicator of zooplankton (Sargent and Falk-Petersen, 1988) and detrital matter (Fahl and Kattner, 1993) is C18:1(n-9).

## 2.5. Statistical analysis

### 2.5.1. Environmental variables

Two-way ANOVAs were used to test the effect of site (2 levels) and month (17 levels) in SPM, Chl  $\alpha$ , BSi and lipids. A t-test was done for BSi in the sediment to check for site differences. Statistical assumptions for normality (Shapiro test) and homoscedasticity (Levene's test) were confirmed prior to all analyses.

#### 2.5.2. Stable isotopes

Two-way ANOVAs were used to assess the effect of site and month on isotopic and C:N molar ratios in food sources and consumers. The Stable Isotope Bayesian Ellipses method in R (SIBER package) was used to investigate isotopic niches by examining the dispersion of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values together with the MixSIAR package as explained in section 2.3 (Jackson et al., 2011; Parnell et al., 2010). A Standard Ellipse Area (SEA), which is to bivariate data as SD is to univariate data, was applied to measure the isotopic niche width using Bayesian inference. SEAs are an alternative to Convex Hull methods since the ellipses are unbiased with respect to sample size and their estimation allows robust comparisons between data sets (Jackson et al., 2011). SEA values were corrected for small sample size ( $\text{SEAC}_c$ ) containing 95% of the data, and Bayesian estimates ( $\text{SEA}_B$ ) were estimated as a measure to trophic width between species-sites. Analyses were performed in R v3.1.3 (R Core Team, 2015).

#### 2.5.3. Fatty acids

Analyses of similarities (ANOSIM) were performed in all detected FA profiles in SPM and Sed to determine resemblance among sampling periods and sites, and among consumer groups across sampling periods (PC-CC, PG-CG, PG-PC and CG-CC, referring to *Callista chione* from Pag (PC) and Cetina (CC) and to *Glycymeris bimaculata* from Pag (PG) and Cetina (CG). All replicated data were used and  $\log(x+1)$  transformed to improve normality prior to analysis. Results based on a global R statistic and p-value were reported. Non-metric multidimensional scaling (nMDS) using a Bray-Curtis similarity matrix were used to visualize the temporal compositional differences of all detected monthly mean FA profiles in each compartment with respect to site. Similarity percentage analysis (SIMPER) and principal component analysis (PCA) were applied to identify the main contributors of dissimilarities in the FA profiles between sites in each compartment. SIBER was also used to analyze feeding niche overlap using the x and y coordinates of the nMDS analysis of FA profiles of consumer groups following Antonio and Richoux (2014). SIBER analyses were

performed using R v3.1.3 (Jackson et al., 2011; R Core Team, 2015) and multivariate analysis (ANOSIM, nMDS, SIMPER and PCA) using PAST 3.0 (Hammer et al., 2001).

**Table 1.** Parameters measured in the different compartments at two study sites, Pag and Cetina between May 2014 and October 2015.

	SPM	Sed	DG
Chl <i>a</i>	✓	✓	
BSi & total Si	✓	✓	
Granulometry		✓	
%OC, %N, $\delta^{13}\text{C}$ , $\delta^{15}\text{N}$	✓	✓	✓
%IC		✓	
FA	✓	✓	✓
Lipids	✓	✓	✓

### 3. RESULTS

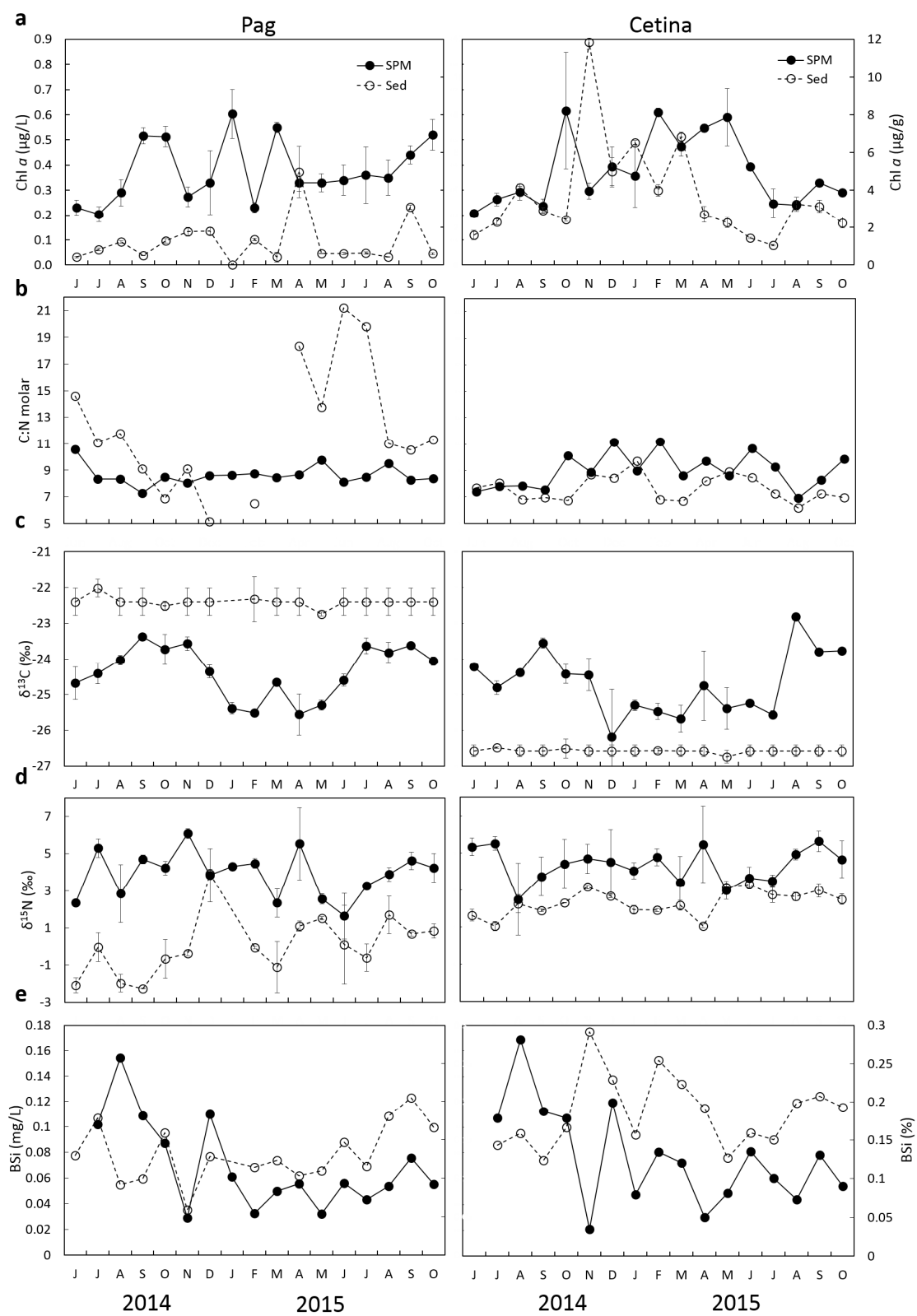
#### 3.1. Environmental variables and food sources

Temperature at both sites followed a significantly correlated temporal trend ( $r = 0.98$ ,  $P < 0.05$ ) with monthly means between 8.9°C and 25.1°C in Pag and 12.7°C and 26.4°C in Cetina. Monthly salinity values ranged between 34.7 and 38.3 in Pag and 32.2 and 38.5 in Cetina. These values are typical in coastal eastern Mediterranean waters (Vilibić et al., 2015). SPM concentrations had means of  $1.02 \pm 0.33$  mg/L in Pag and  $0.92 \pm 0.35$  mg/L in Cetina (Table S1) and didn't significantly differ between sites ( $P > 0.05$ , Table S2). In Pag, monthly values ranged from 0.43 to 3.98 mg/L and highest values were observed in both summers with a peak in March 2015, whereas in Cetina SPM ranges varied from 0.53 to 1.99 mg/L with two peaks observed in February and June 2015. Temporal variation of Chl  $a_{\text{SPM}}$  were similar between sites ( $P > 0.05$ ) ranging from 0.2 to 0.6 µg/L in Pag and 0.2 to 0.7 µg/L in Cetina (Tables S1, S2). In Pag, the maximum Chl  $a_{\text{SPM}}$  values were observed in October 2014 and in January, March and October 2015 whereas the lowest concentration of Chl  $a_{\text{SPM}}$  was observed in both summers. In Cetina, the highest Chl  $a_{\text{SPM}}$  concentration was observed during winter and spring. Between June-September 2014 and June-August 2015, the low values of both Chl  $a_{\text{SPM}}$  and Chl  $a$  % in SPM, suggested that this period was the poorest for Chl  $a$  availability in the water column (Fig 2a, Table S1). A similar annual trend was observed from satellite Chl  $a$  extracted from the MODIS-Aqua sensor with overall low Chl  $a_{\text{SPM}}$  values in summer and fall and high values during spring and winter (Fig S1). Significant differences were observed in Chl  $a_{\text{Sed}}$  between sites ( $P < 0.001$ ) with ranges from 0.4 to 5.0 µg/g in Pag and 1.1 to 11.8 µg/g in Cetina; the latter showed high values between November 2014 and March 2015.

