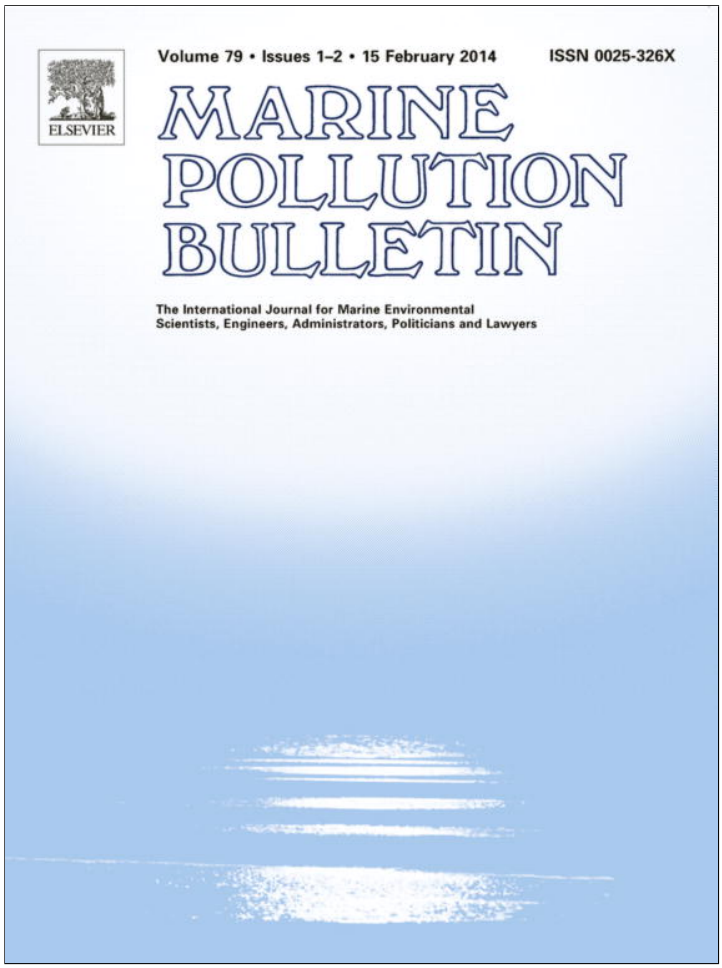

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Effects of sewage discharges on lipid and fatty acid composition of the Patagonian bivalve *Diplodon chilensis*

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ABSTRACT

Lipid and fatty acid (FA) composition and selected oxidative stress parameters of freshwater clams (*Dipolodon chilensis*), from a sewage-polluted (SMA) and a clean site, were compared. Trophic markers FA were analyzed in clams and sediment. Saturated FA (SAFA), and bacteria and sewage markers were abundant in SMA sediments, while diatom markers were 50% lower. Proportions of SAFA, branched FA, 20:5*n* − 3 (EPA) and 22:6*n* − 3 (DHA) were higher in SMA clams. Chronic exposure of *D. chilensis* to increasing eutrophication affected its lipid and FA composition. The increase in EPA and DHA proportions could be an adaptive response, which increases stress resistance but could also lead to higher susceptibility to lipid peroxidation TBARS, lipofuscins (20-fold) and GSH concentrations were higher in SMA clams. FA markers indicated terrestrial plant detritus and bacteria are important items in *D. chilensis* diet. Anthropogenic input in their food could be traced using specific FA as trophic markers.

1. Introduction

Lipid composition can influence growth, reproduction, defense/detoxification systems and physiology of organisms under environmental stress (Bell et al., 1995; Leveille et al., 1997; Parrish et al., 2000). It is known that lipid and fatty acid composition can be altered in the aquatic medium by environmental pollution caused by anthropogenic activities (Cheung et al., 2010; Kainz et al., 2008; Leveille et al., 1997; Penha-Lopes et al., 2009; Perrat et al., 2013; Rocchetta et al., 2006). Trace metals or metal ions, such as chromium and copper (Rocchetta et al., 2012; Sabatini et al., 2009), pesticides, like glyphosate (Romero et al., 2011) and sewage discharges, containing high levels of fecal coliform bacteria (Sabatini et al., 2011) can damage lipids by oxidative processes. Not all fatty acids (FA) react equally and the susceptibility of individual FA to peroxidation increases exponentially with the number of double bonds on the carbon chain (peroxidation index) (Holman, 1954). In addition, comparative studies have shown that phospholipid composition can be readily modified by the food quality or pollution (Ayala et al., 2007; Leveille et al., 1997).

