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Seasonal variation of stable isotope ratios of size-fractionated zooplankton in the Bay of Marseille (NW Mediterranean Sea)

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Stable isotope ratios of six size fractions of zooplankton (80 to >2000 μm) were analyzed seasonally in 2009–2010 at the SOMLIT site in the Bay of Marseille. Isotopic ratios generally increased with zooplankton size. The highest $\delta^{15}N$ values were observed in the 1000–2000 μm fraction. The largest size class (>2000 μm), dominated by gelatinous plankton, had lower $\delta^{15}N$ values due to the low isotopic signatures of most of these organisms. In the larger size fractions (>1000 μm), isotopic ratios were measured at the taxon level. Brachyuran, stomatopod, teleost and cephalopod larvae showed the highest $\delta^{15}N$ values, and salps and pteropods the lowest ones. Lower values of both $\delta^{13}C$ and $\delta^{15}N$ were recorded in winter and spring than in summer and autumn for all fractions. Seasonal variations were consistent with the fluctuations of environmental parameters (temperature, nutrients, Chl *a* concentration) and were related to phytoplankton and seawater particulate organic matter (POM) composition. Stable isotope and flow cytometry analysis of water POM indicated that sewage wastewater particles were mixed with marine phytoplankton at the SOMLIT site and transferred up into the zooplanktonic food web.

KEYWORDS: plankton; $\delta^{13}C$; $\delta^{15}N$; size classes; flow cytometry

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INTRODUCTION

The Mediterranean Sea is considered an oligotrophic sea, with low nutrient concentrations and relatively moderate levels of primary production (Estrada, 1996). Fertilization processes that may take place locally are mostly related to wind-driven upwelling events and land runoff due to river discharge and seasonal storms. Coastal environments, highly variable and complex, are exposed to various anthropogenic and land-related influences (sewage discharges, rivers, etc.). The marine coastal area near Marseille, south of France, is severely affected by organic matter inputs and industrial contamination linked to the human activities of this large conurbation ($>1.8 \times 10^6$ inhabitants) (Syakti *et al.*, 2012). This area is also influenced by the Northern Current (Millot, 1999) and the coastal Huveaune River inputs.

These local environmental conditions influence both phytoplankton and zooplankton production and their seasonal variations (Calbet *et al.*, 2001). Composition, biomass and seasonal variations of zooplankton have been well studied in the NW Mediterranean Sea (Razouls and Kouwenberg, 1993; Champalbert, 1996; Calbet *et al.*, 2001; Gaudy *et al.*, 2003; Saiz *et al.*, 2007; Fanelli *et al.*, 2011). Champalbert (Champalbert, 1996) shows that zooplankton diversity is usually lower in coastal neritic waters than in oceanic waters, whereas biomass is higher due to terrestrial inputs.

Zooplankton play a prominent role in marine pelagic systems due to their trophic position and their role in organic matter transfer from phytoplankton to upper level consumers (Cushing, 1989; Saiz *et al.*, 2007). However, they also represent a major pathway for contaminants in the food web and act as biomagnification agents of most contaminants between low and high trophic levels, as shown in the European hake food web (Cossa *et al.*, 2012; Harmelin-Vivien *et al.*, 2012). In these studies, zooplankton is considered as a unique homogeneous compartment, including all organisms with sizes between 200 and >2000 μm. A size-based approach has been proposed as a useful means to get obtain insight into the structure and function of marine food webs (Kerr and Dickie, 2001) and is now commonly used in plankton studies (Rau *et al.*, 1990; Rolff, 2000; Saiz *et al.*, 2007; Carlotti *et al.*, 2008; among many others). Body size determines potential predator–prey interactions (Cohen *et al.*, 1993), rates of production and natural increase in abundance (Banse and Mosher, 1980), energy requirements and metabolism (Fenchel, 1974; Boudreau *et al.*, 1991) and mortality rates (Hirst and Kiørboe, 2002).

Provided that primary producers have distinct stable isotope ratios, carbon and nitrogen stable isotopes constitute a powerful tool for discriminating among organic matter sources (Vizzini and Mazzola, 2006). Stable isotopes have been used successfully to trace the transfer of organic matter of different origins through aquatic food webs (Fry and Sherr, 1984).

The nitrogen isotope ratio is used to help define the trophic levels of organisms, as $\delta^{15}N$ usually increases by 2.5–4.5‰ (mean 3.4‰) from prey to predator (Minagawa and Wada, 1984; Post, 2002). As an increase in $\delta^{13}C$ of 0.4–2‰ only occurs from diet to consumer (De Niro and Epstein, 1978; Post, 2002), a consumer's carbon isotope composition can give clues about the sources of its diet, particularly in systems with distinct organic matter sources (Fry and Sherr, 1984). The relative importance of these sources may vary in space and time, and stable isotope ratios at the base of food webs in the marine environment can be highly variable (Vizzini and Mazzola, 2006).

Stable isotope ratios have been widely used to analyze marine food webs and many studies have been performed on isotopic signatures of zooplankton (Rau *et al.*, 1990; Fry and Quiñones, 1994; Rolff, 2000; Kibirige *et al.*, 2002; Sommer and Sommer, 2004; Gentsch *et al.*, 2009; among many others). However, few of them concern the Mediterranean Sea (Koppelmann *et al.*, 2009; Fanelli *et al.*, 2011). Most of these studies analyzed zooplankton stable isotope ratios by size classes (Fry and Quiñones, 1994; Rolff, 2000; Calbet *et al.*, 2001; Kibirige *et al.*, 2002; Koppelmann *et al.*, 2009), but only a few give detailed analysis on taxonomic groups or species (Sommer and Sommer, 2004; Fanelli *et al.*, 2011).

Thus, a size-fractionation study of zooplankton was undertaken within the general framework of the COSTAS (Contaminants in the trophic system: phytoplankton, zooplankton, anchovy, sardine) multidisciplinary program aimed at studying contaminant transfer along the food webs of the two dominant planktivorous teleosts in the Mediterranean Sea, the sardine *Sardina pilchardus,* and the European anchovy, *Engraulis encrasicolus*.