The annual patterns of  $\text{Chl } a_{\text{SPM}}$  and  $\text{Chl } a_{\text{Sed}}$  were significantly correlated in Cetina ( $r = 0.49$ ,  $P < 0.05$ ) but not in Pag ( $r = 0.15$ ,  $P > 0.05$ ).

The concentration of  $\text{BSi}_{\text{SPM}}$  showed high temporal variability ranging from 0.03 to 0.20 mg/L in Pag and from 0.02 to 0.20 mg/L in Cetina and non-significant differences between sites (Tables S1, S2). Summer 2014 presented the highest  $\text{BSi}_{\text{SPM}}$  values and no significant correlation was found between  $\text{BSi}_{\text{SPM}}$  and  $\text{Chl } a_{\text{SPM}}$  at any site (all at  $r < 0.28$ ,  $P > 0.05$ ) (Fig 2). The concentrations of  $\text{BSi}_{\text{Sed}}$  ranged from 0.06 to 0.2% in Pag and 0.1 to 0.3% in Cetina and showed significant differences between sites (t-test,  $P < 0.05$ ).  $\text{BSi}_{\text{SPM}}$  and  $\text{BSi}_{\text{Sed}}$  were coupled during most of the study period (Fig 2e).

Sediment grain size was relatively constant with high content of sand and gravel, and low content of silt and clay (fine sediment), which accounted for  $< 2\%$ . Sand (2 mm – 63  $\mu\text{m}$ ) represented a higher portion in Cetina (87%) than in Pag (74%) whereas gravel ( $> 2\text{mm}$ ) was more abundant in Pag (26% vs 11%). No significant relationships were found among grain size and OC content ( $r = 0.12$ ,  $P > 0.05$ ).



**Fig 2.** Temporal variations at Pag and Cetina sampling sites (a) Chlorophyll *a* concentration (Chl *a*) - note different units in two vertical axis, (b) C:N molar ratio, (c)  $\delta^{13}\text{C}$ , (d)  $\delta^{15}\text{N}$  and (e) biogenic silica (BSi) in suspended particulate matter (SPM) and sediment (Sed) - note different units in two vertical axis. Error bars represent standard deviations. Three replicates were used for each data point.

### 3.2. Stable isotopes

#### 3.2.1. Food sources

The isotopic temporal variation ranged from -25.6 to -23.4‰ for  $\delta^{13}\text{C}_{\text{SPM}}$  and 1.6 to 6.1‰ for  $\delta^{15}\text{N}_{\text{SPM}}$  in Pag and from -26.2 and -22.8‰ for  $\delta^{13}\text{C}_{\text{SPM}}$  and 0.03 to 5.6‰ for  $\delta^{15}\text{N}_{\text{SPM}}$  in Cetina (Fig 2c, 2d). Two-way ANOVAs indicated significant differences between month and the interaction with site in  $\delta^{13}\text{C}_{\text{SPM}}$  (at  $P < 0.001$ ) but not between sites ( $P > 0.05$ ), whereas for  $\delta^{15}\text{N}_{\text{SPM}}$  there were significant differences between sites, month and their interaction (all at  $P < 0.001$ ; Table S3). Greater isotopic values were observed during summer and fall at both sites. In the sediment, the isotopic temporal variation ranged from -22.7 to -22.0‰ for  $\delta^{13}\text{C}_{\text{Sed}}$  and -2.3 to 3.9‰ for  $\delta^{15}\text{N}_{\text{Sed}}$  in Pag, and from -26.7 to 26.5‰ for  $\delta^{13}\text{C}_{\text{Sed}}$  and 1.1 to 3.3‰ for  $\delta^{15}\text{N}_{\text{Sed}}$  in Cetina. There were significant differences in  $\delta^{13}\text{C}_{\text{Sed}}$  between sites (at  $P < 0.001$ ), whereas neither significant temporal differences nor in their interaction with site ( $P > 0.05$ ; Table S3). More negative values in  $\delta^{13}\text{C}_{\text{Sed}}$  than  $\delta^{13}\text{C}_{\text{SPM}}$  were observed in Cetina whereas the contrary was observed in Pag. Significant differences in  $\delta^{15}\text{N}_{\text{Sed}}$  (all at  $P < 0.001$ ) made evident the lower and variable values in Pag and greater and constant in Cetina (Fig 2d). On the other hand,  $\delta^{15}\text{N}_{\text{SPM}}$  showed more positive values than in  $\delta^{15}\text{N}_{\text{Sed}}$  at both sites.

The C:N<sub>SPM</sub> molar ratios did not significantly differ between sites ( $P = 0.055$ ), with ranges from 7.3 to 10.6 in Pag and from 6.9 to 11.2 in Cetina; however, temporal differences within site were significant (at  $P < 0.001$ ), and Pag values showed a lower annual mean (Table S3). There was no evident annual pattern at any site (Fig 2b). A significant correlation was shown between C:N<sub>SPM</sub> and SPM at both sites ( $r = 0.50$ ,  $P < 0.05$ ) and between C:N<sub>SPM</sub> and Chl *a*<sub>SPM</sub> in Cetina ( $r = 0.60$ ,  $P < 0.05$ ), illustrated with the coincidence of peaks in October 2014 and February and April-June 2015 (Fig 2). BSi<sub>SPM</sub> and C:N<sub>SPM</sub> were not significantly correlated at any site (all at  $r < 0.47$ ,  $P > 0.05$ ). In all cases, POC<sub>SPM</sub>/Chl *a*<sub>SPM</sub> values were  $> 200$  (Table S1).

C:N<sub>Sed</sub> molar ratios in Pag varied from 5.2 to 21.2 whereas in Cetina from 6.2 to 9.7. In Pag, high values were recorded from spring to mid-summer. The range of C:N<sub>Sed</sub> values in Cetina was similar

to that observed in  $C:N_{SPM}$  (Fig 2b, Table S3). Inorganic carbon represented 10.7 to 12.1% of sediment weight in Pag and from 7.6 to 11.6% in Cetina (Table S1). The values in Pag were closer to the weight % of Carbon in the  $CaCO_3$  molecule, suggesting that  $CaCO_3$  is the main inorganic carbon constituent in this area. Overall, more than 93% of the TC was inorganic in both sites. Total Nitrogen values ranged from 0 – 0.05% at both sites (Table S1).