It is worth noticing that essential FA (not biosynthesized effectively by the animal) are highly conserved in aquatic food chains (Arts et al., 2001). FA have therefore been utilized to identify trophic interactions between the dominant taxa in food webs, mainly due to their biological specificity and their characteristic of being transferred from primary producers to higher trophic levels (Leveille et al., 1997; Parrish et al., 2000). These compounds have recently been used as markers to follow the transfer of organic matter within food webs (Abdulkadir and Tsuchiya, 2008; Meziane and Tsuchiya, 2002). Although FA have been extensively used as trophic markers in marine and estuarine systems (Carreira et al., 2011; Costa et al., 2011; Koussoroplis et al., 2011; Napolitano et al., 1995; Napolitano et al., 1997; Parrish et al., 2000), not much attention has been paid to their use in freshwater studies (Desvilettes et al., 1994; Gomes et al., 2010; Leveille et al., 1997; Perga et al., 2009; Sushchik et al., 2003).

The freshwater clam *Diplodon chilensis* (Bivalvia: Hyriidae, Gray, 1828) plays an important role in maintaining the aquatic ecosystems equilibrium in Andean water bodies of Southern Argentina and Chile, by its ability to reduce chlorophyll and nutrient loads, due to its remarkable filter-feeding capacity (Lara et al., 2002; Parada et al., 2008; Sabatini et al., 2011; Valdovinos and Pedreros, 2007).

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The lipid class composition of *D. chilensis* shows seasonal variations with no changes in the FA pattern within each class. However, its FA composition is affected by food quality (Pollero and Brenner, 1981).

A population of *D. chilensis* living in the shore of Lacar lake, at San Martín de los Andes Bay (SMA) in North Patagonia (40°10′S, 71°20′W), are exposed to treated and untreated domestic effluents with an important load of fecal bacteria. Adults of this population show an enhanced antioxidant defence system without signs of neither protein oxidative damage nor changes in the total lipid content. However, they present significant oxidative damage to lipids, suggesting that oxidative stress, caused by the increased eutrophication process of the place, is not fully compensated (Sabatini et al., 2011). Under this stressing conditions, lipid composition and the unsaturated/saturated FA ratio may be altered, but this has not been tested yet. Besides, changes in specific FA could contribute to reduce lipid peroxidation (Pamplona, 2008).

We propose to evaluate the effect of sewage pollution on the lipid classes and FA composition in the freshwater clam *D. chilensis* and to elucidate if the anthropogenic input in their food could be traced by using specific FA as trophic markers. Selected morphometric and oxidative stress parameters are also analyzed in order to discuss the results within a metabolic frame.

2. Materials and methods

2.1. Sample collection

Individuals of *D. chilensis* (length 55.4–62.0 mm) and sediment (triplicates) were collected at two different sites in the Lacar lake shore during winter. Control samples were obtained from the area of Yuco (40°10′S, 71°31′30″W), about 20 km from the city of San Martín de los Andes, where no pollution has been detected (Sabatini et al., 2011). The other sampling site, also described in our previous paper, was next to the city of San Martín de los Andes, at approximately 50 m from the discharge of the sewage treatment plant (SMA) (40°10°S, 71°20°60°W). The sampling sites have been characterized before, showing an increase in the total nitrogen and phosphorous content, higher total suspended solid and organic matter content and higher total and fecal coliform bacteria for SMA area, compared to Yuco (control) (Sabatini et al., 2011). Clams and sediments were randomly sampled by a diver from the two banks, located between 5 and 8 m of depth, as was previously describe (Sabatini et al., 2011).