In the present study, stable isotope ratios of six different size classes of zooplankton, along with those of particulate organic matter (POM) sources, were analyzed seasonally in the Bay of Marseille and combined with zooplankton taxonomic composition and flow cytometry sorting. Our results were then related to environmental parameters and microphytoplankton composition provided by the SOMLIT program (a long-term observation survey at the sampling site).

The aims of this study were: (i) to determine the influence of season and size class on the C and N isotopic signatures of zooplankton, (ii) to relate the observed variations to zooplankton taxonomy and POM composition, (iii) to determine the influence of environmental parameters on both POM and zooplankton isotopic

signatures and eventually (iv) to demonstrate, or not, the integration of sewage-derived POM in zooplankton food webs.

METHOD

Site and sampling procedure

Zooplankton was collected in the Bay of Marseille at the SOMLIT (Service d'Observation en Milieu LITtoral) site (43.24°N; 5.29°E) (Fig. 1). Nutrients, temperature, salinity, oxygen, pH, chlorophyll *a* and suspended particulate matter (SPM) have been recorded at this site every 2 weeks since 1994 within the frame of a long-term national program on littoral observation (http://somlit.epoc.u-bordeaux1.fr). The SOMLIT site is mainly influenced by the Northern Current (Millot, 1999; Petrenko *et al.*, 2005), but is also subjected to anthropogenic and terrestrial inputs from the Marseille sewage treatment plant located at Cortiou and the coastal Huveaune River (Fig. 1), depending on winds and rain events (Cresson *et al.*, 2012). Thus, three main potential sources of POM are available to zooplankton (seawater, sewage water and river runoff).

Zooplankton was vertically sampled, from 0 to 50 m depth, using a WP2 zooplankton net with a 200 μm mesh size and a PVC cod-end during the day, in spring (March 2009), summer (July 2009), autumn (October 2009) and winter (February 2010). Six zooplankton size classes (80−200, 200−300, 300−500, 500−1000, 1000−2000 and >2000 μm) were separated onboard the vessel with sieves of decreasing mesh size. The first size class (80−200 μm) covers some small organisms caught by the 200 μm net because of clogging. Six to eight tows were performed on each date to collect enough material in all size classes for stable isotope analysis. Surface water samples for POM analysis were collected at the SOMLIT site in each season and in the Cortiou sewage plume only in summer and winter for logistical reasons. The Huveaune River flow is deflected to the Marseille sewage outfall most of the time, but flows directly to the sea through its natural bed after heavy rain events. Stable isotope ratios from Huveaune River POM during the study period were taken from Cresson *et al.* (Cresson *et al.*, 2012).

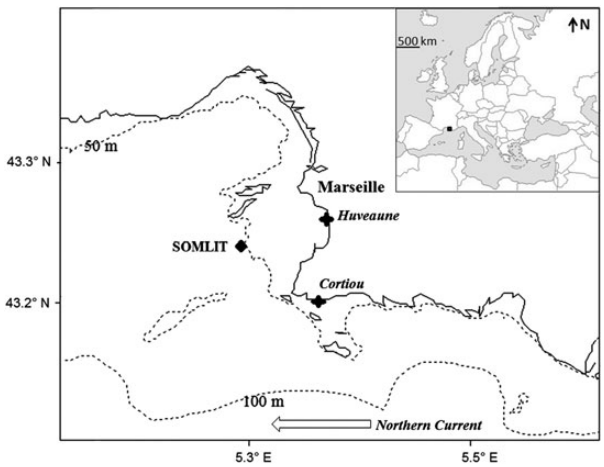

Fig. 1. Localization of the SOMLIT site, Cortiou sewage outfall and Huveaune River mouth in the Bay of Marseille, South of France.

Sample processing

Water samples were prefiltered on a 200 μm mesh-sieve to remove large detritus. POM samples were obtained by filtering 6 L of surface water, on pre-weighed Whatman GF/F glass micro-fibre filters pre-combusted for 4 h at 500°C, to obtain a minimum of six filters per season. They were then dried at 70°C, weighed to quantify SPM content and kept in dessicators until analysis. Zooplankton samples were analyzed in the laboratory under a binocular microscope 2 h after sampling to determine the taxonomic composition of each size class. For the two largest size classes (1000−2000 and >2000 μm), organisms were separated into broad taxonomic groups to analyze them separately. The time lag between sampling and sorting allowed gut clearance. In the smaller size classes animals were pooled together for isotopic analysis. Subsequently samples were frozen at −20°C and freeze-dried.

Stable isotope analysis

Salt was separated from the freeze-dried samples which were ground into a fine powder using an agate mortar and pestle. As POM and zooplankton may contain carbonates, an acidification step using the drop-by-drop technique was used to remove ^{13}C-enriched carbonates (De Niro and Epstein, 1978). Thus, a subsample received 1% HCl treatment before rinsing and drying, and was used for δ^{13}C determination, whereas the other untreated subsample was used for δ^{15}N analysis. POM was collected by scraping the surface of acidified and non-acidified filters. Three replicates were performed on POM and on each zooplankton size class per season for both δ^{13}C and δ^{15}N.

Stable isotope measurements were performed with a continuous-flow isotope-ratio mass spectrometer (Delta V Advantage, Thermo Scientific, Bremen, Germany) coupled to an elemental analyzer (Flash EA1112 Thermo Scientific, Milan, Italy). Results are expressed in δ notation relative to Vienna PeeDee Belemnite and atmospheric N_2 for δ^{13}C and δ^{15}N, respectively, according

to the equation: $\delta X\ (‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X is ^{13}C or ^{15}N and R is the isotope ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. For both $\delta^{13}C$ and $\delta^{15}N$, measurement precision is $<0.1‰$ (replicate measurements of internal laboratory standards, acetanilide).