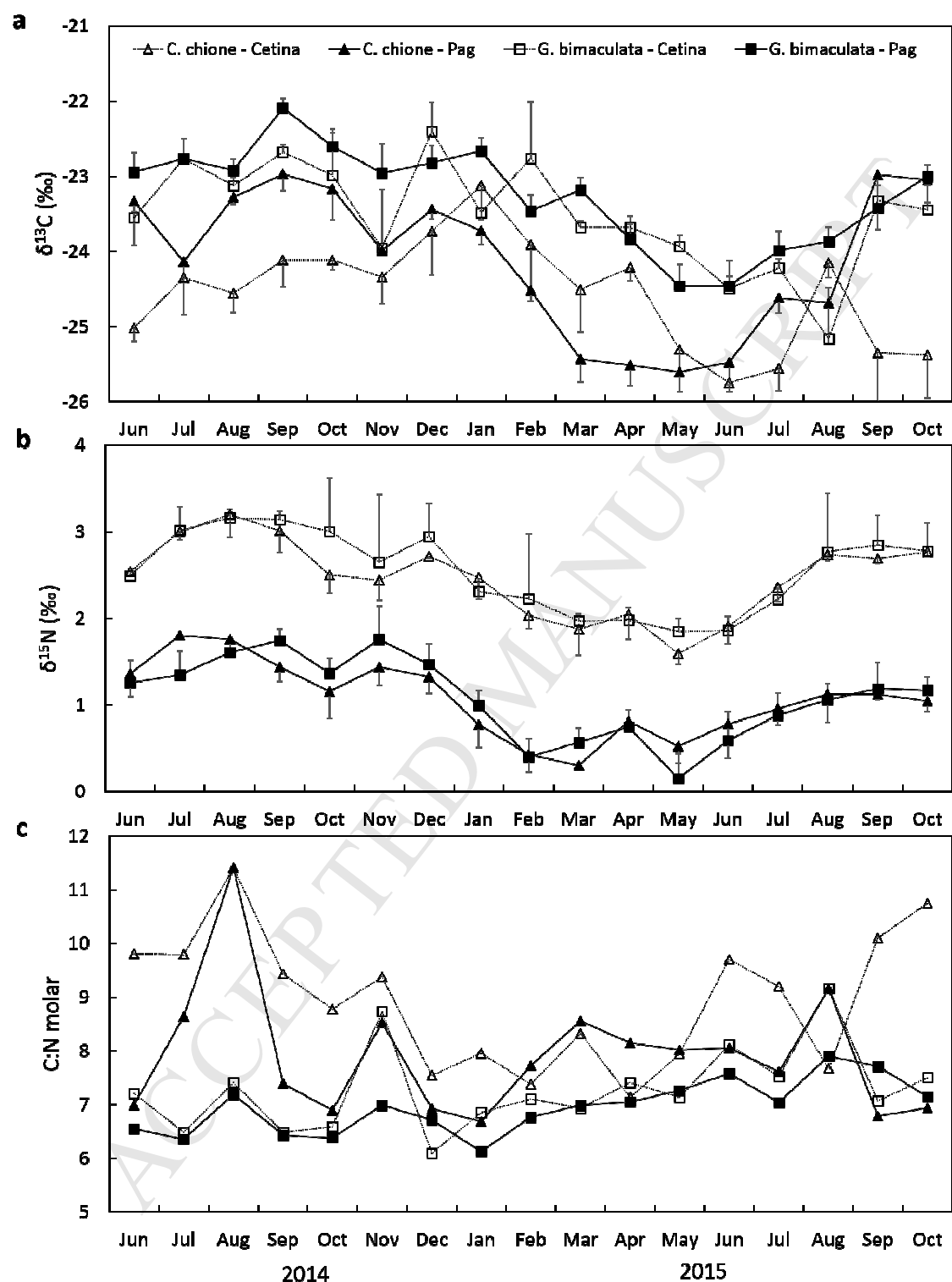
### 3.2.2. Consumers

The DG isotopic carbon signature showed a temporal variation that ranged from -25.6 to -23.0‰ in Pag and -25.8 to -23.1‰ in Cetina in *C. chione*, and slightly higher  $\delta^{13}C_{DG}$  in *G. bimaculata*, from -24.5 to -22.1‰ in Pag and -25.2 to -22.4‰ in Cetina (Fig 3a). In *C. chione* the  $\delta^{15}N_{DG}$  varied from 0.3 to 1.8‰ in Pag and 1.6 to 3.2‰ in Cetina and in *G. bimaculata* from 0.2 to 1.8‰ in Pag and 1.9 to 3.2‰ in Cetina (Fig 3b). Two-way ANOVAs revealed significant differences in  $\delta^{13}C_{DG}$  between sites, month and their interaction for both species (at  $P < 0.001$ ), same for  $\delta^{15}N_{DG}$  values except for temporal changes between sites for *C. chione* ( $P > 0.05$ ) (Table S3). These results indicated that  $\delta^{13}C_{DG}$  variation was species-dependent with slightly more  $^{13}C$ -depleted values in *C. chione*. In addition, a temporal offset of weeks in  $\delta^{13}C_{DG}$  values relative to  $\delta^{13}C_{SPM}$  values, observed in both sites (Figs 2c, 3a). On the other hand,  $\delta^{15}N_{DG}$  values were site-dependent, with more  $^{15}N$ -enriched values corresponding to Cetina samples. Overall, enriched isotopic values were observed between June 2014 to January 2015 and July to October 2015, whereas lower values occurred from February to June 2015. The  $C:N_{DG}$  molar ratio showed significant differences between sites, month and their interaction for *C. chione* ( $P < 0.001$ ) but they were less evident for *G. bimaculata* ( $P \geq 0.001$ ) (Fig 3c; Table S3).

The proportion of each food source on the consumer diets estimated by MixSIAR models revealed that bivalves used both SPM and Sed; the Gelman-Rubin diagnostic for each model  $< 1.05$ , supported the result. However, relative importance of each food source differed between sites. Sed was more important for Pag populations with a small contribution of SPM during summer, whereas in Cetina SPM contributed the most to the bivalves diet (Fig 4). The output from SIBER analysis revealed a large overlap from both convex hull and SEA between species isotopic niche at each site, indicating an overall proximity in their isotopic niche (Fig 5a). According to the temporal isotopic distribution results, feeding niche overlap occurred just in November and March,

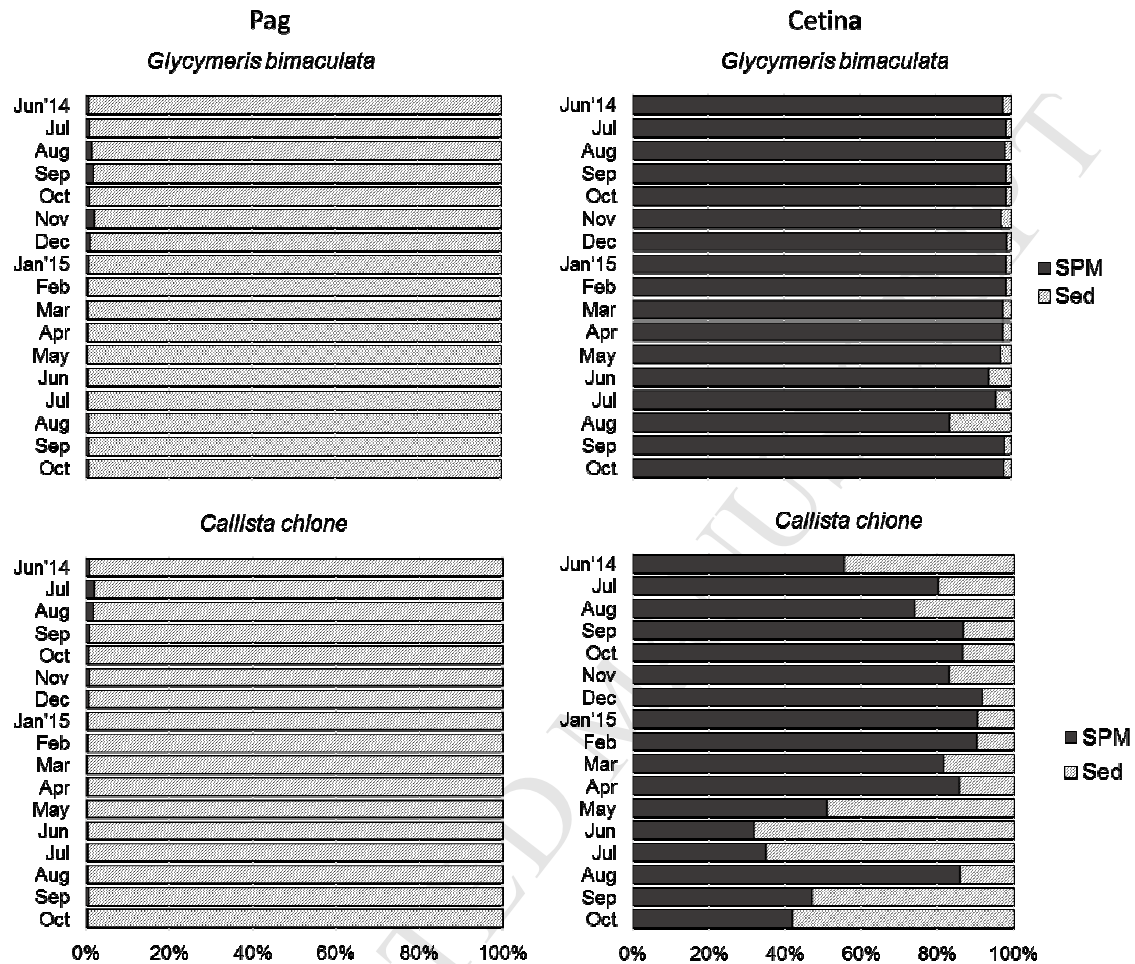


376 revealing different composition of isotopic food sources throughout the year (Fig 5a, Fig S2) and  
377 making the temporal variation observed in Fig 3 clearer. Individuals of Gly<sub>pag</sub> showed the smallest  
378 isotopic feeding niche with a complete SEA overlap and a convex hull overlap with Cal<sub>pag</sub>. Cal<sub>pag</sub> had  
379 a wider niche, where the most enriched values overlapped with those from Gly<sub>pag</sub>. Based on SEA<sub>B</sub>  
380 results, Cal<sub>pag</sub> and Gly<sub>Cetina</sub> have a greater plasticity than the other species at these locations (Fig  
381 5c).