2.2. Morphometric parameters and sample preparation

Twenty animals from each site were processed. Morphometric parameters were recorded (shell length, width, height and mass, and total and soft tissue mass). Mussels were anesthetized by placing them on ice before sacrifice. The ratios between total soft tissue mass and shell length (STM/SL), STM and shell height (SH), and STM and shell mass (SM) were calculated. Total digestive mass (digestive gland plus digestive tubes) and part of the gonad, which in this species cannot be separated from the digestive tube, was weighed and homogenized in 134 mM KCl solution (ratio 1:5 g tissue mass/mL) containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and 0.2 mM benzamidine (protease inhibitors) to study lipid and FA composition, and oxidative stress and antioxidant/detoxifying defense parameters. In order to avoid the effects of reproductive condition, samples were taken during winter (no reproductive activity) and only males were analyzed to avoid sex variability, according to previously observed differences in total lipid content (see data in Results, Section 3). Aliquots of the homogenates were centrifuged at 11,000×*g* for 20 min. Oxidative stress and antioxidant parameters were measured in the supernatant (Sections 2.3 and 2.4). Total soluble protein content was measured by the method of Bradford (1976), using bovine serum albumin as standard. Total glycogen was measured using Van Handel (1965) method. Lipid and FA analyses are described in Section 2.5. Lipofuscins were analyzed on histological preparations (Section 2.4).

2.3. Reduced glutathione and glutathione-S-transferase

Reduced glutathione (GSH) levels were measured following Anderson (1985) procedure. Absorbance at 412 nm was read after 30 min incubation at room temperature with a UV/VIS JAS-CO 7850 spectrophotometer. Results were expressed as nmol GSH/mg proteins.

Glutathione-S-transferase (GST) activity was measured by the technique of Habig et al. (1974). One GST Unit was defined as the amount of enzyme needed to catalyze the formation of 1 μmol of the conjugate of CDNB (1-chloro-2,4-dinitrobenzene) and reduced glutathione, GS-DNB per minute at 25 °C.

2.4. Oxidative damage

Lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS) method, according to Fraga et al. (1988). TBARS concentration was estimated using an extinction coefficient of 156 mM^{-1} cm^{-1} and reading absorbance at 535 nm. Results were expressed as μmol TBARS/mg wet mass.

Protein oxidation was quantified as carbonyl groups according to Reznick and Packer (1994). Carbonyl content was calculated from the peak absorbance (355–390 nm) using an extinction coefficient of 22,000 M^{-1} cm^{-1}. Results were expressed as nmol carbonyl/mg proteins.

For lipofuscin analysis, small pieces of digestive gland tissue were transferred to histocettes and fixed in Baker's formalin (100 mL 40% formaldehyde and 20 g $CaCO_3$ in 1 L distilled water). Samples were then dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin. Lipofuscins were detected in 5 μm thick sections by Schmorl-staining as described by Strahl et al. (2007). For lipofuscins identification, we previously checked unstained thin sections for auto-fluorescent lipofuscin-like granules (Lomovasky et al., 2002). The histochemical properties of these granules were assessed by PAS-Alcian blue-hematoxylin (Moore et al., 1980) and by Sudan Black B and Oil Red O techniques (Bluhm et al., 2001). A Leica ICC50 light microscope attached to a digital camera and the Image J 1.43u program were used to detect and quantify lipofuscins. For each individual, 10 digital color images were randomly taken from the region around the digestive gland and the intestine. The mean individual lipofuscin area was obtained by averaging results from these 10 images. The lipofuscin area was measured and expressed as percentage of the total tissue area analysed for each image. Results are expressed as $Lipofuscin_{CT}$ (area of lipofuscin granules per total area fraction, multiplied by 100).

2.5. Lipid and fatty acid composition

Total lipids of digestive tissue and sediments were extracted with chloroform/methanol (2:1, v/v) according to Bligh and Dyer (1959) and measured gravimetrically. Lipid classes were separated by thin-layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany), using a series of two-solvent systems. First, the plate was developed with chloroform: methanol: acetic acid: water (65:25:4:4 v/v/v/v), for polar lipids (PL), followed by development in the same TLC plate with hexane: ethyl ether: acetic acid (80:20:1 v/v/v), for neutral lipids (NL).

Standard lipids, iodine vapor, and specific reagents were used to identify lipid classes. Preparative HP-TLC, developed with the solvent system described above for neutral lipids, was used to isolate PL and NL for FA analysis (Heras et al., 2000). Lipid class analysis was also performed by thin-layer chromatography (TLC) on silica gel Chromarods (type S-III) with quantitation by flame-ionization detection using an Iatroscan TH-10, Mark III (Iatron Laboratories Inc., Tokyo, Japan). The separation was conducted with a sequence of three different solvent systems according to Ackman and Heras (1997) with modifications described in Heras et al. (1998).