Flow cytometry analysis

Water samples from the SOMLIT site, Cortiou plume and Huveaune River were analyzed at the PRECYM flow cytometry platform (http://precym.com.univ-mrs.fr) to quantify the type and size of particles <20 μm present in POM.

Cell counts were assessed on a FACSCalibur using the CellQuest software (both from BD Biosciences), while sorting was performed with a BD Influx Mariner (BD Biosciences) cell sorter equipped with a 488 nm laser (200 mW, Coherent Sapphire). A posteriori analysis of ultraphytoplankton groups was performed with the SUMMIT v4.3 software (Beckman Coulter).

Standard protocols were used to enumerate phytoplankton (Marie *et al.*, 2001). The red fluorescence (>640 nm or 630LP) related to chlorophyll *a* was used as trigger signal. Cells were enumerated according to their SSC and FSC properties (related to cell size and structure, respectively), and their orange fluorescence (564–606 nm—PE or 580/30 nm). TruCount beads (BD Biosciences®) were added to the samples to determine the volume analyzed, and for both analysis and sorting, 2 μm beads were used to discriminate picoplankton (<2 μm) from nanoplankton (>2 μm) populations, and 6 μm beads to estimate the nanoplankton particle size (both Fluoresbrite YG, Polyscience). All data were collected in log scale and three sorting gates were determined: picoplankton cells (Pico), nanoplankton with low (Nano low) and higher chlorophyll *a* content (Nano high). Data were stored in list mode using the BD FACS™ software (BD Biosciences).

Sorted populations were then prepared for scanning transmission electron microscopy (STEM, Hitachi S570) in order to determine their nature.

Statistical analysis

Statistical analysis was with the Statististica 9.1 software. Two-way ANOVAs were performed to assess the effect of season and size on C and N stable isotope ratios of zooplankton after testing for assumptions. One-way ANOVA was used to test for differences in isotopic signatures of water POM at the three sites sampled. *Post hoc* comparisons of means were performed with Student–Newman–Keuls tests (significance level P-value < 0.05). When possible, differences in mean stable isotope values of individualized zooplankton groups between size classes (1000–2000 and >2000 μm) were tested by the *t*-test.

RESULTS

Zooplankton composition by size class

Zooplankton composition differed between size classes (Table I). The first (80–200 μm) was dominated by detritus and small copepod stages (eggs, nauplii, copepodits), but phytoplankton and small larvae were also abundant. Copepods (>80%) and cladocerans largely dominated the 200–300 and 300–500 μm size classes. Copepods were always the dominant organisms in the 500–1000 and 1000–2000 μm size classes, but teleost eggs, crustacean larvae and gelatinous organisms such as appendicularians, siphonophores, salps and chaetognaths, increased in abundance in larger size classes. The largest size class (>2000 μm) was dominated by gelatinous organisms (siphonophores, salps, chaetognaths), pteropods and macrurid larvae, whereas large copepods were less abundant.

Low seasonal differences in zooplankton composition were recorded in the three smaller size classes. In the 80–200-μm size class, detritus was more important in winter, whereas between 200 and 500 μm copepods largely dominated the zooplankton in all seasons. Copepods dominated the 500–1000-μm size class, except in summer and autumn when invertebrate larvae were more abundant. In the two largest size classes, crustacean larvae and gelatinous organisms were dominant. Siphonophores and teleost eggs were more abundant in winter, whereas invertebrate and teleost larvae along with

Table I: Composition of zooplankton size classes

Size class (μm)	Main groups
80–200	**Detritus, copepods** (**eggs**, nauplii and copepodits), phytoplankton (diatoms, dinobionts), larvae (gastropods, annelids)
200–300	**Copepods, cladocerans**, larvae (gastropods, bivalves, annelids, brachyurids)
300–500	**Copepods, cladocerans**, larvae (gastropods, bivalves, annelids, brachyurids), appendicularians
500–1000	**Copepods, teleost eggs, larvae** (**brachyurids, macrurids**, bivalves), euphausiids, appendicularians, chaetognaths, pteropods
1000–2000	**Copepods, pteropods, chaetognaths, siphonophores, macrurid larvae, brachyurid larvae**, euphausiids, amphipods, teleosts larvae and eggs, appendicularians
>2000	**Siphonophores, salps, chaetognaths, pteropods, macrurid larvae, brachyurid larvae**, teleost larvae, copepods

Dominant groups are indicated in bold characters by decreasing order of importance.

chaetognaths and pteropods were relatively more numerous in summer and autumn.

Effect of season and size on zooplankton stable isotope ratios

Significant differences in isotopic signatures occurred between zooplankton size classes with generally lower values in smaller size classes (from 80 to 500 μm) than in the larger ones (>1000 μm), whatever the season (Tables II and III). However, the increase in isotopic ratios was not regular since the highest mean $\delta^{15}N$ value was recorded in the 1000–2000-μm size class (4.08 ± 0.68‰). The 1000–2000 and >2000-μm size classes were highly heterogeneous in terms of taxonomic composition (Table I). They were composed of groups of organisms which exhibited different stable isotope ratios. Salps and pteropods had the lowest $\delta^{15}N$ (<2‰), whereas larvae of stomatopods, brachyurans, teleosts and cephalopods exhibited the highest $\delta^{15}N$ (>4‰) (Table IV). Euphausiids, brachyuran and macruran larvae and chaetognaths had higher $\delta^{15}N$ in the >2000 μm compared with the 1000–2000-μm size class, but this difference was not significant due to high standard deviations (*t*-tests, $P > 0.05$). Nevertheless, a decrease in $\delta^{15}N$ values was observed in the largest size class (>2000 μm) as a whole, because it was dominated by organisms with low nitrogen isotopic signatures (Table IV).