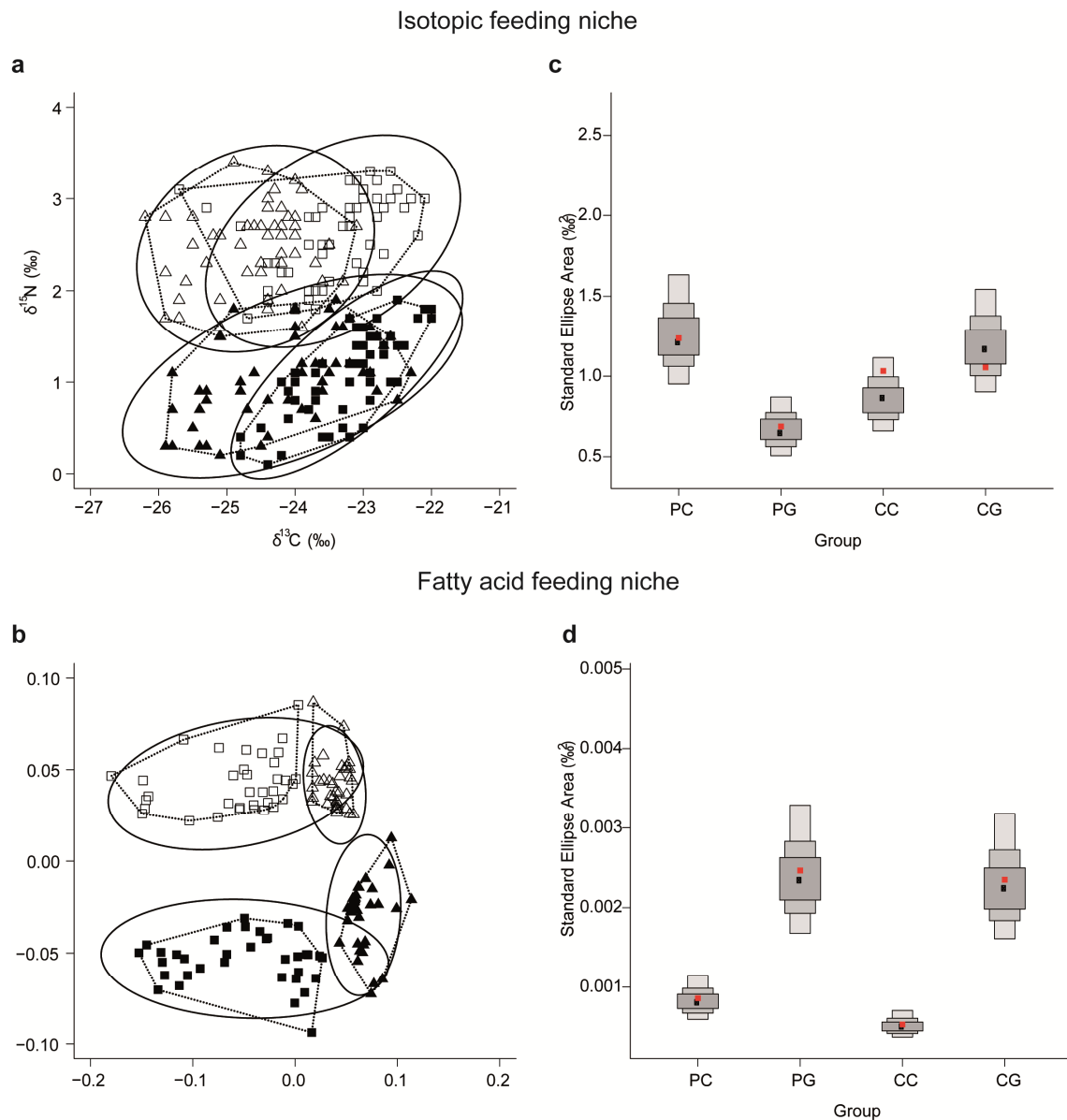


**Fig 3.** Temporal variation in (a)  $\delta^{13}\text{C}$  (‰), (b)  $\delta^{15}\text{N}$  (‰) and (c) C:N molar ratio in the consumer's digestive

gland from both sites. *Callista chione* (*C. chione*) and *Glycymeris bimaculata* (*G. bimaculata*) from Pag and Cetina.



**Fig 4.** Contribution of food sources: suspended particulate organic matter (SPM) and sediment (Sed) to the diet of *Glycymeris bimaculata* and *Callista chione* at two sites: Pag and Cetina. Output from a two-source MixSIAR model for each population.



**Fig 5.** Isotopic (SI) and fatty acid (FA) feeding niche of two benthic suspension feeders at two sampling sites during 17 months. SIBER output using (a) pooled stable isotope bi-plot and (b) nMDS x-y ordinates of pooled FA profiles. *Glycymeris bimaculata* (squares) and *Callista chione* (triangles) in Pag (filled) and Cetina (empty). The continuous line represents the small sample size corrected standard ellipse areas (SEA<sub>c</sub>) and the convex hulls areas are the dotted lines of calculated feeding niche widths for each species. Mean standard ellipse area (SEA) estimates for each group (PC= *Callista chione* from Pag; CC= *C. chione* from Cetina; PG= *Glycymeris bimaculata* from Pag and CG= *G. bimaculata* from Cetina) for SI (c) and FA (d). Boxed areas indicate the SEA<sub>B</sub> with Bayesian 50, 75 and 95% credible interval with the mode indicated by black squares.

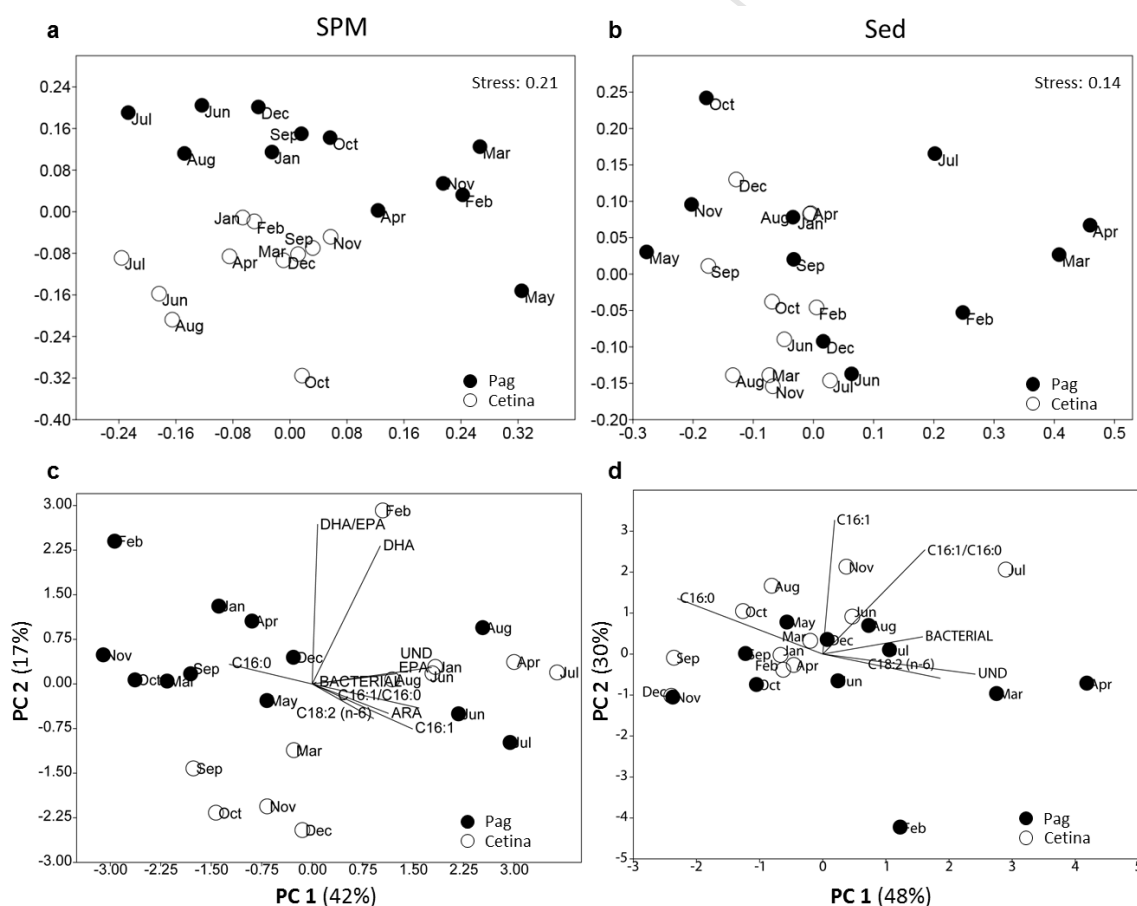
The maximum likelihood estimate for the corresponding SEAc as depicted in Fig 5 is indicated by red squares.

### 3.3. Fatty acids

FA with values  $> 0.01$  (1% of total FA) are represented in Table S5 for SPM and Sed and in Table S6 for DG. All compartments showed the higher proportions on saturated fatty acids (SFA), compared to MUFA and PUFA, with higher TFA% during fall and in DG also during winter. The lowest SFA values were observed during the summer, when PUFA was more abundant.

#### 3.3.1. Food sources

Considering all  $FA_{SPM}$  ( $n = 33$ ) and  $FA_{Sed}$  ( $n = 24$ ), a two-way ANOSIM and nMDS were performed showing significant differences between sites and month ( $r > 0.99$ ,  $P < 0.001$ , and  $r > 0.84$ ,  $P < 0.001$ , respectively) and nMDS revealed the large temporal spread of the data between sites, which was more evident in SPM than in Sed (Fig 6a,b).