Fatty acid methyl esters were prepared from the total, neutral and polar lipids fractions, using a base-catalyzed transesterification microscale procedure with sodium methoxide in methanol (Christie, 1982), without previous saponification. FA methyl esters (FAME) were analyzed by gas–liquid chromatography (GC) in a HP-6890 capillary GC (Hewlett Packard, Palo Alto, CA, USA), fitted with an Omegawax 250 fused silica column, 30 m × 0.25 mm, with 0.25 µm phase (Supelco, Bellefonte, CA, USA) equipped with a flame ionization detector (FID). The column temperature was programmed for a linear increase of 3 °C/min from 175 to 230 °C. Fatty acids were tentatively identified by comparing their characteristic retention times with those from a mixture of standard methyl esters run under the same conditions (Pasquevich et al., 2011) and a FAME mixture of menhaden oil with established composition.

2.6. Statistical analyses

Differences in biochemical variables between sampling sites were assessed by analysis of variance (ANOVA). Normality and homogeneity of variances were tested by Lilliefors' and Bartlett's tests, respectively (Sokal and Rohlf, 1984). Graph Pad Prism 3 software was used for statistical analysis.

3. Results

3.1. Morphometric parameters and glycogen content

STM/SL was higher in clams from SMA (polluted area) than in those from Yuco (2.12 ± 0.34 vs. 1.63 ± 0.15, $p < 0.05$). Similar results were obtained relating STM and SH (2.39 ± 0.37 for SMA vs. 1.81 ± 0.18 for Yuco, $p < 0.05$). In contrast, no significant differences were observed in STM/SM (1.34 ± 0.18 and 1.11 ± 0.07, for SMA and Yuco, respectively). Glycogen levels were significantly lower in SMA clams (155 ± 12 mg/g wet mass) compared to those from Yuco (228 ± 21 mg/g wet mass) ($p < 0.01$).

3.2. GSH and GST

GSH levels showed significant differences between sites, being higher in SMA clams (160 ± 14 nmol/mg protein) than in those collected from Yuco (135 ± 13 nmol/mg protein) ($p < 0.05$). No significant differences were detected for GST activity (0.19 ± 0.02 and 0.18 ± 0.02 U/mg protein), for SMA and Yuco, respectively.

3.3. Oxidative damage

Total protein content showed no significant variation between sites (23.00 ± 1.75 and 21.75 ± 2.56 mg/g wet mass, for SMA and Yuco respectively). Futhermore, total lipid content did not show significant differences (73.81 ± 8.97 and 71.13 ± 9.68 mg/g wet mass for SMA and Yuco, respectively).

No difference in oxidative damage to proteins was detected (carbonyl groups level 0.85 ± 0.05 and 1.05 ± 0.10 nmol/mg protein for SMA and Yuco, respectively) while both, lipid peroxidation (TBARS content for SMA = 0.76 ± 0.05 and Yuco = 0.47 ± 0.09 µmol/g wet mass) and lipofuscin content (5.00 ± 0.73 for SMA and 0.23 ± 0.05) were significantly higher in SMA than in Yuco ($p < 0.001$) (Fig. 1a and b). In particular, lipofuscin area was 20-fold increased in SMA. Lipofuscin granules were mostly concentrated in the connective tissue surrounding the digestive tube, the digestive gland tubules and the gonad (Fig. 2).

3.4. Lipid composition of sediments and clams

No variation between sites in the total lipid content was observed either in sediment (0.24 ± 0.03 and 0.27 ± 0.01 mg/g wet mass, for SMA and Yuco, respectively) or in clam tissues. However, significant differences in the total lipid content were detected between sexes (79.90 ± 7.04 and 81.42 ± 5.05 mg/g wet mass (females) and 66.59 ± 9.40 and 60.7 ± 4.54 mg/g wet mass (males), for SMA and Yuco, respectively, $p < 0.01$). Only males were further analyzed for fatty acid and lipid composition.