C and N stable isotope ratios of zooplankton significantly differed with season and size, and the absence of interaction between factors indicated that the pattern of difference with size was similar in each season (Table III). Mean $\delta^{13}C$ values were significantly higher in autumn and lower in spring, whereas mean $\delta^{15}N$ values were significantly higher in summer and lower in winter (Table II). As a general pattern, zooplankton isotopic signatures were characterized by higher values of both $\delta^{13}C$

Table II: Mean (± SD) stable isotope ratios of zooplankton size classes per season

	Spring	Summer	Autumn	Winter	Mean
$\delta^{13}C$ (‰)					
80–200 μm	−24.79 ± 0.13	−22.73 ± 0.12	−19.30 ± 0.17	−23.13 ± 0.03	−22.49 ± 2.30
200–300 μm	−25.58 ± 0.33	−22.93 ± 0.03	−20.30 ± 0.20	−23.52 ± 0.02	−23.08 ± 2.18
300–500 μm	−26.00 ± 0.13	−22.70 ± 0.04	−20.18 ± 0.10	−23.12 ± 0.37	−23.00 ± 2.38
500–1000 μm	−25.24 ± 0.25	−23.77 ± 0.11	−20.20 ± 0.48	−22.98 ± 0.40	−23.05 ± 2.12
1000–2000 μm	−23.04 ± 0.38	−22.44 ± 0.01	−19.00 ± 0.31	−22.26 ± 0.29	−21.69 ± 1.82
>2000 μm	−22.59 ± 0.31	−21.77 ± 0.28	−18.70 ± 0.14	−21.70 ± 0.40	−21.19 ± 1.71
Mean	*−24.54 ± 0.99*	*−22.72 ± 1.42*	*−19.61 ± 1.42*	*−22.79 ± 1.57*	*−22.42 ± 2.00*
$\delta^{15}N$ (‰)					
80–200 μm	1.38 ± 0.07	3.94 ± 0.04	2.08 ± 0.47	2.27 ± 0.04	*2.42 ± 1.08*
200–300 μm	1.29 ± 0.13	4.22 ± 0.10	2.29 ± 0.10	2.08 ± 0.04	*2.47 ± 1.24*
300–500 μm	1.08 ± 0.16	4.21 ± 0.16	2.32 ± 0.06	2.45 ± 0.43	*2.52 ± 1.29*
500–1000 μm	1.78 ± 0.08	4.51 ± 0.08	3.44 ± 0.53	2.82 ± 0.03	*3.14 ± 1.14*
1000–2000 μm	3.36 ± 0.41	4.83 ± 0.23	3.68 ± 0.35	4.46 ± 0.77	*4.08 ± 0.68*
>2000 μm	2.74 ± 0.34	4.20 ± 0.26	2.56 ± 0.32	2.08 ± 0.44	2.90 ± 0.91
Mean	*1.94 ± 1.03*	*4.32 ± 0.84*	*2.73 ± 0.88*	*2.69 ± 0.90*	*2.92 ± 1.12*

Mean values were calculated using three replicates per size class and season.

Table III: Results of two-way ANOVAs on C and N stable isotope ratios of plankton at the SOMLIT site according to season and size class

	F	*P*-value	*Post hoc*
$\delta^{13}C$			
Season	58.79	<0.001	Autumn > Summer = Winter > Spring
Size class	9.52	<0.001	2000 = 1000 > 500 = 80 = 300 = 200
Season × Size class	0.64	0.840	n.s.
$\delta^{15}N$			
Season	13.23	<0.001	Summer > Autumn = Spring = Winter
Size class	4.18	0.001	1000 > 500 = 2000 = 300 = 200 = 80
Season × Size class	0.56	0.732	n.s.

F, statistics; *P*, level of significance; *Post hoc*, results of NKS *post hoc* tests of comparison of means. n.s., not significant.
The lower limit only of each size class was indicated for clarity. 80 = 80–200 μm, 200 = 200–300 μm, 300 = 300–500 μm, 500 = 500–1000 μm, 1000 = 1000–2000 μm, 2000 ≥ 2000 μm

and $\delta^{15}N$ during the warm season (summer–autumn) compared with the cold season (winter–spring). In all size classes, the highest $\delta^{13}C$ was recorded in autumn and the highest $\delta^{15}N$ in summer, whereas the lowest values of both elements were recorded in spring, except for $\delta^{15}N$ in the >2000 μm size class (winter) (Table II).

Time (season) represented the main factor of isotopic variability in zooplankton (Table III). Differences in stable isotope ratios were higher between seasons ($\Delta\delta^{13}C \pm 4.93 + 1.21‰$, $\Delta\delta^{15}N = 2.38 + 0.94‰$) than between size classes ($\Delta\delta^{13}C = 1.89 + 1.95‰$, $\Delta\delta^{15}N = 1.66 + 0.88‰$).

POM composition and seasonal variation of isotopic ratios

There were strong differences between quantitative and qualitative composition of water POM at the SOMLIT site, Cortiou plume and Huveaune River. A much greater quantity of small particles (2–6 μm) was recorded in the Cortiou plume (212×10^3 cells μm L^{-1}) and Huveaune River (117×10^3 cells μm L^{-1}) than in SOMLIT POM (21 cells μm L^{-1}). These small particles were mainly detritus and bacteria, as was observed on STEM photos. They represented 100% of cells found in Huveaune River water, 94% in Cortiou plume and only 25% in SOMLIT water. Excepting these detrital particles, Cortiou plume contained more pico- and nanoplankton cells than SOMLIT water (12×10^3 and 15 cells μm L^{-1}, respectively). However, in spite of such a difference in cell abundance, the composition of the pico- and nanoplankton community was similar at the two sites, with a dominance of the cyanobacteria *Prochlorococcus* and *Synechococcus*, and two groups of non-identified picoeukaryotes (picoeukaryotes 1 and 2) (Fig. 2). Thus, while the Huveaune River water was entirely composed of detritus and bacterial particles, the Cortiou plume and SOMLIT POM were composed of both detritus and living phytoplankton cells, but in different proportions. Detritus dominated the sewage plume, whereas living pico- and nanoplankton cells dominated in SOMLIT water.

