



# The Depleted Carbon Isotopic Signature of Nematodes and Harpacticoids and Their Place in Carbon Processing in Fish Farm Sediments

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Fish farm-originating organic matter can modify the ecological processes in a benthic ecosystem. This was investigated in the sediments of the northern Adriatic Sea by measuring  $\delta^{13}\text{C}$  signature of nematodes, harpacticoids, and sedimentary organic matter, and by assessing pore water nutrients and bacterial composition. In a mesocosm experiment,  $^{13}\text{C}$ -labeled diatoms were added on top of sediment cores and  $^{13}\text{C}$  enrichment was measured as a proxy of diatom uptake by meiofauna. The  $\delta^{13}\text{C}$  signatures were depleted under fish farming cages compared to the reference site, as observed for sedimentary organic matter ( $-24.4\text{‰}$  vs.  $-21.8\text{‰}$ ), for nematodes ( $-22.5\text{‰}$  vs.  $-17.7\text{‰}$ ), and for harpacticoids ( $-25.3\text{‰}$  vs.  $-20.8\text{‰}$ ). The direct consumption of fish feed ( $-22.2\text{‰}$ ) was not traced in meiofauna taxa. Nematodes from the farm site likely reflect a diet comprising sedimentary organic matter, as they were enriched by  $2\text{‰}$  relative to the sedimentary organic matter. The nematodes from the reference site were enriched by  $4.2\text{‰}$  relative to the sedimentary organic matter, which implies that they rely on more enriched food sources, like diatoms, which was confirmed by their uptake of  $^{13}\text{C}$ -labeled diatoms. The nematode assemblage incorporated more diatom  $^{13}\text{C}$  than harpacticoids, making them more important players in the carbon flux from diatoms to higher trophic levels at the reference site. Harpacticoids from the reference site were enriched by  $1.1\text{‰}$  compared to sedimentary organic matter, implying that this was their primary food source. Harpacticoids from the farm site were depleted by  $0.9\text{‰}$  relative to the sedimentary organic matter, indicating they were influenced by a very depleted food source like bacteria. Harpacticoids from both the cage and reference sites consumed  $^{13}\text{C}$ -labeled diatoms, which implies their diet might span a broad  $\delta^{13}\text{C}$  range, from bacteria to diatoms. Pore water nutrients with high dissolved inorganic carbon, phosphate, and ammonium concentration indicated an elevated microbial degradation of organic compounds under the fish farm. The denaturing gradient gel electrophoresis analysis showed a 70% similarity between

sediment bacteria communities from the fish farm and reference site. The study demonstrated that fish farm-originating organic matter enters the meiofauna food chain, and that nematodes and harpacticoids use different food sources under the fish farm and at the reference site.

**Keywords:**  $\delta^{13}\text{C}$ , sedimentary organic matter, nematodes, harpacticoid copepods,  $^{13}\text{C}$ -labeled diatoms, sediment bacteria, fish farming, northern Adriatic Sea

## INTRODUCTION

Aquaculture is a fast-growing industry worldwide, with top production in China, India, and Indonesia, and is in a production parallel with capture fisheries FAO (2019b). In 2017, 80.1 million tons of aquatic animals were produced, of which 45.6 million tons were finfish in freshwaters and 7.8 million tons were finfish produced in marine environment (FAO, 2019a). In the Mediterranean Sea, the seabass *Dicentrarchus labrax* and the seabream *Sparus aurata* are the most important commercial coastal fish species cultured (FAO, 2005–2019). In some coastal areas, fish farming contributes markedly to the total nutrient discharges in line with the municipal, industrial, and agricultural wastes (Porrello et al., 2005; Pitta et al., 2006; Kutti et al., 2007). The fish production typically increases the carbon, nitrogen, and phosphorus budget in the environment (Sarà, 2007; Holmer et al., 2008) and cause local eutrophication (Mirto et al., 2010). Although the operations spatially take place in the water column in and around employed hanging cages, its impact is not restricted to the pelagic realm (Gao et al., 2005). It was revealed that the finfish culture affects the sediment biogeochemistry and dynamic of benthic organisms, including microbial communities via sedimentation of uneaten feed and fish feces (La Rosa et al., 2004; Bongiorno et al., 2005; Holmer et al., 2007; Hornick and Buschmann, 2018). Bacterial abundance, biomass carbon production, and enzymatic activities all increase beneath fish cages (La Rosa et al., 2001; Caruso, 2014 and references therein). Those changes in benthic ecosystem are especially pronounced in shallow waters and sheltered areas where most fish farms are located (Borja et al., 2009). Biogeochemical changes of the sediment under fish cages have a negative impact on macrofauna composition, as shown by a lower abundance and a decrease in biomass (Hargrave et al., 1997; Gao et al., 2005), but also by the community shifts toward deposit-feeders (Dempster et al., 2002).

The close association of meiofauna with the sediment matrix (Kennedy and Jacoby, 1999) results in their lower density under cages (Mazzola et al., 1999; Mazzola et al., 2000; La Rosa et al., 2001). The same was confirmed for our study site, where harpacticoids and kinorhynchs abundance was lower under fish farms compared to the reference, while the nematode abundance was not altered (Grego et al., 2009). The impact of fish farming activities on the energy transfer toward the receiving meiofauna community, however, is far from well known. To define carbon fluxes in marine ecosystems ecologists use natural isotopic signatures (Fry, 2006; Callier et al., 2013; Leduc et al., 2020) which can be combined

with the uptake of pre-labeled food in incubation experiments (Middelburg et al., 2000; Carman and Fry, 2002; De Troch et al., 2007). About 1–2‰ of  $^{13}\text{C}$  enrichment is mostly observed per trophic level through the marine food web (DeNiro and Epstein, 1981; Ostrom and Fry, 1993; Soreide et al., 2006). The food-labeled experiments, in contrast, focus on an exact food source, e.g., diatoms, excessively enriched in  $^{13}\text{C}$ , which can be traced in the grazers, e.g., meiofauna, resulting as higher values of  $\delta^{13}\text{C}$  (Wyckmans et al., 2007; Franco et al., 2008; Maria et al., 2011; Lammers et al., 2016). The natural abundance of isotopes reflect the accumulation of it over a longer period of time and allows trophic level estimates. However, the difference among sources or organisms might be too small to make conclusions, while added tracers provide conclusive results from large differences among isotope ratios, but only in a short time span (Middelburg, 2014). As cited from Glibert et al. (2019) “the power of isotopes is that small differences allow great insights” (sources of nutrients, food web structure, and rate process), but the technique is also very sensitive to errors.

The stable isotopes were used to reveal the impact of fish rearing on the environment in several studies (Ye et al., 1991; Sarà et al., 2004; Sara et al., 2006; Holmer et al., 2007; Landrum and Montoya, 2009). According to Mayor et al. (2017) the analysis of the carbon stable isotopes of bulk sediment represents an alternative and fast approach to quantify the fate of fish farm-derived organic matter. Moreover, the isotopic signature of the fish feed pellets can be distinct from the local sources, as they are composed of terrestrial material and fishmeal originating from other seas (Moreno-Rojas et al., 2008). Using stable isotopes the incorporation of fish feed and their fecal waste into bivalves (Bergvik et al., 2019) and some other macrozoobenthic organisms (Kusche et al., 2017), including larger nematodes (>500  $\mu\text{m}$ ) (Callier et al., 2013), have been published, while to our knowledge no similar study exists for meiofaunal organisms.

The aims of the present study were: (i) to examine whether the fish farm-originating organic matter enters the meiofauna food chain, and (ii) to assess differences in food sources of two meiofauna taxa (nematodes and harpacticoids) under the fish farm and at the reference site. We investigated the natural  $\delta^{13}\text{C}$  signatures in the sedimentary organic matter (OM) and in meiofauna, at the reference and farm sites, together with the  $\delta^{13}\text{C}$  of fish feed used. An experiment with the addition of  $^{13}\text{C}$ -labeled diatoms to meiofauna cores was conducted, to trace the grazing by nematodes and harpacticoids under changed conditions (fish farm vs. reference), to reveal the potential changes in one resource utilization. The natural signatures, together with the experiment, allowed us to study the carbon flux and, thus, overall ecosystem

functioning due to fish farming. Additionally, we tested for differences in pore water nutrients and sediment-associated bacterial communities, to describe changes in the environment of meiofauna, as a result of organic material deposition from the farming activities on the seabed.