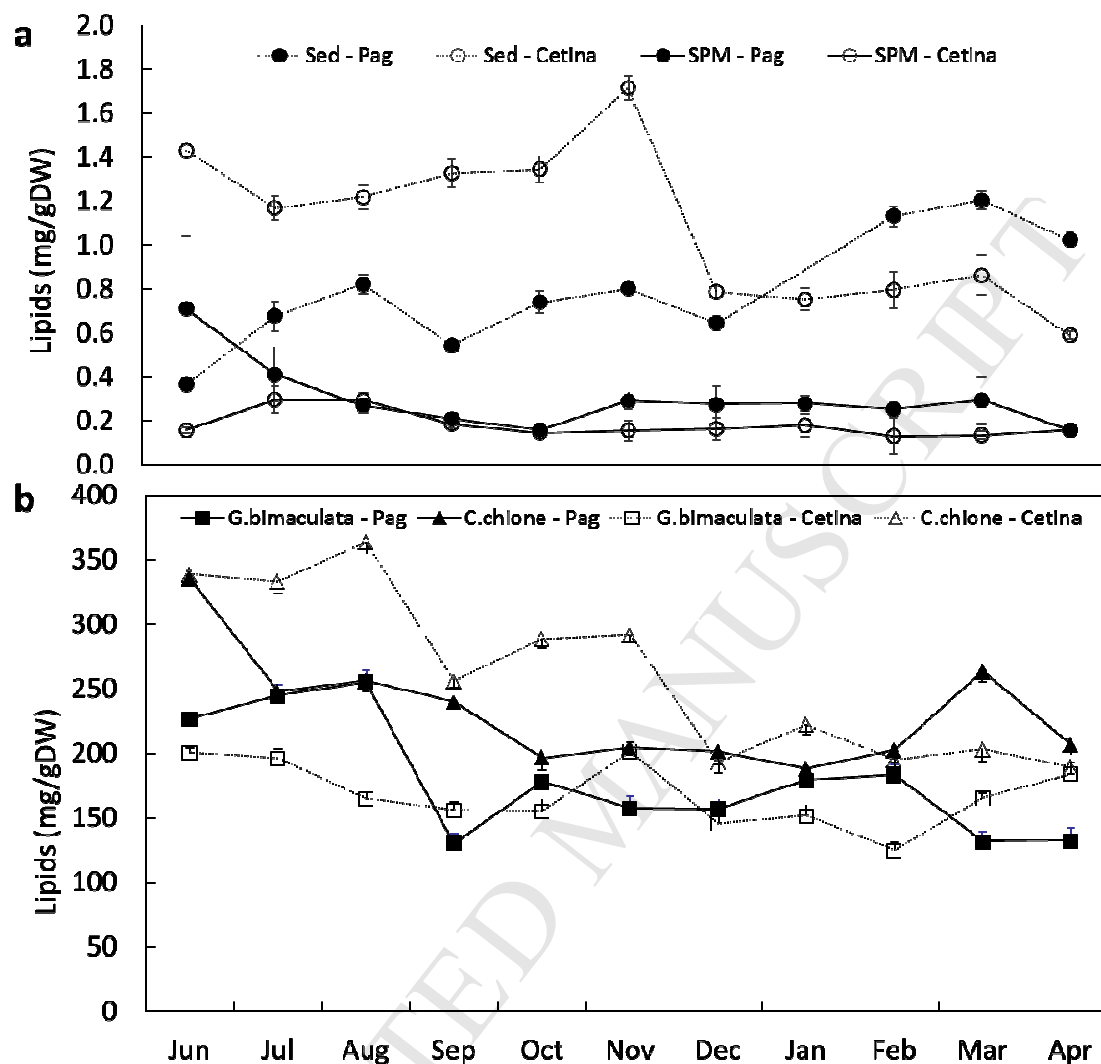


**Fig 6.** The upper panels show the non-metric multidimensional scaling (nMDS) of (a) suspended particulate organic matter (SPM) and (b) sediment (Sed), based on all FA profile averages. The bottom panels show the Principal component analysis (PCA) output using most influential FA profiles (see text) in (c) SPM and (d) Sed. Sampling sites are represented as filled dots for Pag and blank dots for Cetina.

SIMPER analyses revealed that the FA C16:1, C18:2 (n-6) and PUFA were the profiles which better explained dissimilarities between sites for both SPM and Sed, with a cumulative contribution of nearly 50% for both compartments (Table S7).

To observe the spatio-temporal variations of the main FA profiles for each food source (SPM and Sed) identified with SIMPER, PCAs were performed (Fig 6c,d). In the case of FA<sub>SPM</sub>, January and April (only at Cetina) and summer months (at both sites) were positively related to PC1 (explaining 42% of the variance) due to the contribution of EPA, UND, 16:1, 16:1/16:0, 18:2, BACTERIAL and ARA. This statistical relationsuggested thatphytoplankton material was combined with bacterial and terrestrial components in the SPM in those months. In PC2, the contribution of DHA and DHA/EPA showed that SPM sampled in February at Cetina had organic matter with good quality as well. In contrast, the negative relation of FA<sub>SPM</sub> in PC1 indicated by C16:0, showed that the organic matter available in SPM in Pag during fall, winter and spring had relatively poorer quality. However, FA<sub>SPM</sub> from Pag were more enriched with DHA than FA<sub>SPM</sub> from Cetina during the same period (Fig 6c). The spatio-temporal separation of FA<sub>Sed</sub> samples was less evident than the one observed for FA<sub>SPM</sub>, suggested that most of the samples represent a food mixture without predominant components. However, a small prevalence of the terrestrial component could be detected in March and April at Pag, whereas a phytoplankton component was prevalent during summer at Cetina (Fig 6d).

The concentration of lipids<sub>SPM</sub> differed significantly between sites; however, it followed a similar temporal pattern ( $P < 0.001$ ; Table S2) ranging from 0.16 to 0.71 mg/L in Pag and from 0.13 to 0.30 mg/L in Cetina (Fig. 7). At both sites, the lowest concentration of lipids<sub>SPM</sub> took place between August and October (Fig 7a). In lipids<sub>Sed</sub>, values ranged from 0.36 to 1.20 mg/g in Pag and 0.59 to 1.72 mg/g in Cetina with different temporal patterns between sites; the concentration was significantly higher in Cetina ( $P < 0.001$ ; Table S2).



**Fig 7.** Temporal variation of lipid concentration in (a) sediment (Sed; dashed line) and suspended particulate organic matter (SPM; continuous line) and (b) *Glycymeris bimaculata* and *Callista chione* at Pag (fill) and Cetina (blank).

### 3.3.2. Consumers

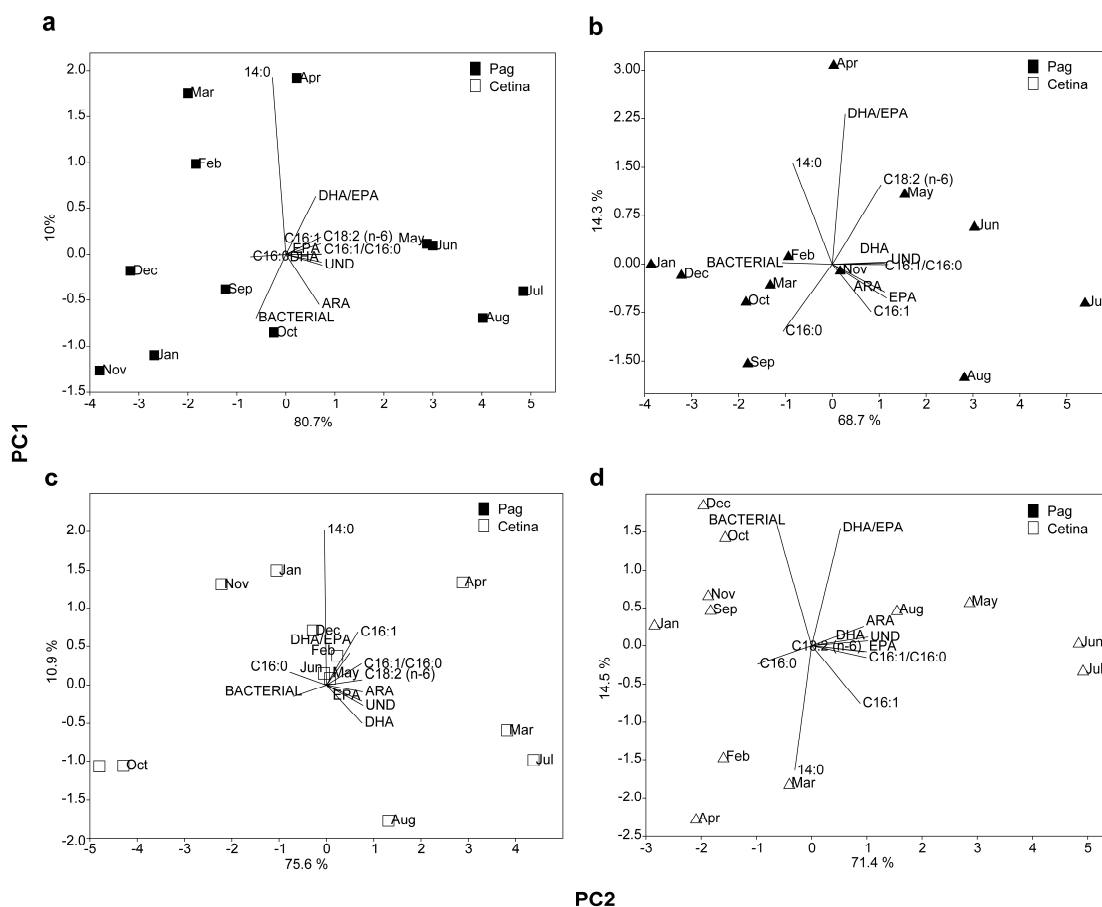
Results for FA<sub>DG</sub> were derived from the same pooled individuals than for SI. Most predominant FA group were SFA (> 58 TFA%), followed by MUFA (> 23 TFA%) and PUFA (> 5 TFA%) (Table S6). ANOSIM revealed significant differences on all FA profiles (n = 38) among each group formed by species-site ( $r = 0.83$ ,  $P < 0.001$ ) and these were more evident between species than sites in the pairwise comparison ( $r = 1$ ,  $P < 0.001$  between species;  $r < 0.53$ ,  $P < 0.001$  between sites). SIMPER analyses and PCA revealed the FA profiles that most contributed to temporal differences for each