Stable isotope ratios also differed between these three types of POM. Both $\delta^{13}C$ (ANOVA, $F = 7.60$, $P = 0.018$) and $\delta^{15}N$ (ANOVA, $F = 17.75$, $P = 0.002$) differed significantly between POM of the SOMLIT site, Cortiou sewage plume and Huveaune River. Huveaune POM was characterized by low $\delta^{13}C$ ($-26.25 \pm 0.51‰$) and high $\delta^{15}N$ ($4.48 \pm 0.41‰$), Cortiou POM by low values of both $\delta^{13}C$ ($-25.50 \pm 0.62‰$) and $\delta^{15}N$ ($-0.59 \pm 0.82‰$) and SOMLIT POM by high $\delta^{13}C$ ($-23.59 \pm 1.37‰$) and intermediate $\delta^{15}N$ ($2.18 \pm 1.39‰$). Stable isotope ratios of the three POM types and zooplankton at SOMLIT showed similar seasonal variations. They were generally lower during the cold period (winter–spring) than during the warm season

Table IV: Mean (± SD) carbon and nitrogen stable isotope ratios of the main groups of large zooplankton organisms (>1000 μm) by increasing $\delta^{15}N$ value

	n	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Salps	11	−22.76 ± 1.29	0.35 ± 1.46
Pteropods	13	−18.82 ± 1.83	1.54 ± 0.47
Ctenophores	4	−22.36 ± 0.39	2.67 ± 1.55
Siphonophores	12	−20.42 ± 0.70	3.23 ± 0.53
Hyperiid amphipods	5	−18.01 ± 1.43	3.43 ± 0.45
Macruran larvae	7	−20.79 ± 0.52	3.60 ± 0.60
Euphausiids	9	−22.68 ± 1.22	3.63 ± 1.03
Copepods	6	−21.45 ± 0.58	3.82 ± 0.07
Chaetognaths	15	−20.78 ± 0.72	3.97 ± 0.82
Brachyuran larvae	9	−22.04 ± 1.59	4.07 ± 1.07
Stomatopod larvae	1	−19.61 ± 0.00	4.91 ± 0.00
Teleost larvae	8	−20.35 ± 0.91	4.97 ± 0.65
Cephalopod larvae	1	−18.83 ± 0.00	9.53 ± 0.00

N, number of samples analyzed.

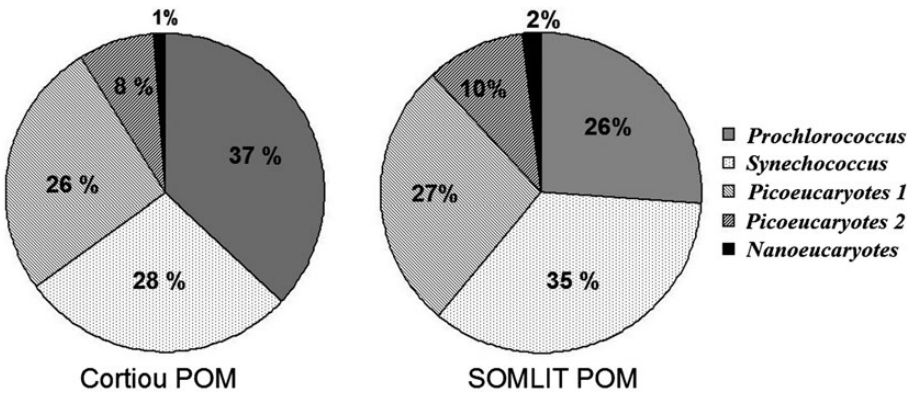

Fig. 2. Composition of pico- and nanoplankton groups (<20 μm) in the POM of Cortiou plume and SOMLIT water, excluding detritus particles.

(summer–autumn). These seasonal variations of zooplankton at SOMLIT clearly reflected those of POM sources at this site (Fig. 3), indicating the POM integration in zooplankton food webs. At the SOMLIT site, the $\delta^{15}N$ difference between POM and zooplankton was higher in winter and spring (mean $\Delta\delta^{15}N \pm 1.29 + 0.67‰$) than in summer and autumn (mean $\Delta\delta^{15}N = 0.21 + 0.77‰$), suggesting higher fractionation factors along the planktonic food web during the cold season.

Seasonal variations of environmental parameters and microphytoplankton composition

The environmental parameters measured at the SOMLIT site showed high seasonal variations (Table V), but two main periods could be highlighted. The cold season (winter–spring) was characterized by temperatures ~13°C and high SPM, nutrient (NO_3 and NO_2) and Chl *a* content. In contrast, the warm season (summer–autumn) had temperatures ~21–22°C and low SPM, nutrient and Chl *a* concentrations. Precipitation was highest during winter and lowest in summer.

Seasonal variations of microphytoplankton composition at the SOMLIT site (Table VI) showed high abundance of large phytoplanktonic cells (20–200 μm) in summer and spring, and lower abundance in winter and particularly in autumn. Flagellates and crytophyceans dominated in spring and winter, whereas summer was the only season when diatoms were abundant. In autumn, the phytoplankton community was largely dominated by crytophyceans, along with some dinobionts.

DISCUSSION

The results of the present study showed seasonal variations in stable isotope ratios of zooplankton size groups (80 to >2000 μm) according to their composition and among taxonomic groups. The original approach of this paper resides in the relationships between these