## MATERIALS AND METHODS

### Study Area and Experimental Design

The experimental units, namely standard meiofauna cores with an inner diameter of 3.6 cm, were collected by means of scuba diving in April 2007 from the inner part of the Bay of Piran, northern Adriatic Sea ( $45^\circ 29' 11.96''\text{N}$ ,  $13^\circ 34' 51.05''\text{E}$ ), where a fish farm, rearing *D. labrax* is located [for details consult Grego et al. (2009)] (**Figure 1a**). The study area is shallow (average water depth: 13 m) and the background granulometric composition of the muddy sediment is clayey silt (Ogorelec et al., 1991; Kovač et al., 2003) with high carbonate content of approx. 30% (Ranke, 1976). The sedimentation rates in the northern Adriatic bays range from 3 to 5 mm yr<sup>-1</sup> (Ogorelec et al., 1991), but sedimentation rates around the fish farm were eight times higher compared to the reference site (Kovač et al., 2004).

In total, 10 meiofauna cores were retrieved under fish cages, further referred to as 'CAGE' cores and 10 meiofauna cores were collected at about 100 m away from the cages, further referred to as 'OUT' cores (**Figure 1b**). The latter served as a reference cores, since it was proven that 100 m away from fish farm meiofauna main taxa density and diversity were not impacted (Grego et al., 2009). The cores were immediately transported to a thermostatic chamber and incubated at 14°C under a 12:12 h light-dark cycle and 150  $\mu\text{E}/\text{m}^2/\text{s}$  PAR (similar to the *in situ* conditions). The cores were provided with oxygen through an aeration system and fresh GF/F filtered sea water was added in drops during the experiment duration. The excess water in cores was removed through a hole in the core (at which a pipette tip was sealed).

Freeze-dried  $^{13}\text{C}$  labeled diatoms (5.2 mg per core) were dissolved in filtered seawater and were gently applied with a syringe and plastic tube onto the top of the sediment in each of five replicates of the 'CAGE' cores and five replicates of 'OUT' cores. These  $^{13}\text{C}$  enriched cores are referred to as 'CAGE\*' and 'OUT\*' (\*is for enriched samples). Besides, five replicates of 'CAGE' and five replicates of 'OUT' cores were not inoculated with enriched diatoms and served as controls (**Figure 1b**). All 20 cores were incubated for 5 days in the thermostatic chamber under controlled conditions (as described above). The diatoms used in the experiment were freeze-dried *Seminavis robusta* (strain 84A, culture collection of the Laboratory of Protistology & Aquatic Ecology, Ghent University, Belgium) grown in *f/2* medium (Guillard, 1975) with additional  $^{13}\text{C}$  (see **Supplementary Material** for detailed labeling procedure). The labeling technique enriched the  $\delta^{13}\text{C}$  of diatoms from  $-13.07 \pm 0.31$  to  $7191.38 \pm 70.92\text{‰}$  (freeze-dried diatoms). The cells measured  $55.89 \pm 0.94 \mu\text{m}$  in length. The diatoms were composed on average of 30% of carbon, corresponding to approximately 1.56 mg of carbon added into each core. At the end of the experiment, the cores were sliced

into the top (0–1 cm) layer and lower (1–5 cm) layer and stored at  $-20^\circ\text{C}$ .

To extract the meiofauna, the sediment slices were thawed, washed with distilled water on 38  $\mu\text{m}$  sieve, mixed with Levasil® (-distilled water) solution (specific density = 1.17 g/cm<sup>3</sup>), and centrifuged three times for 10 min at 3,000 rpm (de Jonge and Bouwman, 1977; Mc Intyre and Warwick, 1984). Levasil is an aqueous colloidal dispersion of amorphous silica (SiO<sub>2</sub>) with 0.1% of Na<sub>2</sub>O (percentage by weight) and does not contain carbon. Meiofauna was sorted within a few hours after thawing to minimize the leakage of  $^{13}\text{C}$  label (Moens et al., 1999). The natural isotope values of meiofauna were analyzed only from the top sediment layer, as no copepods were present in the deeper layer of the CAGE samples. In some samples we only had few harpacticoid specimens, and we expect that some were lost during experiment due to escapes. Harpacticoids from those samples were pooled to reach enough biomass for a reliable isotope analysis, yielding four (instead of five) CAGE samples, and three (instead of five) CAGE\* samples. The first 150 nematodes of OUT and OUT\* samples (**Figure 2a**) and 100 nematodes from CAGE and CAGE\* samples (**Figure 2b**; larger nematodes) were picked out for isotope analysis. All copepods from the sample were picked out into an embryo dish containing milli-Q water (**Figure 2c**; OUT assemblage and **Figure 2d**; CAGE assemblage). The animals were then washed several times in milli-Q water in order to remove food or detritus from their bodies and then placed in tin capsules (8 mm\*5 mm). The material was then desiccated overnight at 60°C. From each of the 20 cores, an aliquot of sediment was taken to determine the isotope signal of sedimentary OM. The sediment was first dried to constant weight at 60°C and 15 mg was placed in silver capsules (8 mm\*5 mm). The carbonate was removed by the subsequent addition of HCl in increasing concentrations and subsequent drying of the samples at 60°C.

The pellets (ECOLIFE 68 N°3, BioMar) used to feed the fish were also analyzed for  $\delta^{13}\text{C}$ . Five replicates of 25 g of fish pellets were homogenized with agate mortar and pestle, from each 2 g was put into a tin capsule. The samples were desiccated overnight at 60°C, capsules were pinch closed and sent for carbon isotope analysis. These pellets were used to feed the fish since 2004 (Fisfarmers personal comm.).

### Isotope Analytic Techniques and Data Treatment

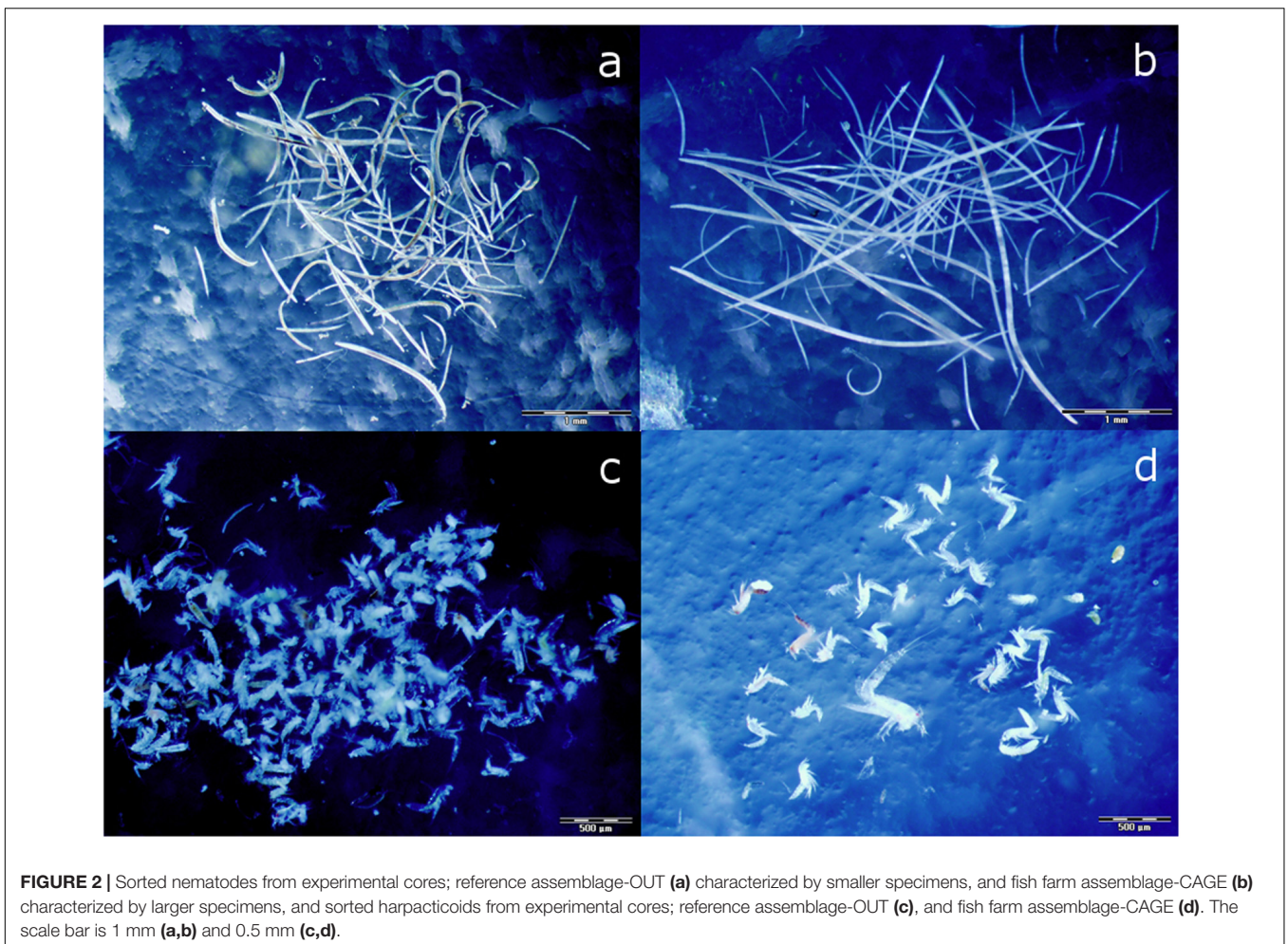
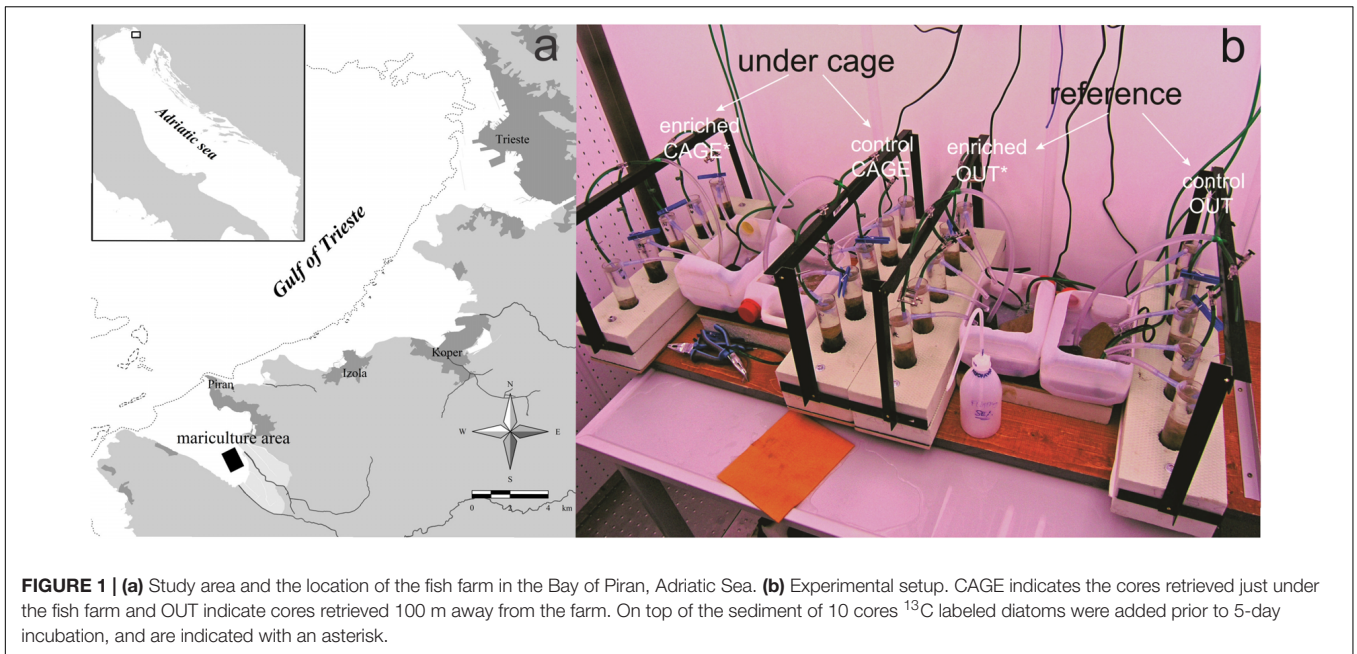
The carbon content and  $\delta^{13}\text{C}$  composition were measured using an isotope ratio mass spectrometer (type Europa Integra) at the UC Davis Stable Isotope Facility (University of California, United States). The carbon isotope ratio was calculated from the measured  $\delta^{13}\text{C}$  values as