Sediments from both sites had higher proportion of neutral (NL) than polar lipids (PL), while the opposite relationship was observed in bivalve samples (Fig. 3).

Differences between sites were detected in the NL/PL ratio in both, sediments and clams. Sediment samples showed higher NL/PL in SMA compared to Yuco, due to a higher proportion of triacylglycerol (TAG) and hydrocarbons (HC), and a lower proportion of the PL phosphatidylethanolamine and phosphatidylcholine (PE, PC) (Fig. 3a). Accordingly, clam tissues from SMA had significantly higher NL/PL than those from Yuco, due to increased proportion of diacylglycerol (DAG), TAG and HC, and lower proportion of PE (Fig. 3b).

3.5. Fatty acid composition of sediments

A total of 53 FA were identified (Tables 1 and S1). The dominant FA in sediments were palmitic (16:0) and palmitoleic (16:1n7) acids, accounting for almost 50% of the total FA in Yuco. FA composition showed significant differences between sites, being the proportions of saturated fatty acids (SAFA), particularly 16:0 and 18:0, branched FA and short chain fatty acids (SCFA) higher in SMA. Besides, 18:1 ($n-9+n-7$) and 20:1$n-9$ mono-unsaturated fatty

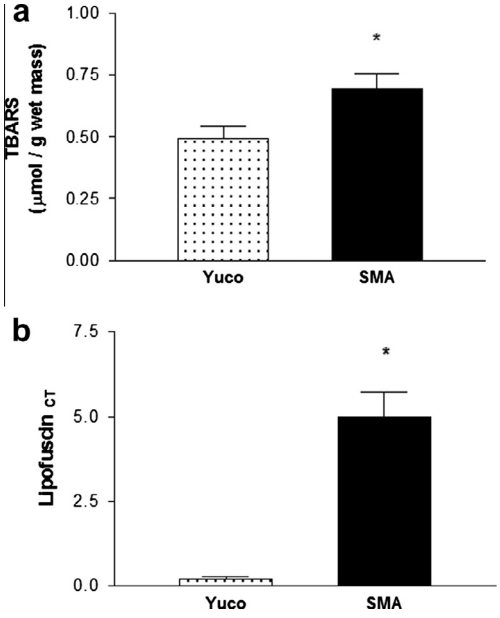

Fig. 1. Lipid peroxidation content (µmol TBARS/mg protein) and lipofuscin$_{CT}$ concentration in control and SMA samples. Data are expressed as means ± S.D. ($n = 14$). *Significant differences between control and SMA ($P < 0.001$).

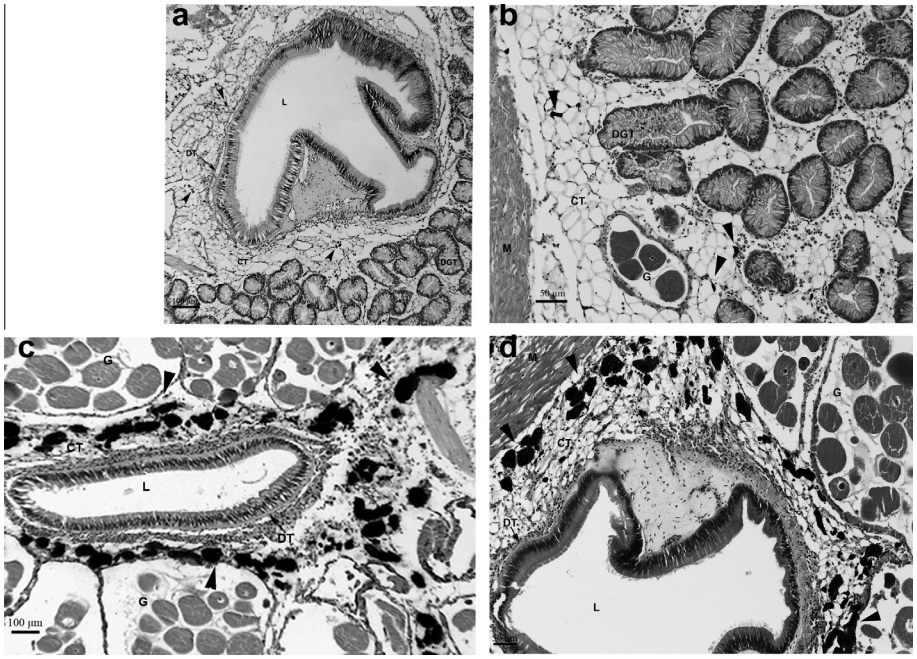

Fig. 2. *Diplodon chilensis* connective tissue round the digestive system sections from (a and b) Yuco and (c and d) SMA. Lipofuscin granules were visualized using Schmorl-stained as irregular dark blue spots (arrowheads). CT, connective tissue; DGT, digestive gland tubules; DT, digestive tube; G, Gonad, L, Lumen; M, muscle.