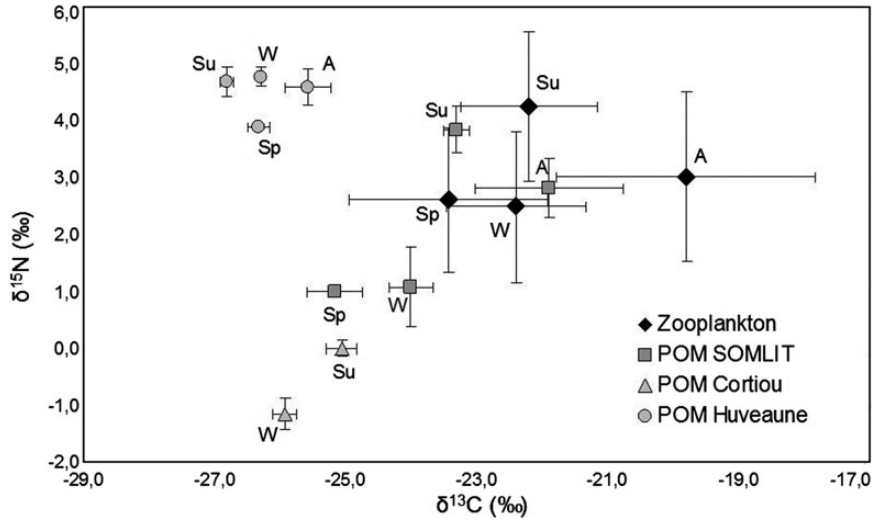

Fig. 3. Mean (±SD) seasonal variations of $\delta^{13}C$ and $\delta^{15}N$ of water POM at SOMLIT site, Cortiou sewage plume and Huveaune River, and mean seasonal values of zooplankton at SOMLIT site. Sp, spring; Su, summer; A, autumn; W, winter.

Table V: Mean (±SD) seasonal values of environmental parameters at the SOMLIT site during the zooplankton sampling months

Parameters	Spring	Summer	Autumn	Winter	Data sources
SPM mg L^{-1}	0.53 ± 0.0	0.40 ± 0.1	0.50 ± 0.1	6.5 ± 0.6	SOMLIT national 2009–2010; this study
Chl *a* μg L^{-1}	0.57 ± 0.1	0.24 ± 0.1	0.30 ± 0.2	0.50 ± 0.0	SOMLIT national 2009–2010
NO_3μmol L^{-1}	1.26 ± 1.0	0.43 ± 0.4	0.27 ± 0.2	1.54 ± 0.2	SOMLIT national 2009–2010
NO_2μmol L^{-1}	0.12 ± 0.1	0.13 ± 0.1	0.06 ± 0.1	0.20 ± 0.0	SOMLIT national 2009–2010
T°C surface	13.3 ± 0.1	22.0 ± 3.1	20.9 ± 1.3	12.5 ± 0.1	SOMLIT national 2009–2010
Salinity	38.09 ± 0.0	37.92 ± 0.1	38.13 ± 0.0	38.02 ± 0.0	SOMLIT national 2009–2010
Rain mm	51 ± 21	1 ± 0.1	83.5 ± 0.5	121 ± 34	www.meteofrance.fr (2009–2010 Martigues-Toulon)

Mean values were calculated using eight samples per season. SPM, suspended particulate matter; Chl *a*, chlorophyll a; T, surface water temperature.

Table VI: Composition of microphytoplankton (20–200 μm) at the SOMLIT site in 2009–2010, during the zooplankton sampling months, in cell abundance percentages (from Database Phytocom courtesy, Becker, personal communication)

Phytoplankton groups	Spring (%)	Summer (%)	Autumn (%)	Winter (%)	Main genera
Diatoms	0.2	40.2	0	0.9	*Chaetoceros*
Dinobionts (dinoflagellates)	4.5	2.8	15.7	13.5	*Gymnodinium, Heterocapsa*
Dinobionts (flagellates)	74.1	45	0	49.9	
Cryptophyceans	21.2	2.8	84.3	35.5	*Cryptomonas*
Prasinophyceans	0	4.1	0	0	
Monades	0	5.1	0	0	
Other groups	0	0	0	0.2	Silicoflagellates
Number of cells L^{-1}	147 863	231 600	2235	36 727	

variations and those of POM sources, environmental parameter fluctuations (temperature, nutrients, Chl *a* concentration) and phytoplankton and seawater POM composition in Marseille Bay.

Influence of composition and size on zooplankton stable isotope ratios

Zooplankton composition at the SOMLIT site differed among size classes. Detritus and small copepod stages dominated the smallest size class (80–200 μm). The dominance of detritus in this size class could be related to the proximity of the large city of Marseille, the constant inputs of Cortiou sewage wastewaters and sporadic river water runoff. Rolff (Rolff, 2000) showed that phytoflagellates and ciliates dominated in the 50–100-μm size class, and ciliates, copepod nauplii, rotifers and diatoms in the 100–200 μm size class. The three following larger size classes (200–300, 300–500, 500–1000 μm) were dominated by copepods at SOMLIT and by cladocerans to a lesser extent, as already observed in different regions (Champalbert, 1996; Rolff, 2000; Calbet *et al.*, 2001; Saiz *et al.*, 2007; Koppelmann *et al.*, 2009). Copepods usually account for 45–95% of these size classes in the Mediterranean Sea (Razouls and Kouwenberg, 1993; Champalbert, 1996). The 1000–2000 and >2000-μm size classes were very diverse and composed, along with large copepods, of pteropods, gelatinous organisms (appendicularians, salps, siphonophores, chaetognaths), euphausiids and large crustacean, cephalopod and teleost larvae.