$$R_{\text{sample}} = (\delta^{13}\text{C}/1000 + 1) \times R_{\text{VPDB}}$$

where  $R_{\text{VPDB}} = 0.0112372$  as  $\delta^{13}\text{C}$  is expressed relative to Vienna Pee Dee Belemnite standard (VPDB).

Incorporation of  $^{13}\text{C}$  is reflected as the specific uptake, i.e.,  $\Delta\delta^{13}\text{C}$  (Middelburg et al., 2000).

$$\Delta\delta^{13}\text{C} = \delta^{13}\text{C}_{\text{enriched}} - \delta^{13}\text{C}_{\text{control}}$$



Since we had five independent replicates of the control, and five independent replicates of the treatment, we calculated the specific uptake using the average of control to pair with each of the five enriched samples. Next, the fractional abundance of heavy isotope ( $F$ ) was calculated:

$$F = {}^{13}\text{C}/({}^{13}\text{C} + {}^{12}\text{C}) = R/(R + 1)$$

Total uptake ( $I$ ) of the nematode or harpacticoid community was calculated by subtracting the fraction  ${}^{13}\text{C}$  of the  $F_{\text{sample}}$  and the  $F_{\text{control}}$ , multiplied by the organic carbon of nematodes or copepods of the enriched samples (Middelburg et al., 2000):

$$I = (F_{\text{enriched}} - F_{\text{control}}) \times C_{\text{organic}}$$

The data were statistically analyzed in R software (R Development Core Team, 2014). Prior to the analysis, all data were checked for normality using the Anderson-Darling test (Sokal and Rohlf, 1995; Dytham, 2003; Zuur et al., 2010). The differences among natural  ${}^{13}\text{C}$  signals were tested with a Student  $t$ -test. The differences in uptake ( $\Delta\delta^{13}\text{C}$  and  $I$ ) by nematodes and harpacticoids in CAGE and OUT samples were tested with 2-way ANOVA and Tukey HSD on non-transformed ( $\Delta\delta^{13}\text{C}$ ) and on square root transformed ( $I$ ) data. The sample marked with \* on **Figure 4** was omitted from statistical testing, because it shows much higher values than the rest of samples. This sample is a composite of three replicates (see section “Natural  ${}^{13}\text{C}$  Values of the Sediment, Meiofauna and Fish Feed”).

The carbon content of analyzed nematode samples was  $15.1 \pm 5.3 \mu\text{gC}/10 \text{ cm}^2$  in OUT\*, and  $45.8 \pm 15.9 \mu\text{gC}/10 \text{ cm}^2$  in CAGE\*. The carbon content of analyzed harpacticoid samples varied from  $26.4 \pm 16.3 \mu\text{gC}/10 \text{ cm}^2$  to  $9.9 \pm 9.0 \mu\text{gC}/10 \text{ cm}^2$  at OUT\* and CAGE\*, respectively. The number of nematodes in tin cupsules was 100 at CAGE\* and 150 at OUT\*. The number of copepods in a sample varied from  $144 \pm 47/10 \text{ cm}^2$  at OUT\* to  $22 \pm 12/10 \text{ cm}^2$  at CAGE\*. As the whole community from experimental cores was not counted,  $C_{\text{organic}}$  was estimated from the number of animals analyzed for isotopes (and their biomass) and the factor to reach the average abundance of nematodes and harpacticoids sampled at same locations in the previous year (see Grego et al., 2009, CAGE = 0 m, OUT = 100 m NE, two seasons).

## Bacterial Community Analysis

In addition to cores used for the experiment, six cores (3.6 cm inner diameter) were retrieved, three at CAGE and three at the OUT site (referred to as ‘field’). From the sediment surface and aliquote was scratched for denaturing gradient gel electrophoresis (DGGE). At the end of the experiment, the aliquots of sediment were taken on top of three CAGE\* and three OUT\* cores (referred as ‘experiment’). After the sediment was sliced, one sample was taken on top of 1–5 cm layer of CAGE\* sediment. Prior to the DGGE analysis, bacterial DNA was prepared using the FastDNA<sup>®</sup> SPIN Kit for Soil (MP Biomedicals). From each DNA extract an internal 194 bp fragment of the V3 region of the 16S rRNA gene (Yu and Morrison, 2004) was amplified using the primer set

357f and 518r (Van Hoorde et al., 2008) with a GC-clamp (5'-GCCCGCCGCGCGCGCGGGCGGGGCGGGGCGGGGCGGGGCGGGG-3') (Temmerman et al., 2003) coupled to the forward primer. On the PCR mixtures (Temmerman et al., 2003), a touchdown PCR (Van Hoorde et al., 2008) with 10 cycles of decreasing annealing temperature ( $0.5^\circ\text{C}$  cycle<sup>-1</sup> decrement, from 61 to  $56^\circ\text{C}$ ), followed by 25 cycles of regular PCR was performed with a Bio-Rad DNA thermal cycler. DGGE analysis using a 35–70% gradient and staining of the gel was performed as described by Van Hoorde et al. (2008). Digitized DGGE gels were normalized and analyzed in BioNumerics software (version 4.61, Applied Maths, Sint-Martens-Latem, Belgium). Calculation of the Dice correlation coefficient and application of UPGMA clustering method (Unweighted Pair Group Method with Arithmetic) resulted in a dendrogram visualizing the similarity between the banding patterns of the sediment samples.