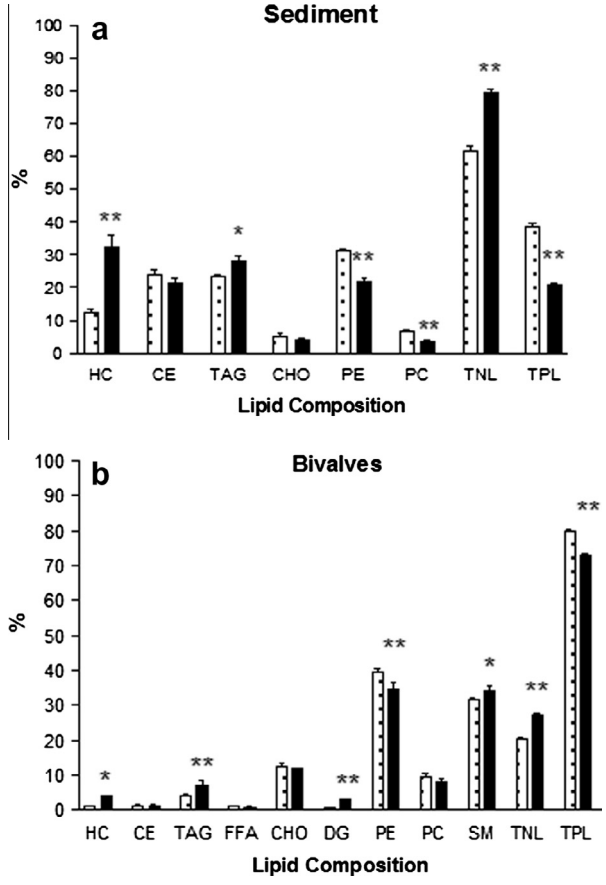

Fig. 3. Lipid composition in sediment samples (a) and *D. chilensis* tissue (b) collected from Yuco (dotted bar) and SMA (full bar) place. Data are expressed as means ± S.D. ($n = 14$). *Significant differences between control and SMA ($p < 0.001$).

acids (MUFA) were also increased in SMA sediments ($p < 0.05$). However, total MUFA proportion was lower in SMA than in Yuco due to an almost 50% decrease in 16:1$n-7$ ($p < 0.05$). The proportion of several $n-3$ and $n-6$ FA differed significantly between sites, resulting in a lower proportion of polyunsaturated fatty acids (PUFA) in SMA, due to an increase in the total $n-6$ FA proportion.

3.6. Fatty acid composition in D. chilensis

Total FA composition from clam tissues showed few variations between sites. Only EPA (20:5$n3$) and branched FA were increased in SMA clams respect to those of Yuco ($p < 0.05$). The predominant fatty acids were 16:0 > 20:4$n-6$ (AA) > 20:0 > 22:2 NMI > 20:5$n-3$ > 18:0 > 18:2$n-6$ > 18:1$n-9$ > 18:1$n-7$, which constituted 64% of the total FA (Table 2). The minor FA (<1%) are presented as Supplementary material in Table S2. Total PUFA were more abundant than SAFA while the $n6/n3$ ratio was always >1.

FA compositions of NL and PL fractions are shown in Table 2. SAFA, MUFA and long chain fatty acids (LCFA, $\geqslant$C24) were more abundant in the NL than in the PL fraction while PUFA were more abundant among the PL. The PL fraction had lower contents of C18 PUFA and higher contents of 20- and 22-carbon highly unsaturated FA (HUFA), such as 20:4$n-6$, 22:2NMI, 22:4$n-6$ and 22:6$n-3$ (DHA), compared to the NL fraction.