Both $\delta^{13}C$ and $\delta^{15}N$ of zooplankton generally increased with size at the SOMLIT site, though not following a constant pattern. The lowest $\delta^{13}C$ was observed in the 200–300-μm size class, while the abundance of terrestrial detritus in the 80–200-μm size class should have resulted in lower $\delta^{13}C$ values, as observed in front of the Rhone River (Harmelin-Vivien *et al.*, 2008). However, this might be due to a high proportion of ^{13}C-enriched exuviae and feces as indicated by Checkley and Entzeroth (Checkley and Entzeroth, 1985) and Klein Breteler *et al.* (Klein Breteler *et al.*, 2002). In addition, $\delta^{15}N$ of the largest size class (>2000 μm) was lower than that of the 1000–2000 and 500–1000-μm size classes. In the literature, an increase in zooplankton $\delta^{15}N$ with size is generally recorded (e.g. Rau *et al.*, 1990; Malej *et al.*, 1993; Waite *et al.*, 2007; Koppelmann *et al.*, 2009; among others). Enrichment in zooplankton $\delta^{15}N$ with size is usually explained as reflecting size-related consumption patterns in marine plankton food webs (Rolff, 2000). Several authors report a large degree of trophic level overlap among zooplankton size classes (Fry and Quiñones, 1994; Koppelmann *et al.*, 2009). A large range of $\delta^{15}N$ was recorded among the different groups present in the two largest size classes at SOMLIT, which was related to feeding differences. Microplankton filter feeders (salps, pteropods) had much lower $\delta^{15}N$ than predators (chaetognaths, large crustacean larvae, teleost and cephalopod larvae). Wide differences of isotopic signatures, particularly $\delta^{15}N$, among zooplankton groups or even species have been recorded in many studies, and are related to differences in diet (Schell *et al.*, 1998; Hobson *et al.*, 2002; Strzelecki *et al.*, 2007; Waite *et al.*, 2007; Fanelli *et al.*, 2011). The lowest isotope ratios are found in filter feeders (numerous copepods, ostracods, pteropods, salps and thaliaceans) which feed on POM and small planktonic cells, and the highest $\delta^{15}N$ values were recorded in carnivores (chaetognaths, large crustaceans, micronekton) (Fanelli *et al.*, 2011). Costalago *et al.* (Costalago *et al.*, 2012) analyzed the stable isotope ratios of some zooplankton groups in the Gulf of Lions, close from Marseille Bay. The comparison of their results from the summer season with our data shows similar $\delta^{15}N$ and higher $\delta^{13}C$ values for microplankton, copepods and cladocerans (dominant in 200–500-μm size classes).

Effect of composition and environmental parameters on POM isotopic signatures

Although located a few miles offshore, the SOMLIT site can be influenced by material of terrestrial and

anthropogenic origin washed into the sea by the Cortiou sewage outfall and the Huveaune River (Fig. 1), as shown by hydrodynamic studies performed in the Bay of Marseille (Pradal and Millet, 2006). This is particularly the case after heavy rains and with strong winds blowing from the south-east. Detritus, along with some bacteria, were the only components of the Huveaune River POM when it was flowing to the sea. POM collected in the Cortiou sewage plume was a mixture of wastewater POM and marine phytoplankton, but was largely dominated by anthropogenic detrital material (>75%). In contrast, SOMLIT water POM was mainly composed of phytoplankton, but also included a considerable proportion of small detritus particles. Cresson *et al.* (Cresson *et al.*, 2012) demonstrated with mixing models that POM sampled further inshore in the Bay of Marseille is composed of POM of various origins, including Cortiou sewage and Huveaune River POM. However, coastal and offshore marine phytoplankton was dominant in seawater POM. These authors recorded higher POM $\delta^{15}N$ in winter as their sites are more influenced by the Huveaune River than Cortiou sewage waters. The lower $\delta^{15}N$ recorded in SOMLIT POM in winter indicated a strong influence of Cortiou sewage waters ($-1.17 + 0.3$ ‰) during winter sampling that occurred just after a heavy rainy event. Higher quantities of POM with lower $\delta^{15}N$ from Cortiou mixed with marine POM and decreased the $\delta^{15}N$ of the SOMLIT POM in winter.

Thus, both flow cytometry analysis and seasonal variations of POM isotopic signatures strongly suggest that the detrital particles observed in SOMLIT water POM originated from Cortiou sewage inputs.

The low $\delta^{13}C$ values (<−25‰) recorded in Huveaune and Cortiou waters agree with their continental origin, as terrestrial plant detritus exhibits much lower $\delta^{13}C$ than marine phytoplankton (Fry and Sherr, 1984; Riera and Richard, 1996; Harmelin-Vivien *et al.*, 2008). Schell *et al.* (Schell *et al.*, 1998) also found depleted isotopic values in coastal waters due to the Mackenzie River inputs of terrestrially derived carbon and nitrogenous nutrients with low ^{13}C and ^{15}N values. The low $\delta^{13}C$ observed in SOMLIT POM in winter (−24.00‰) and spring (−25.16‰) confirmed the influence of terrestrial matter at this site during these seasons. While the high $\delta^{15}N$ of the Huveaune River water (>4‰) agrees with those found in other rivers (Kendall *et al.*, 2001; Harmelin-Vivien *et al.*, 2010), the very low $\delta^{15}N$ value recorded in the Cortiou plume (∼0‰) is surprising and could be related to wastewater treatment and denitrifying bacteria which may be responsible for these low $\delta^{15}N$ ratios (Kendall *et al.*, 2007). Blooms of diatoms and their diazotrophic cyanobacterial symbiont responsible of N_2 fixation (diazotrophy) were noticed in some plume areas where organic matter containing recently fixed nitrogen had a $\delta^{15}N = -1$‰ (Yeung *et al.*, 2012). However, the flow cytometry analysis did not confirmed the presence of these organisms in our study area. Previously, Darnaude *et al.* (Darnaude *et al.*, 2004) found similar stable isotope ratios for marine phytoplankton in Marseille Bay ($\delta^{13}C = -22.36 + 0.24$‰; $\delta^{15}N = 2.33 + 0.11$‰). In the Gulf of Lions, in the western part of Marseille, Harmelin-Vivien *et al.* (Harmelin-Vivien *et al.*, 2008) found higher stable isotope ratios for phytoplankton ($\delta^{13}C = -20.08 + 0.78$‰; $\delta^{15}N = 4.45 + 0.75$‰) that in SOMLIT POM. This proves that even these areas are rather close (∼200 km), the local baseline of a food web is very important.

Seasonal variations of stable isotope ratios of POM were similar at the three sites, with generally lower values during the cold season (winter–spring) and higher values during the warm season (summer–autumn), except for high $\delta^{15}N$ in the Huveaune River in winter. Environmental parameters also differed between these two main climatic periods. Both seasonal variations of environmental parameters and POM composition could explain the observed seasonal differences in POM isotopic signatures. The cold season is characterized by high Chl *a* and nutrient levels (Table V). Temperature seems to influence stable isotope ratios and $\delta^{13}C$ values decrease with decreasing temperatures according to Goericke and Fry (Goericke and Fry, 1994). However, in our case the mean $\delta^{13}C$ values of zooplankton in winter and summer were not statistically different.