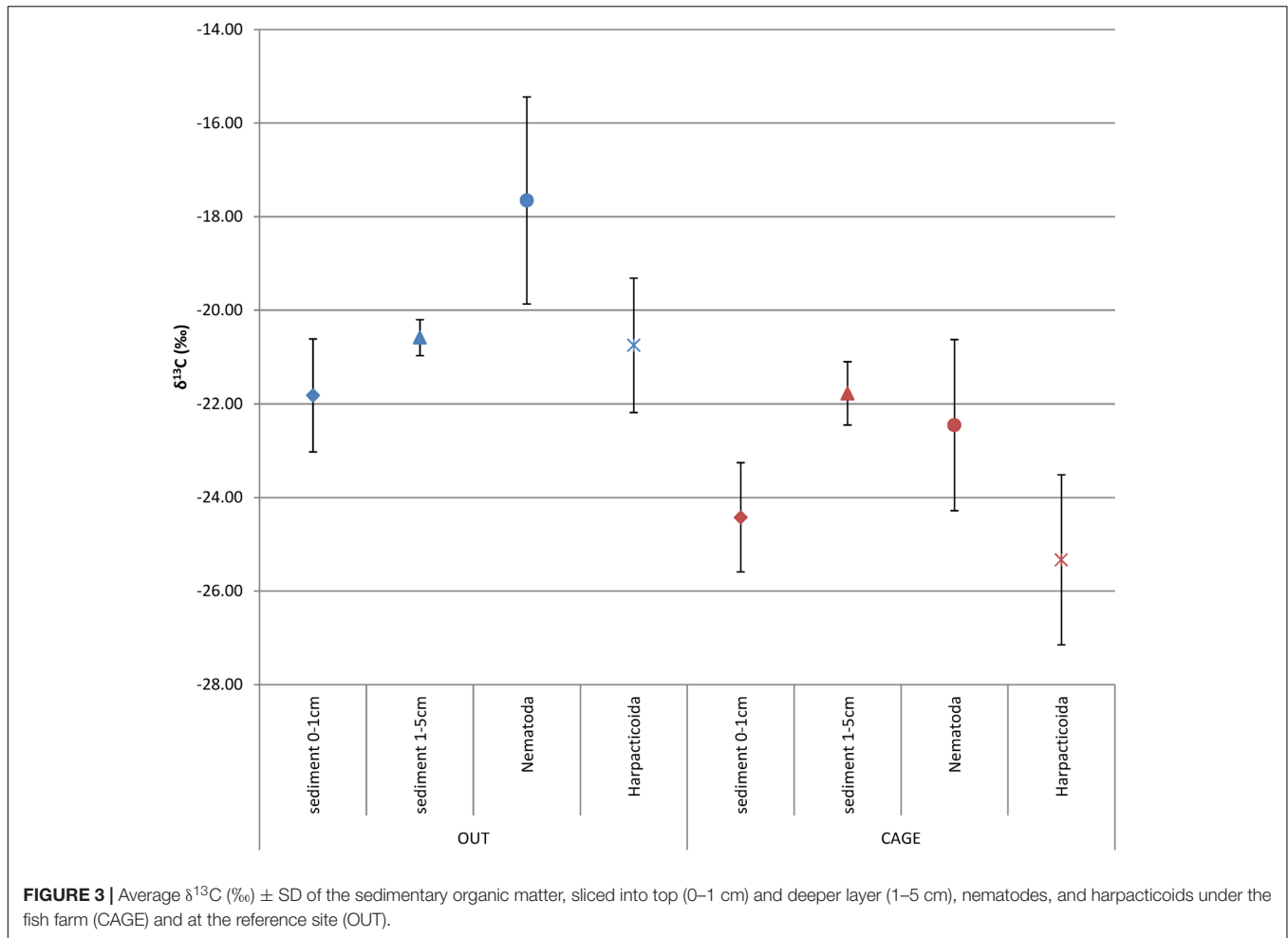
## Pore Water Analysis

In addition to meiofauna cores, the plexiglass cores with an inner diameter 6 cm were collected for pore waters by means of scuba diving, three at the CAGE and three at the OUT site. The overlying water was also analyzed for nutrients. The sediment cores were sliced according to a standard routine at 0.5 cm interval in the top layer, where most changes are expected, and 1–5 cm layer. Sediment slices from three replicates were pooled to achieve 10–15 ml of pore water. Sediment samples were packed into 50 ml polyethylene centrifuge tubes and centrifuged at 11,500 rpm for 20 min. After centrifugation,  $P_{\text{tot}}$  and  $N_{\text{tot}}$  were analyzed in unfiltered samples by wet oxidation described in Grasshoff et al. (1999). Under  $\text{N}_2$  atmosphere, the interstitial waters were filtered through 0.45  $\mu\text{m}$  pore size (Millipore HA) membrane filters and analyzed for solutes. The pH measurements were carried out and corrected for *in situ* temperature ( $13.4^\circ\text{C}$ ) and salinity (37.7) (Millero, 1995). Interstitial water alkalinity was measured by Gran titration (Edmond, 1970). Dissolved inorganic carbon (DIC) was calculated from alkalinity and pH. Nitrite ( $\text{NO}_2^-$ ), nitrate ( $\text{NO}_3^-$ ), ammonium ( $\text{NH}_4^+$ ), phosphate ( $\text{PO}_4^{3-}$ ), and silicate ( $\text{SiO}_4^{4-}$ ) were measured photometrically (Grasshoff et al., 1983), and sulfate was measured turbidimetrically (Tabatabai, 1974) with UV/VIS spectrometer PerkinElmer, Lambda 14.

## RESULTS

### Natural ${}^{13}\text{C}$ Values of the Sediment, Meiofauna and Fish Feed

The sedimentary OM from the top sediment layer (0–1 cm) was significantly more depleted in  $\delta^{13}\text{C}_{\text{org}}$  under the fish farm compared to reference site (CAGE samples  $-24.42 \pm 1.17\text{‰}$  vs. OUT samples  $-21.82 \pm 1.21\text{‰}$ ) (**Figure 3** and **Table 1**). The OM in the deeper sediment layer (1–5 cm) was less depleted in  $\delta^{13}\text{C}$  than the top sediment layer (0–1 cm), with values of  $-21.78 \pm 0.67\text{‰}$  and  $-20.59 \pm 0.38\text{‰}$  in the CAGE and OUT samples, respectively. A significant difference in sedimentary OM  $\delta^{13}\text{C}$  values was found between the top and the underlying



**FIGURE 3 |** Average  $\delta^{13}\text{C}$  (‰)  $\pm$  SD of the sedimentary organic matter, sliced into top (0–1 cm) and deeper layer (1–5 cm), nematodes, and harpacticoids under the fish farm (CAGE) and at the reference site (OUT).

**TABLE 1 |** Student *t*-test (2 tailed) comparing  $\delta^{13}\text{C}$  differences between pairs from **Figure 3**.

Pair	<i>p</i> -value
Sedimentary OM (0–1 cm) OUT vs. Sedimentary OM (0–1 cm) CAGE	<b>0.021</b>
Sedimentary OM (1–5 cm) OUT vs. Sedimentary OM (1–5 cm) CAGE	0.057
Nematoda OUT vs. Nematoda CAGE	<b>0.006</b>
Harpacticoida OUT vs. Harpacticoida CAGE	<b>0.004</b>
Nematoda OUT vs. Harpacticoida OUT	<b>0.030</b>
Sedimentary OM (0–1 cm) OUT vs. Sedimentary OM (1–5 cm) OUT	0.155
Sedimentary OM (0–1 cm) OUT vs. Nematoda OUT	<b>0.010</b>
Sedimentary OM (0–1 cm) OUT vs. Harpacticoida OUT	0.437
Nematoda CAGE vs. Harpacticoida CAGE	0.051*
Sedimentary OM (0–1 cm) CAGE vs. Sedimentary OM (1–5 cm) CAGE	<b>0.018</b>
Sedimentary OM (0–1 cm) CAGE vs. Nematoda CAGE	0.092
Sedimentary OM (0–1 cm) CAGE vs. Harpacticoida CAGE	0.263

Significant differences are bolded and an almost significant difference is presented by \*.

sediment layers in the CAGE samples (**Table 1**), but not in the OUT samples.