When the FA composition of NL and PL was compared between sites, significant differences were observed in both lipid classes. Among NL, LCFA (mainly 24:1) content was lower in SMA than in Yuco ($p < 0.05$), while branched fatty acids were 47% higher in SMA ($p < 0.05$). The major differences between sites were detected in the PL fraction. In particular, the proportions of SAFA, branched FA and several $n-3$ PUFA, specially 20:5$n-3$ and 22:6$n-3$, were higher in SMA ($p < 0.05$). Total MUFA and 20:4$n-6$ were diminished in SMA respect to Yuco ($p < 0.05$).

3.7. Fatty acid markers of potential food sources

Fatty acids and fatty acid ratios used to identify potential food sources are shown in Table 3. EPA and some 16-carbon FA were

Table 1
Major fatty acid composition of sediment collected from Yuco (clean area) and SMA sites.

	Total Fatty acids	
Fatty acid	Yuco	SMA
10:0	0.7 ± 0.1	0.9 ± 0.1
12:0	1.2 ± 0.2	1.7 ± 0.3
14:0	3.2 ± 0.1	3.5 ± 0.2
15:0	0.6 ± 0.1	0.6 ± 0.1
16:0	14.4 ± 0.4	17.5 ± 1.1*
17:0	1.0 ± 0.0	1.1 ± 0.3
18:0	5.9 ± 0.1	8.8 ± 0.3*
20:0	1.6 ± 0.3	1.4 ± 0.1
i15:0	2.2 ± 0.1	5.5 ± 0.3*
ai15:0	1.5 ± 0.1	1.6 ± 0.0
7Me 16:0	1.1 ± 0.0	1.2 ± 0.2
i17:0	0.9 ± 0.1	2.8 ± 0.9*
16:1$n-7$	30.6 ± 1.1	17.4 ± 0.9*
18:1$n-9$	2.2 ± 0.0	3.4 ± 0.2*
18:1$n-7$	2.7 ± 0.0	3.8 ± 0.2*
22:1$n-9$	0.7 ± 0.3	1.1 ± 0.2
24:1	2.6 ± 0.1	2.8 ± 0.4
16:2$n-4$ + phytanic	0.5 ± 0.0	1.0 ± 0.5
16:3$n-4$	1.4 ± 0.1	1.9 ± 0.4
16:4$n-1$	1.5 ± 0.2	5.6 ± 0.3*
18:2$n-6$	1.3 ± 0.1	3.8 ± 0.2*
18:3$n-6$	1.3 ± 0.2	0.4 ± 0.1*
18:3$n-1$	0.2 ± 0.1	1.1 ± 0.2*
18:3$n-3$	1.1 ± 0.1	0.4 ± 0.1*
20:5$n-3$	4.4 ± 0.1	2.8 ± 0.1*
Others[a]	2.9	5.5
∑SAFA[b]	29.1 ± 1.3	35.9 ± 1.5*
∑Branched	6.6 ± 0.3	13.0 ± 0.6*
∑MUFA	39.5 ± 2.0	28.9 ± 1.7*
∑PUFA	12.8 ± 2.5	25.0 ± 2.1*
∑$n3$	5.7 ± 0.7	5.0 ± 0.2
∑$n6$	3.5 ± 0.8	5.7 ± 0.6*
$n6/n3$	0.6	1.1
SCFA	5.6 ± 0.2	6.5 ± 0.4*
LCFA	2.7 ± 0.2	2.9 ± 0.1

Samples are expressed as means ± SD as % w/w ($n = 5$).
* Significant differences between control and SMA are indicated by asterisks ($P < 0.05$). Samples were obtained during winter. I = iso; AI = anteiso; phytanic = 3,7,11,15-tetramethyldecanoic.
[a] Amount of fatty acids < 1%.
[b] Total include minor fatty acids, listed as supplementary material. Branched = i13:0 + i14:0 + i15:0 + i16:0 + i17:0 + ai15:0 + ai17:0 + 7Me 16:0 + pristanic. Short chain fatty acids (SCFA) = C10 + C12 + C13 + C14. Long chain fatty acids (LCFA) = ⩾ C24:0.