consumer group, with PC1 explaining more than 68% and PC2 up to 14.5% (Table S8; Fig 8). PUFA, C20:5 and C22:6 contributed to nearly 50% of the differences between each group.

Generally, in both sites and for both species, PC1 axis clearly separated spring and summer months from fall and winter, according to the digestion of high quality fresh material or bacterial material enriched with saturated components, respectively (Fig 8; Table S6).

In late spring and summer, Gly<sub>pag</sub>'s digestive glands were strongly positively related to PC1 (EPA, DHA, 16:1, 16:1/16:0, UND) and separated from the fall, winter and early spring according to negative relations to PC1 (16:0). The latter samples were additionally separated according to the influence of BACTERIAL FA (between Sep–Jan) and C14:0 (between Feb–Apr) positively and negatively related to PC2, respectively (Fig 8a). In Cal<sub>pag</sub>'s digestive glands, a positive relationship of fresh material markers to PC1 was found between May and August, with a more pronounced terrestrial component (C18:2) in May and June, and phytoplankton (16:1, EPA) in July and August. A BACTERIAL component (negative PC1) was more digested during fall, winter and early spring, being more enriched with C16:0 or C14:0 (Apr) (Fig 8b). The digestive glands of Gly<sub>Cetina</sub> from March, April, June and August were positively loaded to PC1, according to enrichment with all unsaturated FA components and separated from September, October, November and January, due to enrichment with the BACTERIAL component negatively related to PC1. Samples from February, May, June and December were indifferent to both axis, which suggested the digestion of mixture without prevalence of any food component (Fig 8c). In Cal<sub>Cetina</sub>'s digestive glands, samples were positively related to PC1 (EPA, DHA, ARA, 16:1, 16:1/16:0, UND) between May and August, and separated from the other months according to negative relation of 16:0 to PC1. Additionally, the influence of the BACTERIAL component separated fall and early winter samples (between Sep–Jan) from the late winter and early spring (Feb–Apr) which were enriched with C14:0 (Fig 8d).





**Fig 8.** Principal component analysis (PCA) output using most influential fatty acid profiles in Pag for (a) *Glycymeris bimaculata* (b) *Callista chione* and Cetina for (c) *G. bimaculata* (d) *C. chione*.

Overall, spring and summer were the seasons with the fresher material found in the digestive gland of both specimens, in contrast to fall and winter, where the BACTERIAL component was dominant. The herbivorous calanoid copepod markers (C20:1 and C22:1) were more abundant in spring and summer; oleic acid (C18:1) characteristic of zooplankton was more abundant in *C. chione*, and a diatom marker (C14:0) was more abundant in *G. bimaculata*. In addition, within the SFA pool, the bacteria marker associated to detrital matter (C20:0) was slightly higher in *G. bimaculata*, especially at Cetina (Table S6). SIBER analysis of FA revealed a substantial overlap between SEA but not convex hulls from each consumer group (Fig 5b). The FA feeding niche plot also showed that consumers were grouped by site and the highest niche plasticity corresponded to *G. bimaculata* at both sites (Fig 5d).

The concentration of lipids<sub>DG</sub> ranged from 188.34 to 335.39 mg/g in Cal<sub>Pag</sub>, 189.59 to 363.90 mg/g in Cal<sub>Cetina</sub>, 130.53 to 255.36 mg/g in Gly<sub>Pag</sub> and from 125.07 to 201.78 mg/g in Gly<sub>Cetina</sub>. Overall, there was a decreasing trend from summer to spring (Fig 7b).

#### 4. DISCUSSION

In coastal zones, marine particulate organic matter is composed by a mixture of terrestrial and marine particles and living and detrital material mainly derived from plankton, bacteria, fecal pellets or zooplankton remains such as exoskeletons (Berto et al., 2013; Savoye et al., 2012). The coastal fringe in the eastern Mediterranean is especially important due to its oligotrophic conditions, where environmental variation (e.g. wind, river discharges) may exert stronger influence on the nutrient and biota dynamics in its shallow water column. Within this frame, it is important for ecological studies, particularly for trophic ecology approaches, to disentangle food sources and their variability to gain insights into the relationships between biotic and abiotic factors that shape the ecosystem.

##### 4.1. Characterization of food sources

The  $\delta^{13}\text{C}$  pattern in the SPM showed isotopically lighter carbon during winter and spring (down to -26‰). This was presumably due to high precipitation events associated to river discharge and more positive  $\delta^{13}\text{C}$  values during summer and fall (up to -22.8‰), closer to those related to marine environments (e.g. ~-22‰ in marine phytoplankton) (Fry and Sherr, 1984; Harmelin-Vivien et al., 2008), suggesting a progressive mixing of terrestrial and marine materials (e.g. Berto et al., 2013; Savoye et al., 2003).

Unlike SPM, large spatial rather than temporal variations were observed in  $\delta^{13}\text{C}$  values in Sed, which averaged  $-22\text{‰} \pm 0.4$  at Pag and  $-26\text{‰} \pm 0.2$  at Cetina. These values were within those reported for locations in the Adriatic Sea (Faganeli et al., 2009; Ogrinc et al., 2005; Žvab Rožič et al., 2015) receiving marine and terrigenous inputs and suggested that the isotopic signature in Sed may reflect a longer time-integrated signal than that in SPM. The upper sediment layer in Pag showed more positive  $\delta^{13}\text{C}$  values which could be attributed to microphytobenthic production, which is most likely contributing to primary production in other areas in the Adriatic Sea (Šestanović et al., 2009). In contrast, the proximity to the river mouth suggests that the influence

of terrestrial matter in Cetina should be responsible of the  $^{13}\text{C}$ -depleted signal as shown in other estuarine areas (Hedges et al., 1997; Sarma et al., 2014).

The interpretation of the nitrogen signal requires considering the potential contribution of several sources, namely  $\text{N}_2$ ,  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  or DON (e.g. Berto et al., 2013; Currin et al., 1995; Peterson, 1999). On the one hand, the phytoplankton and bacterioplankton  $\delta^{15}\text{N}$  signature ranges in the order of -2 to 5‰ (Rees et al., 2006), whereas on the other, the Eastern Mediterranean basin is  $^{15}\text{N}$ -depleted due to its oligotrophic nature (Pantoja et al., 2002; Sachs and Repeta, 1999). Given that our  $\delta^{15}\text{N}_{\text{SPM}}$  data ranged from 1.6 to 6.1‰ in Pag and from 2.5 to 5.5‰ in Cetina, it is hard to distinguish the drivers of the  $\delta^{15}\text{N}_{\text{SPM}}$  signal; however, a combination of factors modulating the observed results seem more feasible. The ~4‰ temporal variation observed along the study period suggests that a mixture of freshwater and marine water influenced both study sites (Fig 2d). The higher values coincided in months with high precipitation events (typically fall) that increased river discharges (Cetina area) and pulses of underwater springs (characteristic from Pag area) (Surić et al., 2015) which potentially enhanced the enrichment in the nitrogen isotopic composition signal (Xue et al., 2009).