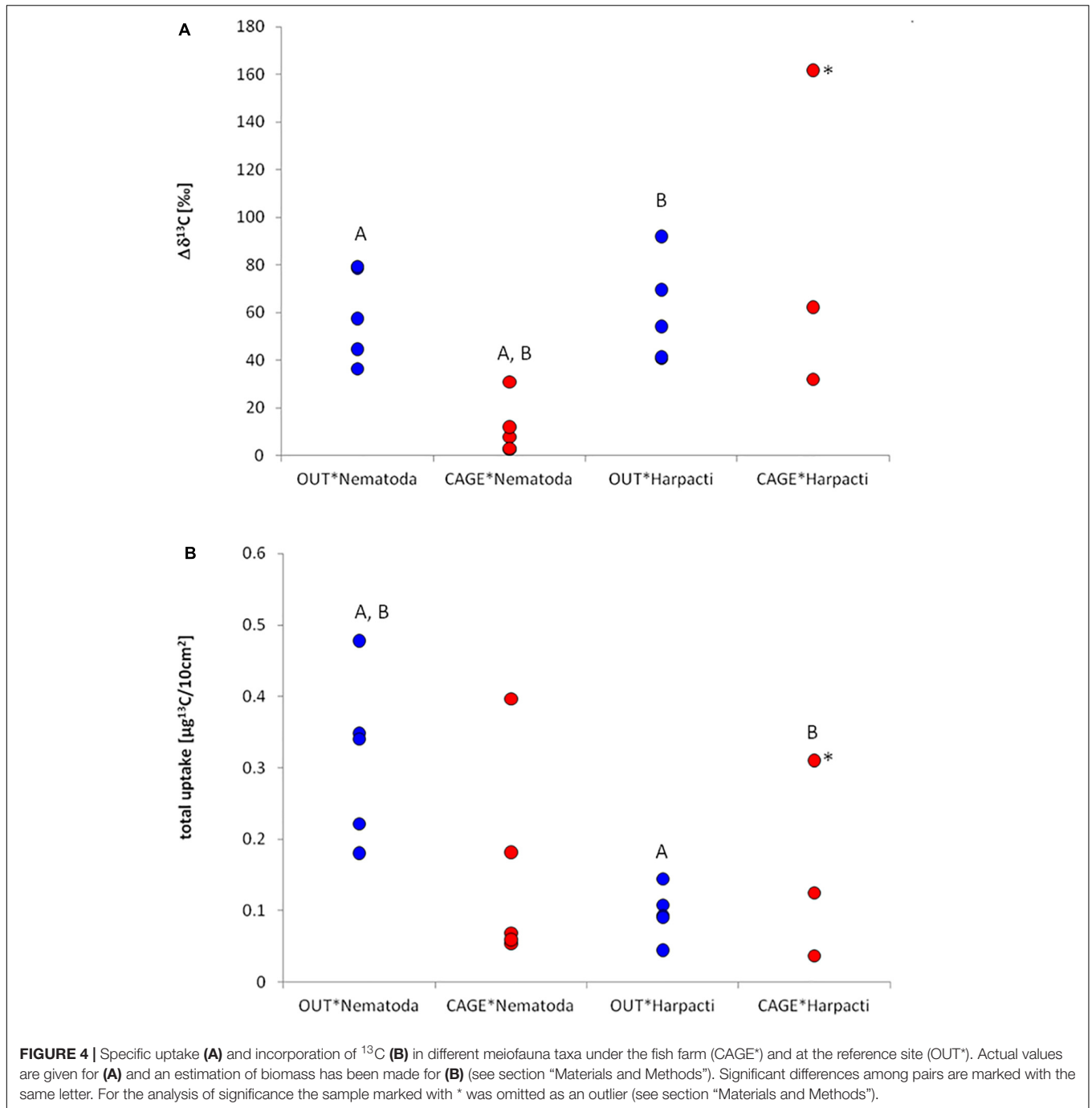
The meiofauna assemblage was represented by Nematoda, Harpacticoida, Polychaeta, Kinorhyncha, Turbellaria, Bivalvia,

Gastropoda, Ostracoda, Amphipoda, Acarina, Isopoda, Cumacea, Mysidacea, Decapoda, and Ophiuroidea, listed in order of decreasing abundance (Grego et al., 2009). For this study we focused on the two most abundant meiofauna taxa Nematoda and Harpacticoida.

The natural isotope values of nematodes were significantly depleted in CAGE samples ( $-22.46 \pm 1.83\text{‰}$ ) compared to OUT samples ( $-17.65 \pm 2.21\text{‰}$ ) (**Figure 3** and **Table 1**). Likewise, the harpacticoids  $\delta^{13}\text{C}$  were significantly depleted in CAGE ( $-25.33 \pm 1.82\text{‰}$ ) compared to the OUT samples ( $-20.75 \pm 1.43\text{‰}$ ) (**Figure 3** and **Table 1**). The comparison between nematodes and harpacticoids showed that harpacticoids were always more depleted in  $\delta^{13}\text{C}$ , independent of the site (**Table 2**). This difference between nematodes and harpacticoids was significant at the OUT site and marginally not significant ( $p = 0.051$ ) at the CAGE site (**Figure 3** and **Table 1**).

The sedimentary OM was depleted by 2.6‰ at CAGE samples compared to the OUT samples, while the fauna showed more intense depletion in CAGE samples, i.e., by 4.8‰ nematodes and by 4.6‰ harpacticoids, compared to OUT samples (**Table 2**).

Fish pellets that were used to feed the fish had  $\delta^{13}\text{C}$  value of  $-22.2 \pm 0.1\text{‰}$ .



### $^{13}\text{C}$ -Labeled Diatom Addition Experiment

After the incubation with  $^{13}\text{C}$  labeled diatoms, specific uptake ( $\Delta\delta^{13}\text{C}$ ) was measured in all nematode and harpacticoid samples (Figure 4A) in the range from 2.6 to 161.8‰. The two-way ANOVA resulted in significant ( $p = 0.001$ ) influence of location (CAGE\* and OUT\*) but not taxa (nematodes, harpacticoids) on the specific uptake. The nematode specific uptake was significantly higher in OUT\* samples comparing to CAGE\* samples (Tukey HSD,  $p = 0.006$ ). The harpacticoid specific

uptake was not different between OUT\* and CAGE\* samples. In OUT\* samples the specific uptake among harpacticoids and nematodes was also very similar, and also in CAGE\* samples the specific uptake between taxa was not different. The nematodes from CAGE\* samples and the harpacticoids from OUT\* samples showed significant difference in specific uptake (Tukey HSD,  $p = 0.005$ ).

The estimated (see section “Materials and Methods”) nematode abundance was  $2859 \pm 836/10 \text{ cm}^2$  at OUT\* and  $2809 \pm 649/10 \text{ cm}^2$  at CAGE\*. The estimated

harpacticoid abundance was  $665 \pm 119/10 \text{ cm}^2$  at OUT\* and  $208 \pm 175/10 \text{ cm}^2$  at CAGE\*. The nematode biomass was  $504 \pm 209 \mu\text{gC}_{\text{org}}/10 \text{ cm}^2$  at OUT\* and  $1,399 \pm 433 \mu\text{gC}_{\text{org}}/10 \text{ cm}^2$  at CAGE\* site. The harpacticoid biomass was  $149 \pm 41 \mu\text{gC}_{\text{org}}/10 \text{ cm}^2$  at OUT and  $114 \pm 57 \mu\text{gC}_{\text{org}}/10 \text{ cm}^2$  at CAGE\*. The consumption of nematodes and harpacticoids was in the range from 0.04 to  $0.49 \mu\text{g}^{13}\text{C}/10 \text{ cm}^2$ . The two-way ANOVA revealed a significant ( $p = 0.009$ ) influence of taxa (nematodes and harpacticoids), but not location (CAGE\* and OUT\*) on the incorporation of  $^{13}\text{C}$ . The  $^{13}\text{C}$  isotope incorporation (I) into nematode biomass from sampling unit OUT\* and CAGE\* samples did not result in significant differences (Figure 4B). Similarly, the  $^{13}\text{C}$  isotope incorporation in harpacticoid biomass was not different at the OUT\* and CAGE\* sites. The incorporation of  $^{13}\text{C}$  was larger for nematodes than for harpacticoids at the OUT\* site (Tukey HSD,  $p = 0.029$ ). There was also a significant difference in incorporation between nematodes from OUT\* and harpacticoids from CAGE\* ( $p = 0.038$ ). From the carbon biomass of labeled diatoms (resulting in 8.4%  $^{13}\text{C}$ ), we added approximately 131  $\mu\text{g}^{13}\text{C}$  into each experimental core. From the average incorporation in nematodes and harpacticoids from the OUT\* cores we estimated they incorporated about 0.2% of added  $^{13}\text{C}$ , while the nematodes and harpacticoids from CAGE\* incorporated 0.1% of added  $^{13}\text{C}$  to the experimental unit.

Adding  $\delta^{13}\text{C}$  enriched diatoms to the sediment resulted into a significant increase of the isotope values of the sedimentary OM in the top sediment layer from  $-21.82 \pm 1.21\text{‰}$  to  $43.34 \pm 22.79\text{‰}$  for OUT\* ( $p = 0.001$ ) and from  $-24.42 \pm 1.17\text{‰}$  to  $18.10 \pm 24.05\text{‰}$  for CAGE\* samples ( $p = 0.023$ ), as measured at the end of the experiment (when some diatoms were already eaten by meiofauna). However, the labeled diatoms did not reach the deeper sediment layers (1–5 cm), as documented by similar values of the sedimentary OM ( $-19.77 \pm 0.73\text{‰}$  for OUT\* and  $-21.90 \pm 0.71\text{‰}$  for CAGE\*) to non-enriched samples.