Higher quantities of ^{13}C depleted terrestrial inputs and ^{15}N depleted sewage wastewater in winter, due to rainy events, might explain in part the lower $\delta^{13}C$ and $\delta^{15}N$ observed. The abundance of small planktonic cells (flagellates and dinoflagellates) during the cold season also contributed to low stable isotope values, as dinobionts have lower stable isotope values than diatoms (Fry and Wainwright, 1991; Sommer and Sommer, 2004). In addition, Grégori *et al.* (Grégori *et al.*, 2001) observed a massive bloom of picophytoplankton cells (0.2–2 μm) in spring in the Bay of Marseille, and small cells are markedly ^{13}C depleted compared with larger microphytoplankton particles (Rau *et al.*, 1990).

In contrast, the warm season was characterized by low nutrient and Chl *a* content. The growth of phytoplankton in an impoverished pool of nitrates, where only ^{15}N-enriched compounds remain, may be responsible for the high $\delta^{15}N$ values observed for POM in summer as shown by Savoye *et al.* (Savoye *et al.*, 2003) and Montoya (Montoya, 2007). Fry and Wainwright (Fry and Wainwright, 1991) also report a ^{13}C enrichment of phytoplankton at the end of a bloom. During summer, the bloom of large diatoms was probably responsible for

nutrient consumption and limitation, resulting in an increase in stable isotope values. Moreover, diatoms are isotopically heavier than dinobionts (Sommer and Sommer, 2004; Waite *et al.*, 2007). Thus, both composition of the phytoplankton community (diatoms) and nutrient limitation contributed to increase POM stable isotope ratios in summer along with high temperatures. As observed by Cresson *et al.* (Cresson *et al.*, 2012), the fact that high concentrations of Chl *a* (winter–spring) did not match with the maximum abundance of large microphytoplankton, and particularly diatoms (summer), also reflects the predominance of small phytoplankton cells at the SOMLIT site, as at many other sites in the Mediterranean Sea (Arin *et al.*, 2005).

Seasonal variation of isotopic signatures of zooplankton and trophic functioning

Mean isotopic signatures of zooplankton at SOMLIT showed the same seasonal tendency in $\delta^{13}C$ and $\delta^{15}N$ as seawater POM, with higher values in summer and autumn, and lower values in winter and spring. Seasonal variations of zooplankton isotopic signatures are observed by many authors with also generally higher $\delta^{13}C$ in summer or autumn (Wainright and Fry, 1994; Bouillon *et al.*, 2000; Kibirige *et al.*, 2002; Fanelli *et al.*, 2011; among others). Kibirige *et al.* (Kibirige *et al.*, 2002) also recorded minimum values of stable isotopes in winter and maximum values in summer for both food sources and dominant zooplankton species.

Seasonal variations in isotopic signatures of zooplankton size classes reflect the seasonal variation of different parameters, (i) temperature and environmental parameters, (ii) composition and isotopic ratios of phytoplankton, (iii) zooplankton composition and (iv) most likely variation in zooplankton diet. Differences in stable isotope values among zooplankton size classes at the SOMLIT site were more dispersed during the cold than the warm season, particularly for $\delta^{15}N$. A $\Delta\delta^{15}N$ of 2.4 and 2.3‰ was observed among zooplankton size classes in winter and spring respectively, and a difference of 0.9 and 1.6‰ in summer and autumn, respectively. In addition, differences between POM and zooplankton were higher during the cold than the warm season. These results suggest that food overlap between zooplankton size classes could be higher in summer–autumn than in winter–spring. A higher nitrogen fractionation was particularly evident in the 1000–2000-μm size class during the cold season, suggesting more specific predatory diets in winter, while lower fractionation in summer indicated wider omnivorous diets. This would imply that food sources available to zooplankton were more diversified and abundant during the cold season, while a food shortage occurred during the warm season with higher food overlap, omnivory and probably competition for resources. Such a hypothesis agrees with higher SPM and Chl *a* contents in the environment in winter and spring. Poulet *et al.* (Poulet *et al.*, 1986) demonstrated that POM has distinct chemical characteristics and nutritional values varying with categories, size fractions and biogenic or terrestrial origin of the particles. Recently, Cresson *et al.* (Cresson *et al.*, 2012) demonstrated that the biochemical and nutritional value of POM further inshore in the Bay of Marseille varies with season, with lowest values being recorded in autumn. Marine trophic pathways differ according to water characteristics and thus seasons (Azam *et al.*, 1983). In the Mediterranean Sea, they range from the prevalence of microbial loop in oligotrophic environments to the classical food chain in meso- or eutrophic environments like neritic zones (Christaki *et al.*, 1996; Saiz *et al.*, 2007). At the SOMLIT site, the food web is probably an intermediate situation between classical food chain and microbial loop (Legendre and Rassoulzadegan, 1999). A strong coupling between the microbial food webs and upper trophic levels is mediated by ciliates and other microheterotrophs that are preyed on by small copepod species and juvenile stages (Calbet *et al.*, 2001; Saiz *et al.*, 2007). To validate or refute the hypothesis of a difference in zooplankton diet with season at the SOMLIT site, more accurate studies at specific and size level, including diet description, taxonomic and biochemical composition, would be necessary.

Thus, from the present study, it can be concluded that a complex zooplankton food web was present at the SOMLIT site in the Bay of Marseille, intermediate between microbial and herbivorous food webs. The small detritus and bacterial particles brought to the sea by the Cortiou sewage outfall and the Huveaune River were mixed with natural marine phytoplankton populations, dominated most of the year by pico- and nanoplankton cells, and consumed by zooplankton populations. It is important that this transfer of highly contaminated organic matter (Wafo *et al.*, 2006; Guigue *et al.*, 2011) be taken into account in modeling approaches and estimation of pelagic food web contamination.

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