In the sediment, more negative and variable values (ranging from -2.3 to 3.9‰) were found in Pag; negative values are likely associated to benthic diazotroph community. For example, in oligotrophic water bodies, diazotroph biomass is responsible of atmospheric nitrogen fixation (Rees et al. 2006, Pantoja et al. 2002, Wada et al. 2012), particularly by blue-green algae (Currin et al., 1995; Minagawa and Wada, 1986). Studies on cultured cyanobacteria revealed that nitrogen isotope ratios ranging from -1.5 to -3.0‰ during  $\text{N}_2$  fixation were associated to high  $\delta^{13}\text{C}$  in cyanobacteria cells (Wada et al., 2012). Alike, studies on cyanobacteria in sedimentary organic matter in the NW and Eastern Mediterranean showed  $\delta^{15}\text{N}$  ranges from -2 to 2‰, suggesting that the observed values were the result of bacterial organic matter remineralization (Kerhervé et al., 2001; Möbius, 2013). This association would imply that bacterial nitrogen fixation is a main contributor to the  $\delta^{15}\text{N}$  signal in PON in the coastal system off Pag island. On the other hand, comparatively more constant but also relatively low values in Cetina (averaging  $2.3\pm 0.2$ ), were similar to those in Mediterranean estuarine areas (Carlier et al., 2007).

Based on the live phytoplankton C:N molar ratio of 6, the Redfield ratio is a traditional indicator to discriminate fresh relative to degraded marine and to distinguish marine from terrestrial organic matter (Redfield, 1958). Bacterial cells have lower C:N molar ratio than phytoplankton due to preferential mineralization of N (Newton et al., 1994), therefore, in periods of food scarcity C:N ratios may decrease due to bacterial presence. The  $C:N_{SPM}$  values (between 7 and 11, Fig. 2b) in Pag suggest a high proportion of phytoplanktonic material (e.g. Schubert and Calvert, 2001) throughout the year and a particular contribution of bacterial material in December and February, what seems to be in concordance with the isotopic signals previously discussed. At Cetina,  $C:N_{SPM}$  ranged from 7 to 11 indicating a mixture of phytoplankton with marine degraded and/or terrestrial OM in agreement with other estuarine systems, probably enhanced by riverine inputs (Dias et al., 2016; Hedges et al., 1997; Savoye et al., 2003). The presence of several C:N ratio peaks (e.g. October 2014, December, February and June 2015; Fig 2b) in Cetina site was most likely related to resuspension events that incorporated degraded material into the water column. The coincidence with high suspended particulate matter values (Table S1) provides further support to this idea. The low relationship between C:N and biogenic silica suggest that the non-siliceous phytoplankton community could be more important than diatoms, radiolarians and silicoflagellates in the carbon pool in this shallow water column.

In the sediment at Pag, the overall higher  $C:N_{sed}$  ratios (between 5 and 31) suggested higher abundance of bacteria during fall and winter, in agreement with the observed N isotopic signature (Fig 2b,d). The  $C:N_{sed}$  ratios in Cetina were more constant (averaging 7.7) and coexisted with comparatively higher percentage of biogenic silica, implying a higher concentration of fresh OM with diatom abundance. However, the FA marker for diatom was quite low (as discussed next) suggesting that the presence of empty frustules explains the high BSi values and providing further support to the presence of bacteria in the OM pool.

In general, the low %C and %N in the sediment were within the range of other reports from the Adriatic, e.g. 0.5-1.3 %C and 0.1-0.17 %N (Ogrinc et al., 2005) and 0.02-0.15 %N (Matijević et al., 2009) corroborating the poor nutritional quality of the sediment in this oligotrophic basin. In addition, the POC/Chl *a* values were > 200 indicating high chlorophyll *a* degradation most likely due to the strong presence of heterotrophic/mixotrophic organisms (Bentaleb et al., 1998; Galois et al., 1996) as reported in estuarine areas in the Adriatic Sea (Šolić et

al., 2015), which have also been found to be key bacterial grazers in coastal oligotrophic waters (Unrein et al., 2013).

Seasonal FA patterns in the SPM also indicated different sources of OM at two sites. The most important components of SPM and Sed lipids were 14:0, 18:0, 18-1(n-9), 20:1, 20:0 and BACTERIAL FA (Fig 6, Table S6). Within SFA, C20:0, which could be linked to bacteria associated to detrital matter collected from the sediment (Galap et al., 1999) or suspended particulate matter was higher during winter and it was more abundant in Pag, coincident with SI interpretations. The high proportion of MUFA, 18-2(n-6) and BACTERIAL FA in Cetina suggested that terrestrial inputs predominated in the OM in SPM, also supporting the observations of SI results. Usually, the FA marker C18:2(n-6) is associated with terrestrial sources (Budge and Parrish, 1998; Fischer et al., 2014; Parrish et al., 1996), seagrass (Kharlamenko et al., 2011) or protozoa (Zhukova and Kharlamenko, 1999). The FA 18:1(n-9) was particularly abundant during spring and summer, and it has been associated with zooplankton (Sargent and Falk-Petersen, 1988) and cyanobacteria (Antonio and Richoux, 2016). Relatively high PUFA concentrations (particularly EPA's; Fig 6) suggested that the best OM quality in SPM took place during summer at both sites. However, in Pag, slightly higher values coincident with relatively low C:N and high BSi values, indicated a larger contribution of fresh siliceous phytoplankton biomass in that site. Further, herbivorous calanoid copepod markers were more abundant in Cetina during fall and winter, presumably following phytoplankton biomass peaks. In the Sed, high PUFA concentrations were also observed in summer, suggesting an effective pelagic-benthic coupling along the study area.

Phytoplankton FA markers,  $\delta^{13}\text{C}$ , C:N ratios and BSi<sub>SPM</sub> values suggested that SPM is of better quality and has more phytoplankton influence during spring and summer. Often,  $\delta^{13}\text{C}$  is positively correlated to Chl *a* concentrations (e.g. Miller and Page, 2012) and probably this relationship is more evident in shallow coastal areas, such as the present study area. However, the temporal mismatch between Chl *a* and the rest of measured parameters in the present study was most likely influenced by the high amount of resuspended material different to phytoplankton (e.g. bacteria and empty diatom frustules) that may obscure the signal of Chl *a*, which is related solely to phytoplankton biomass.

The combined SI and FA temporal variation profiles in the SPM and Sed described an alternation between a freshwater dominated system in winter to a marine-water dominated one in summer. Indeed, the particulate matter in Pag appeared of better quality due to abundance of EFA within PUFA in both compartments and by the lighter values of  $\delta^{13}\text{C}$  and less SFA observed in the sediment. However, the nitrogen isotopic signature better distinguished the sources contributing to the OM pool at each site resulting in a better tracer of environmental changes than the carbon isotope (Fig 3a). Overall, results suggested that bacteria seemed to play a major role in the organic carbon and nitrogen budgets in the particulate matter in the coastal system off the island of Pag. BACTERIAL FA showed that bacteria were abundant in both compartments at both sites, making evident their importance as food source (Galois et al., 1996; Šestanović et al., 2009).

#### 4.2. Spatial and temporal variation in bivalve diet

The isotopic composition of the digestive gland of *Callista chione* and *Glycymeris bimaculata* revealed a pronounced temporal variation at two sampling sites. The carbon isotopic variation indicated to be species-specific, with slightly higher values in *G. bimaculata* while large differences in the nitrogen isotope were site-specific, with higher values shown in Cetina (Fig 3a,b). The temporal variation in  $\delta^{13}\text{C}_{\text{DG}}$  was coupled to that in  $\delta^{13}\text{C}_{\text{SPM}}$ , indicating that bivalves adjusted their dietary shifts depending on the availability of food mainly in SPM (Figs 2,3). This coupling suggested that SPM is an important food source what is in agreement with other studies in coastal systems (Antonio and Richoux, 2014; DeNiro and Epstein, 1978; Kang et al., 1999; Kasai et al., 2004; Riera and Richard, 1996). Bivalves increase their  $\delta^{13}\text{C}$  values due to the preferential release of  $^{12}\text{C}$  during respiration (Kang et al., 1999; Kharlamenko et al., 2001; Page and Lastra, 2003), and our results showed a slight  $^{13}\text{C}$ -enrichment in DG with respect to SPM, presumably reflecting this process. In our study, *G. bimaculata* presented slightly heavier  $\delta^{13}\text{C}$  values than *C. chione* indicating that they feed more upon autochthonous particles (i.e. phytoplankton derived organic matter) showing a stronger dependence on primary producers, as seen in other studies (France, 1995; Kang et al., 2003; Kharlamenko et al., 2008; Nadon and Himmelman, 2006). Another stable isotope study conducted on *C. chione* from the NW Mediterranean showed a more enriched isotopic signature ( $\delta^{13}\text{C}$ :  $-19.4\text{‰} \pm 0.5$ ,  $\delta^{15}\text{N}$ :  $4.7\text{‰} \pm 0.4$ ,  $n=5$  in Carlier et al., 2007), suggesting that same species may reflect local environmental differences along the Mediterranean Sea.