## Bacterial Communities in the Field Samples and in the Experimental Samples

The DGGE gel showed a large number of bands in the sediments of the CAGE and OUT samples (Figure 5a). The bacterial community in experimental cores is very similar to the ones in field cores. The dark bands on the DGGE gel, especially in experimental units of CAGE samples, may indicate enhanced bacterial growth during the experiment. The dendrograms revealed at least 80% similarity between the triplicate subsamples of each site. The CAGE and OUT samples were different from each other before and after the experiment, and clustered at 70% (field, Figure 5b) and 74% (experiment, Figure 5c) similarity levels. There is a difference between the top layers and the single lower layer (one replicate of 1–5 cm) of the CAGE samples. The lower layer of the CAGE sample groups together with the upper layers of OUT samples (Figure 5b), indicating the fish farm impact is more pronounced in the top sediment layer.

## Pore Waters of the Field Samples

The pore water analysis showed highly elevated  $A_{\text{tot}}$ , DIC,  $\text{NH}_4^{4+}$ , and  $\text{PO}_4^{3-}$  values (Table 3) under the fish cages (CAGE) compared to the control (OUT). The top sediment layer (0–1 cm) of CAGE samples showed the highest values of  $\text{NH}_4^{4+}$ ,  $\text{PO}_4^{3-}$ , and  $\text{SiO}_4^{4-}$  (Figure 6). Furthermore, in CAGE samples almost 100% of the total P is represented by  $\text{PO}_4^{3-}$ . Among the three dissolved N forms, the  $\text{NH}_4^{4+}$  was by far the dominant at both sites, but especially at the CAGE site. The  $\text{NO}_3^{2-}$  concentration was higher in the OUT samples. The silicon values in pore waters are elevated in the CAGE samples, compared to the control. The pH values, are uniform in the CAGE and OUT samples.

## DISCUSSION

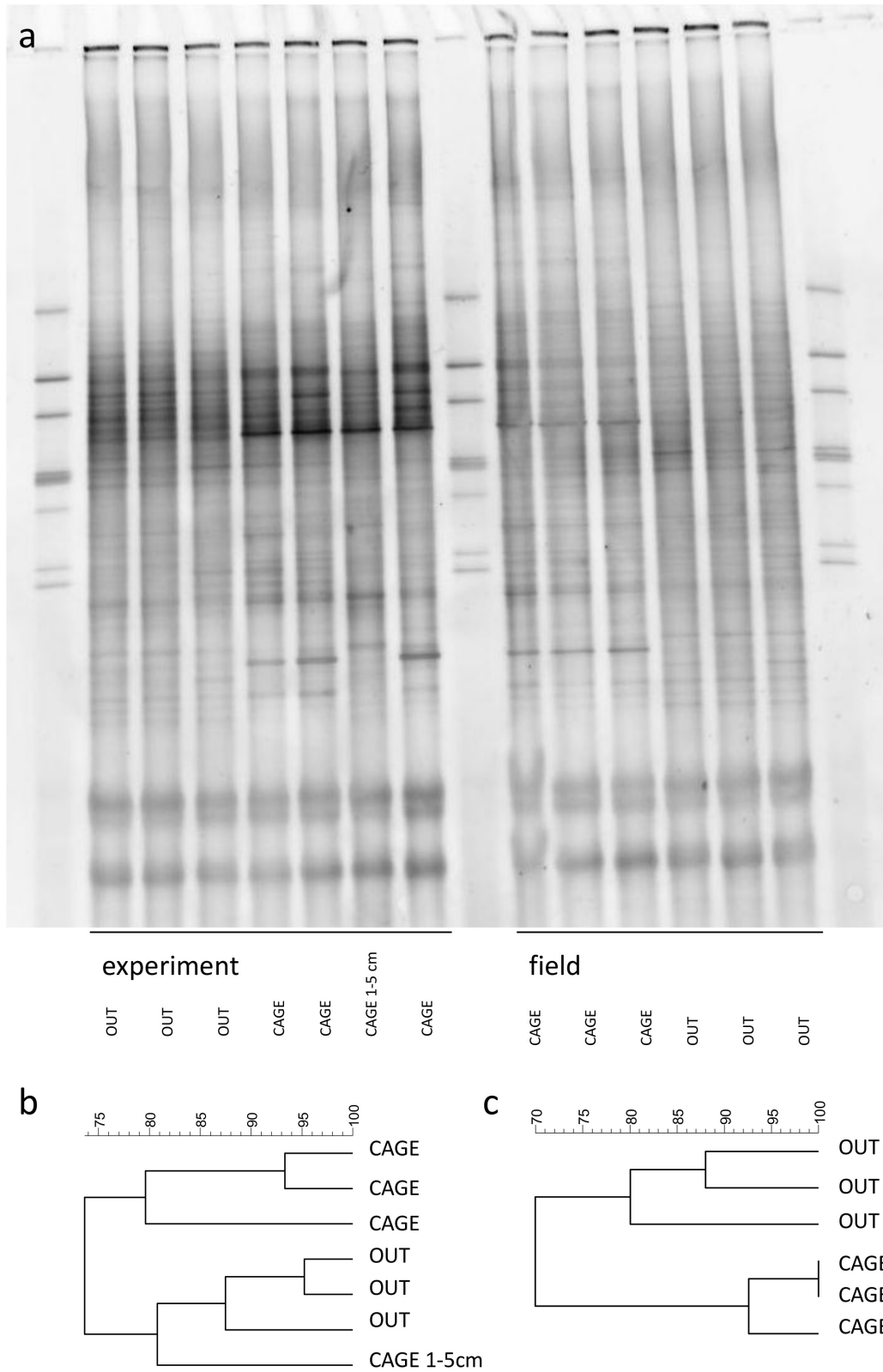
### Meiofauna $\delta^{13}\text{C}$ Values and Uptake of $^{13}\text{C}$ -Labeled Diatoms in Relation to Fish Farming

The fish farm originating organic matter enters the meiofauna food web, as reflected in depleted  $\delta^{13}\text{C}$  of nematodes and harpacticoids at the farm site compared to the reference site (by 4.8 and 4.6‰, respectively). Similar shifts to more depleted  $\delta^{13}\text{C}$  values (for 2‰) were also observed for nematodes and several other invertebrates (polychaetes and anemones) under salmon farming cages in fjordic inlets in Ireland (Callier et al., 2013). Izquierdo-Gomez et al. (2015) also reported on more depleted  $\delta^{13}\text{C}$  values of wild penaeid prawns under fish farms compared to the reference site.