The  $\delta^{15}\text{N}$  in Cetina populations was ca. 1‰ higher than in Pag, allowing a clear distinction between sites (Fig 3b, Table S3). The ranges of  $\delta^{15}\text{N}$  in specimens at Cetina fell within those from other filter-feeders in the Adriatic Sea (Ezgeta-Balić et al., 2014), whereas the more negative  $\delta^{15}\text{N}$  signature from Pag populations has not been previously reported for bivalves in the Adriatic Sea. These results together with the low isotopic signal observed in the Sed in Pag suggested that there are particular environmental conditions in that site. The temporal  $\delta^{15}\text{N}$  signal variation showed more negative values during winter and spring, coincident with the carbon isotope signal. In Pag populations, these values were closer to those of Sed, suggesting an important contribution of the Sed pool to their diet (further discussed in the next paragraph). These results emphasize the importance of studies on temporal variation due to the rapid imprint that environmental changes have on animal tissues. The observed spatio-temporal intraspecific variation from SI data support the use of the digestive gland as a rapid OM turnover tissue (Deudero et al., 2009; Ezgeta-Balić et al., 2014).

FA profiles complemented the information on the spatio-temporal variation of the diet composition provided by isotopic signatures. For all populations, temporal differences on FA profiles were more evident than the spatial variation. In *G. bimaculata*, SFA values were similar to those reported in *Glycymeris nummaria* (up to 82% TFA) in the south Adriatic (Najdek et al., 2016) suggesting these FA greatly contribute to the diet of *Glycymeris* sp. in the eastern Adriatic, perhaps reflecting specific selection patterns, in contrast to *C. chione* and other bivalves (Ezgeta-Balić et al., 2012; Najdek et al., 2013). Within the SFA pool, the bacteria marker associated to detrital matter (C20:0) was slightly higher in *G. bimaculata*, especially at Cetina. PUFA were higher in *C. chione* especially during summer, particularly in Pag (Table S6). PUFA, especially EFA, are essential for growth but also a high degree of unsaturation (UND) is characteristic of healthy mollusks (Dupčić Radić et al., 2014). Overall, diatom, dinoflagellate and herbivorous calanoid copepod markers contributed most to the bivalves' diet during spring and summer at both sites, particularly in *C. chione*. Further, since oleic acid, a marker of zooplankton, was more abundant in *C. chione*, it seems that zooplankton is preferential food for this bivalve. All these observations suggested that *C. chione* preferentially uptakes food with better quality than *G. bimaculata*.



Overall detritus, diatom, dinoflagellate, zooplankton and EFA markers were the most abundant in studied bivalve's diet in agreement with other studies in the south-eastern Adriatic Sea. In these studies, other filter-feeders such as *Mytilus galloprovincialis*, *Ostrea edulis* and *Modiolus barbatus* (Ezgeta-Balić et al., 2012), *Pinna nobilis* (Najdek et al., 2013), *Arca noae* (Dupčić Radić et al., 2014; Ezgeta-Balić et al., 2012) and *G. nummaria* (Najdek et al., 2016) revealed ingestion of a mixed diet. The higher concentration of lipids in bivalves coincided with those months with the best indicators of food quality in SPM (e.g. spring and summer). *Callista chione* fed more upon fresh material than *G. bimaculata* which relied largely on bacteria-derived detritus, as observed in other species of the same genus, such as *G. glycymeris* (Galap et al., 1999) and *G. nummaria* (Najdek et al., 2016). The importance of bacteria in bivalves' diet has also been reported in other shallow coastal areas (Antonio et al., 2012; Kang et al., 2003). Altogether, our results corroborated the importance of heterotrophic bacteria in the carbon cycle and in the contribution to the maintenance of benthic communities in oligotrophic waters such as the Adriatic Sea (Šantić et al., 2013; Unrein et al., 2013).

#### 4.3. Contribution of food sources to bivalve diet and feeding niche

A two source mixing model was used to elucidate the contribution of each source to bivalves' diet through time (Fig 4). Based on our results, the contribution of the Sed pool dominated in the populations from Pag with a slight contribution of SPM during summer, when a major concentration of fresh material was available in the water column. In contrast, the SPM pool was the main contributor to the diet composition at Cetina especially for *G. bimaculata* (> 97%). Dual isotope and fatty acid analyses characterized each site as different feeding niches (Fig 5). Within sites, the isotopic feeding niches showed an overlap only in November and March at Cetina, revealing different food sources composition during the rest of the year, whereas at Pag, the overlap was evident during fall and winter (Fig S2). A larger contribution of Sed was clear in Pag populations supported by the <sup>15</sup>N-depleted values found in the DG. Isotopic niches alone should be carefully addressed as remarked in other studies (Albó-Puigserver et al., 2015; Fry, 2013; Karlson et al., 2015), especially when analyzed in bulk matter, where the detailed isotopic signature of specific components (e.g. dinoflagellates, diatoms, copepods, bacteria, etc.) could be obscured, particularly in a poorly investigated area (i.e. lacking specific enrichment factors and baseline studies).



At Pag, the highest niche plasticity was observed for *C. chione* suggesting its ability to consume more isotopically diverse food sources than *G. bimaculata*. Niche plasticity allows to reduce intraspecific competition when food availability is scarce (Gutt, 2006) by consuming different fractions of POM (Riera, 2007). The narrower isotopic range in *G. bimaculata* would suggest that both species compete for food in a period of food scarcity, however, the fatty acid feeding niche revealed that despite the overlap, *G. bimaculata* is more opportunistic. This behavior matches with the trophic activity of *C. chione* which appeared to be more selective within the available organic pool. Morphological differences could also play a role in the selection of food particles. The presence of siphon in *C. chione* could restrict its ability to pick particles from the environment. On the contrary, little is known about the feeding habitats of *G. bimaculata* which has no inhalant siphon and presumably has the ability to feed upon particles in the interstitial water.

Our results suggest that both species have the capacity to ingest particles from both SPM and Sed pools. This type of intraspecific variation was also observed in the Pacific oyster *Crassostrea gigas* in France (Riera and Richard, 1996). SPM is a dynamic pool of autochthonous and allochthonous material thus, multiproxy approaches facilitate distinction in the origin of SPM. We can conclude that each species obtained their carbon from different sources and that the coupling of nitrogen isotopic composition between Sed and DG was useful to distinguish populations (Fig 5b). A larger number of bacterial FA markers in *G. bimaculata* indicated that this species fed more upon bacteria than *C. chione*. In agreement with this observation, bacteria just constituted a small proportion of the diet in *C. chione* in the western Mediterranean (Charles et al., 1999). *G. bimaculata* might act as an opportunistic feeder in this respect, incorporating resuspended particles with more detrital-derived material such as seen in *P. magellanicus* (Shumway et al., 1987). Bacteria seems to contribute significantly in periods of food scarcity to satisfy nitrogen bivalve requirements (Langdon and Newell, 1990), which would be the case in the present study, especially for Pag.

## CONCLUSIONS

The temporal variation of particulate matter in the coastal central Adriatic Sea reflected a mixture between marine and terrestrial sources throughout the year, with a clear marine influence during

summer and fall, and terrestrial inputs during spring and winter. Detrital material associated to bacterial FA was more abundant in SPM from Pag, whereas in Cetina, both detrital and terrestrial FA had the highest concentrations. In addition, EFA and zooplankton markers indicated that during spring and summer OM had better quality at both sites. The more negative carbon isotope signature from sediment in Cetina suggested a greater influence of terrestrial matter from the river discharges. It is difficult to draw conclusions on the temporal variation of  $\delta^{15}\text{N}$  signal; however, this signal is most likely related to the activity of diazotroph biomass, particularly in Pag.

The  $\delta^{13}\text{C}$  was species-specific, with more positive values in *G. bimaculata* than *C. chione* whereas  $\delta^{15}\text{N}$  was site-specific, with more positive values in Cetina than in Pag. A small temporal offset was observed in  $\delta^{13}\text{C}_{\text{DG}}$  with respect to  $\delta^{13}\text{C}_{\text{SPM}}$ , indicating the dietary shift adjustment (weeks) of bivalves to available SPM. The observed spatio-temporal intraspecific variation supported the use of digestive gland as a rapid turnover tissue. FA in the digestive gland revealed a mixed diet where *C. chione* fed more upon fresh material than *G. bimaculata*, which relied largely on bacteria-derived material.

Overall, our results suggested that there was little feeding niche overlap between *C. chione* and *G. bimaculata* during the year, strongly indicating resource partitioning between species, typical in a food-limited system such as the central eastern Adriatic. The pronounced temporal variation of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  within populations highlighted interspecific isotopic variation in bivalve populations. In Cetina, SPM greatly contributed to the diet of bivalves whereas in Pag, Sed appeared to have major importance. The study of trophic ecology in co-occurring species allowed identifying species-specific feeding adaptations to environmental variability providing valuable information for other ecological studies and complementing challenging research opportunities, i.e. sclerochronology.

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