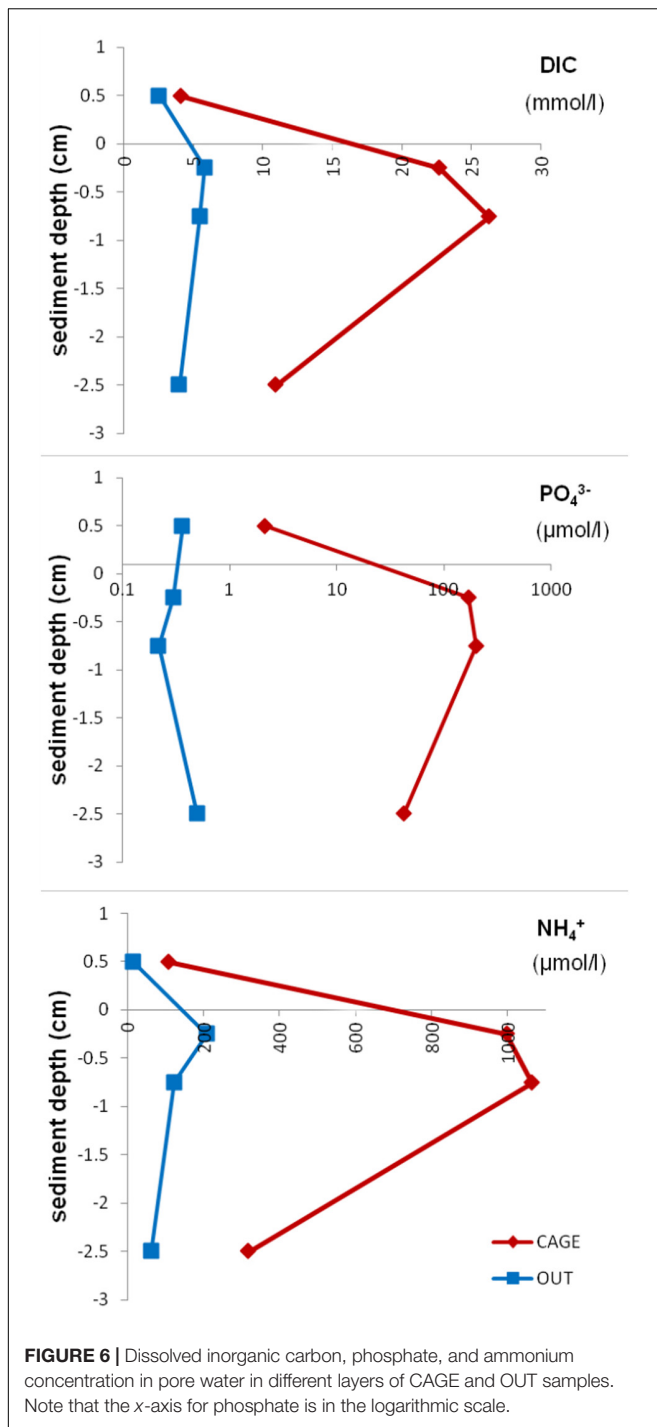
The organic load caused changes in the pathways of material flow in the food web. The nematodes and harpacticoids consume different food sources under the fish farm compared to reference site. Under the fish farm, an important food source for the nematode assemblage was the sedimentary OM, as reflected from  $\delta^{13}\text{C}_{\text{nematoda}}$  that was 2‰ heavier with respect to  $\delta^{13}\text{C}_{\text{sedimentaryOM}}$ . As indicated by  $\delta^{13}\text{C}$  of the fish feed ( $-22.1\text{‰}$ ), it was not directly used by nematodes ( $\delta^{13}\text{C}$ ;  $-22.5\text{‰}$ ) as their carbon isotope signatures were similar. The nematodes from the reference site, on the other hand, displayed 4.2‰ heavier  $\delta^{13}\text{C}$  relative to sedimentary OM. As reported by Nadon and Himmelman (2006), there is a general trend in several taxa of benthic consumers to become enriched by 4.1‰ relative to  $\delta^{13}\text{C}$  particulate OM. Since in the Bay of Piran, two thirds of the sedimentary OM originates from the water column OM and only one third originates from benthic producers (Faganeli et al., 1988), the nematodes from the reference site ( $-17.7\text{‰}$ ) likely reflect a diet comprising a mixture of particulate organic matter (POM) and benthic producers. The  $\delta^{13}\text{C}$  of microphytobenthos was reported to be  $-15.4\text{‰}$  (Oakes et al., 2012),  $-16.3\text{‰}$  (Christiansen et al., 2017), and  $-19.3\text{‰}$  (Herman et al., 2000).

Harpacticoids, on the other hand, consume predominantly the sedimentary OM at the reference site, as shown from their 1.1‰ higher  $\delta^{13}\text{C}$  relative to sedimentary OM, a typical enrichment for a consecutive trophic level (Fry, 2006; Glibert et al., 2019). The harpacticoids from the cage site are influenced by a different and  $^{13}\text{C}$ -depleted carbon source, as shown in their  $\delta^{13}\text{C}$  signal





**FIGURE 5 |** The DGGE gel of the sediment bacterial community **(a)**. Samples were collected on top of the sediment prior to (field) and after 5 days incubation in the thermostatic chamber (experiment). An additional sample of lower sediment layer (1–5 cm) was collected at the end of the experiment. The dendrograms represent the similarity of experimental **(b)** and field **(c)** bacterial community (Dice (Tol 1.0%–1.0%)(H > 0.0% S > 0.0%)[0.0%–100.0%]).



( $-25.3\text{‰}$ ), which was for  $0.9\text{‰}$  depleted relative to sedimentary OM under cages. The fish feed used ( $\delta^{13}\text{C}$ ;  $-22.1\text{‰}$ ) could also not be linked directly to harpacticoid diet as it was distinctly heavy-isotope enriched compared to harpacticoids. The potential food sources for harpacticoids could be non-diatom microphytobenthic groups, (range for combined cyanobacteria and green algae  $-24.8\text{‰}$  to  $-23.8\text{‰}$ ) on which they could be selectively feeding, thus resulting in their depleted  $\delta^{13}\text{C}$

(Evrard et al., 2012). Furthermore, a lower concentration of sulfate in cage sediment compared to the control sediment (Table 3) may indicate a greater abundance or activity of sulfate-reducing bacteria (SRB) under cage (Leloup et al., 2009). The SRB exhibit very low  $\delta^{13}\text{C}$  signals ( $-55\text{‰}$ ) (Londry and Des Marais, 2003) and their densities were found to be positively correlated to organic enrichment associated to fish farming (Kondo et al., 2012). Moreover, the interface between sulfate-reducing sediments and the oxygenated water column may host filamentous bacteria *Beggiatoa* mats, whose biomass is also light in  $^{13}\text{C}$  ( $-27.9\text{‰}$ ) (Sassen et al., 1993). In fact, Gee (2005) reported on the association of filamentous bacteria with specimens of harpacticoid *Bulbamphiascus incus* under a Scottish salmon farm. This genus dominated the harpacticoid community from the studied fish farm sediments (Grego, unpublished).

The harpacticoids under fish cages had the most depleted signal from all analyzed items. For that reason even simple, two end-member isotope mixing models did not work for harpacticoids (Stable Isotope Mixing Models for Estimating Source Proportions by the United States Environmental Protection Agency-IsoError was used). Additionally, the mixing models were not reliable due to low number of replicates given the (high) variability of  $\delta^{13}\text{C}_{\text{nematoda}}$  and  $\delta^{13}\text{C}_{\text{harpacticoida}}$ . But most of all, it would be hard to achieve correct information on the contribution of different sources associated to fish farming on harpacticoid or nematode diet without the addition of fish feces  $\delta^{13}\text{C}$  in the equation, as for example in Ye et al. (1991) and Yokoyama et al. (2010).

The nematode assemblage in reference sediments of northern Adriatic Sea contributes more to carbon flux from benthic primary producers to higher trophic levels compared to harpacticoid assemblage. This can be stated by the fact that the nematode  $\delta^{13}\text{C}$  signal reflects their reliance on diatoms as food source and it was further confirmed by the outcome of  $^{13}\text{C}$ -labeled diatom addition experiment where nematode assemblage exhibited a greater grazing pressure on diatoms compared to harpacticoid assemblage. The nematode specific uptake was similar to harpacticoid specific uptake, but because of higher biomass of nematode assemblage they incorporated more diatom  $^{13}\text{C}$  than harpacticoid assemblage (Figure 4B). The high specific uptake of harpacticoid assemblage (Figure 4A) does not explain their  $\delta^{13}\text{C}$ , that clearly indicates sedimentary OM as their main food source. Similarly, the harpacticoid assemblage from cage sediments at the reference site was feeding on  $^{13}\text{C}$  labeled diatoms, whereas according to  $\delta^{13}\text{C}$  signal they rely on a much depleted food source, potentially bacteria. That could suggest that the harpacticoid food sources (in reference and farm site) range in large  $\delta^{13}\text{C}$  interval, from bacteria to diatoms. It is possible that under fish cages they consume the available food sources, which can also explain their high variation in  $\delta^{13}\text{C}$  (Figure 3). A minor specific uptake of  $^{13}\text{C}$ -labeled diatoms was shown for nematodes at the farm site (Figure 4A), indicating that the diatoms are not their food source under cages, which is in accordance with their  $\delta^{13}\text{C}$  signal, and reflects consumption of sedimentary OM and less enriched food sources. However, given the biomass of nematodes is approximately three times higher under cages, the incorporation of diatom  $^{13}\text{C}$  at cage site

**TABLE 2** |  $\delta^{13}\text{C}$  ( $\pm$ SD) of sedimentary organic matter (SedOM) from different layers, of water column particulate organic matter (POM), of Nematoda and Harpacticoida, and the difference (bold) between OUT vs. CAGE and SedOM vs. fauna.

	OUT	CAGE	Difference OUT-CAGE
SedOM 0–1 cm	–21.8 (1.2)	–24.4 (1.2)	<b>–2.6</b>
SedOM 1–5 cm	–20.6 (0.4)	–21.8 (0.7)	<b>–1.2</b>
Nematoda	–17.7 (2.2)	–22.5 (1.8)	<b>–4.8</b>
Harpacticoida	–20.7 (1.4)	–25.3 (1.8)	<b>–4.6</b>
Fish feed		–22.2 (0.1)	
SedOM*	–21.6 (0.1)		
POM*	–22.6 (1.0)		
Difference Nematoda-SedOM	<b>4.2</b>	<b>2.0</b>	
Difference Harpacticoida-SedOM	<b>1.1</b>	<b>–0.9</b>	

\*Values are from 2002 and 2003 (BIOFAQ: FP5).

was still similar as at the compared to reference site. Due to higher relative biomass of nematodes in meiofauna community, they generally consume more diatom  $^{13}\text{C}$  than harpacticoid copepods (two-way ANOVA). Overall, the meiofauna organisms incorporated approximately 0.2% of the total label added at reference experimental units and 0.1% at cage experimental units. This is in the same order of magnitude (or an order more) as the relative consumption of macroalgae by meiofauna of shallow Antarctic sediments (Braeckman et al., 2019), and about an order of magnitude less than the relative consumption of microphytobenthos by intertidal macrofauna (Herman et al., 2000) in similar time frames (few days). A generally higher specific uptake of diatoms was observed in reference samples (2-way ANOVA) possibly because they are not oversaturated with organic load, as was the case in the farm samples.

## Processes in the Sediment in Relation to Fish Farming

A significant amount of organic carbon from the fish farm is reaching the bottom as illustrated by the  $\delta^{13}\text{C}$  of sedimentary OM (Figure 3), which was 2.6‰ more depleted under the fish farm compared to the reference site (–24.4‰ vs. –21.8‰). Sedimentary OM  $\delta^{13}\text{C}$  of –21.8‰ is a well established value for surface sediments in the Bay of Piran (Faganeli et al., 1991; Ogrinc et al., 2005; Covelli et al., 2006). Using isotope

signatures, it was shown that two thirds of sedimentary OM originate from the water column POM and one third originates from benthic producers (Faganeli et al., 1988). Under fish cages, the sedimentary OM  $\delta^{13}\text{C}$  is also determined by fish feed and fish feces. The fish pellets used at our study site had the carbon isotope value of –22.2‰, similar to the natural POM of the area (–22.6‰, as measured 3 years prior to this study; Table 2), while sedimentary OM under cages was more depleted (–24.4‰). The sedimentary OM appears to be influenced by fish feces rather than fish feed, as it was reported that red sea bream excrete feces that is depleted in  $\delta^{13}\text{C}$  (by 3.5 to 4.4‰) relative to fish feed (Yokoyama et al., 2006). Likewise, Ye et al. (1991) measured  $\delta^{13}\text{C}$  of aquaculture-derived organic carbon that was depleted (–24.1‰) compared to fish feed (–21.5‰). The direct loss of feed is generally considered to be low because large assemblages of wild fish are attracted to farm installations (Dempster et al., 2002, 2010; Fernandez-Jover et al., 2007), and the fouling communities of benthic invertebrates consume the feed leftovers, as was proven before for this study area (Lojen et al., 2003, 2005; Dolenc et al., 2007). Likewise in our study, the sedimentary OM was depleted under fish cages compared to the reference, in Tasmania (Ye et al., 1991). Mayor et al. (2017) reported that phospholipid fatty acid (PLFA) extracted from surficial sediments in five Scottish fish farms also showed a trend to more depleted  $\delta^{13}\text{C}$  values with increasing farm size and production. Contrary, Yokoyama et al. (2006), Holmer et al. (2007), and Callier et al. (2013) did not find a clear trend in  $\delta^{13}\text{C}$  of sedimentary OM in relation to fish farming in Japan, Spain, Greece, Italy, and Ireland. The impact of the cage deposits on the  $\delta^{13}\text{C}$  of sedimentary OM also depends on the  $\delta^{13}\text{C}$  of feed relative to natural POM.

The sedimentary OM under the fish cages is more driven by bacterial activity in contrast to the reference site. Higher DIC,  $\text{PO}_4^{3-}$ , and  $\text{NH}_4^+$  concentrations in pore waters in cage sediments (Table 3 and Figure 6) point to elevated microbial degradation of organic matter compounds. Asami et al. (2005), who studied coastal marine sediments, found a significantly lower sulfate concentration beneath aquacultures. They hypothesized that sulfate-reducing and sulfur-oxidizing bacteria thrived in such an environment, which they confirmed by quantitative competitive PCR. Likewise, a sulfate reduction is observed in our cage sediments by lower  $\text{SO}_4^{2-}$  values compared to reference

**TABLE 3** | Supernatant and pore water analysis of CAGE and OUT samples.

	Layer (cm)	A tot (mM/l)	DIC (mM/L)	$\text{PO}_4^{3-}$ ( $\mu\text{M/L}$ )	P tot ( $\mu\text{M/L}$ )	$\text{SiO}_4^{4-}$ ( $\mu\text{M/L}$ )	$\text{NO}_2^-$ ( $\mu\text{M/L}$ )	$\text{NO}_3^-$ ( $\mu\text{M/L}$ )	$\text{NH}_4^+$ ( $\mu\text{M/L}$ )	N tot ( $\mu\text{M/L}$ )	$\text{SO}_4^{2-}$ ( $\mu\text{M/L}$ )
CAGE	Sup.	4.1	4.1	2.2	2.9	77.9	0.1	2.4	109.0	312.9	27.0
	0–0.5	22.8	22.7	169.3	187.7	276.1	1.6	<1,30	999.1	2866.4	20.7
	0.5–1	26.7	26.3	200.9	219.0	295.9	1.4	<1,22	1063.1	3261.3	19.5
	1–4	11.3	10.9	42.0	43.0	136.4	0.2	0.8	318.5	906.6	20.1
OUT	Sup.	2.7	2.6	0.4	1.5	30.1	0.1	8.6	14.9	166.4	30.4
	0–0.5	5.9	5.8	0.3	1.7	87.5	0.6	12.6	209.2	584.0	29.8
	0.5–1	5.4	5.5	0.2	1.6	81.9	0.3	9.9	122.6	280.6	27.8
	1–4	4.0	4.0	0.5	2.3	116.6	0.3	6.1	62.3	343.4	27.3

site (Table 3) which may indicate the presence of sulfate-reducing bacteria (SRB). The SRB are  $\delta^{13}\text{C}$  depleted, and in marine sediments are expected to have values as low as  $-55\text{‰}$  (Londry and Des Marais, 2003), and may have contributed to lower values of sediment and meiofauna at the farm site. In fact, a different bacterial community was present in the cage sediment compared to the reference sediment (Figure 5). Hollander and Smith (2001) found that lower  $\delta^{13}\text{C}$  values generally point to microbial mediated carbon cycling and processes associated with the intensification of seasonal and long-term eutrophication.

The changes in the sediment under fish cages are reflected mostly in the upper sediment layers of muddy sediments. The top centimeter of sediment of the fish farm sediment had altered  $\delta^{13}\text{C}$  of sedimentary OM compared to the reference site, while the deeper layer (1–5 cm)  $\delta^{13}\text{C}$  was not distinguishable between the cage and reference sites (Figure 3). Also, the bacterial community from deeper sediment layers of fish farm sediment was more similar to the community of the reference sediments (Figure 5).

## CONCLUSION

Fish farm-originating organic carbon reaches the bottom, as illustrated by the depleted  $\delta^{13}\text{C}$  of sedimentary OM in farm compared to the reference site. The sedimentary OM appears to be influenced by fish feces rather than fish feed, and seems to be more affected by bacterial activity in contrast to the reference site. Depleted  $\delta^{13}\text{C}$  of nematodes and harpacticoids under fish cages indicate that fish farm-derived organic matter enters the meiofauna food web. However, the fish feed originated organic matter could not be directly traced in  $\delta^{13}\text{C}$  of nematodes and harpacticoids. The nematodes and harpacticoids consume different food sources under the fish farm compared to the reference site. The nematode assemblage relies on a more  $^{13}\text{C}$  enriched food source at the reference site, while under cages their  $\delta^{13}\text{C}$  seem to be mostly influenced by sedimentary OM. The harpacticoid assemblage, on the other hand, depends predominantly on sedimentary OM as the main source of food at the reference site, while under cages a  $^{13}\text{C}$  depleted food source influenced their  $\delta^{13}\text{C}$ . The combination of background  $\delta^{13}\text{C}$  with the  $^{13}\text{C}$ -labeled diatom addition experiment confirmed the importance of diatoms as a food source for the nematode assemblage at the reference site, making them more important in carbon processing from the benthic primary producers to higher trophic levels in the sediments of northern Adriatic Sea compared to harpacticoids. At the cage site, the nematodes contribute to direct carbon recycling of farm-impacted sedimentary OM. Overall, the carbon stable isotope signatures were shown to be

a useful tool to determine the impact of fish farming activities on the bottom sedimentary OM and meiofauna.

## DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available. Requests to access the datasets should be directed to MG, [mateja.grego@nib.si](mailto:mateja.grego@nib.si).

## AUTHOR CONTRIBUTIONS

AM provided funding from the FP6 ECASA (Ecosystem approach for sustainable aquaculture) project. MD conceived the idea of the feeding experiments. MG and MD performed the experiments. MG analyzed the samples and the data. MG wrote the manuscript with input from all authors.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00572/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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