at lower levels in sediments from SMA than in those from Yuco ($p < 0.05$), probably indicating lower diatom input in the former. Interestingly, regardless of its low proportion in sediments, EPA was selectively enriched in TL of SMA clams. Only one green algae marker (18:3$n-3$) and one Dinophyta marker (long chain PUFA/16-carbon PUFA) showed lower levels in SMA than in Yuco sediments ($p < 0.05$). However, no significant changes were observed of these markers either in the TL fraction or in the NL fraction. Detritus (particulate organic matter) markers, $n3$ type, were present at lower levels SMA than in Yuco sediments while $n6$ type markers showed higher values in SMA than inYuco. No differences were observed in sediment samples for terrestrial plant markers, while 24:1 FA marker was increased in NL fraction of SMA clam tissue, which could indicate a selection. No differences were observed for the zooplankton markers, neither in clams nor in sediments. Two out of three bacteria markers (branched FA and branched/15:0 FA) were at higher proportion in SMA, both in sediments and in clams TL and NL ($p < 0.05$). Two urban discharge markers (18:1$n-9$ + 18:1$n-7$ and 18:1$n-9$ + 18:2$n-6$) were increased in sediments of SMA ($p < 0.05$) but only the latter was increased in SMA clams NL fraction ($p < 0.05$).

4. Discussion

We have found an increase in soft tissue mass (STM/SL) in clams exposed to sewage water pollution (SMA), with higher levels of fecal coliform bacteria (Sabatini et al., 2011) compared to those collected at the clean area of Yuco. This is in agreement with other works, which associate a tendency to higher STM/SL with increased food availability at polluted sites (Honkoop and Beukema, 1997; Sarà et al., 2012). Although glycogen is by far the main energy storage molecule detected in *D. chilensis*, the increase in soft tissue mass cannot be accounted for by augmented glycogen reserves, which are, in fact, 32% lower in SMA than in Yuco. Glycogen, between other macromolecules, can be consumed to maintain energy balance under pollutant-induced stress, or damaged under oxidative stress (Luca-Abbott, 2001; Ribeiro et al., 2001; Whyte et al., 1990). However, we have not found significant differences either in total lipid or protein contents or in protein oxidation between sites, as it was previously observed (Sabatini et al., 2011). The only signs of oxidative damage detected in SMA clams are increased lipid oxidation, reflected by TBARS and lipofuscins. This, together with glycogen depletion, suggests that carbohydrates could also be providing energy for replacing damaged lipids. Accordingly, changes in lipid turnover induced by pollutants have been reported for other freshwater organisms (Lavarías et al., 2007; Lavarías et al., 2006).

4.1. Lipid and FA composition

The differences in sediment lipid composition between sites are reflected in clam lipids. Pollero and Brenner (1981) have observed changes in the neutral to polar lipids ratio (NL/PL) associated to environmental changes in *D. chilensis*, mainly related to diet quality or food availability. Accordingly, we have observed an increase of NL/PL ratio in SMA due to augmented storage lipids (DAG and TAG) and reduced membrane lipids such as PE. The lower proportion of PE could help to moderate the lipid oxidative process and lipid radical generation (Mitchell et al., 2007; Portero-Otin et al., 2001).

The PL fraction showed an increase of $n-3$ FA in SMA bivalves. Increased proportions of these FA (e.g. C18-$n3$) have been previously related to eutrophication processes (Desvilettes et al., 1994). In addition, Pamplona et al. (2002) have reported that long periods of dietary manipulation could alter $n-6/n-3$ ratios of membrane lipids and affect membrane properties. Despite changes in specific PUFA, (more EPA and DHA, less AA in SMA) the percentage contribution of PUFA to PL does not differ between clams from both sites. The SAFA/PUFA ratio remains unchanged, indicating that these bivalves are capable of counteracting the oxidative damage caused by pollution (Sabatini et al., 2011). It is worth noticing that both DHA and EPA, are involved in important biological functions at various trophic levels, like the diapause in copepods (Pond and Tarling, 2011), brain and skeleton development, and schooling behavior in fish (Masuda and Tsukamoto, 1999), growth in shellfish (Wacker et al., 2002), or immunomodulation and resistance to stress and disease in fish (Montero et al., 2003; Montero et al., 2004). Furthermore, a preferential retention of these FA in fish membranes has been observed even under dietary essential fatty acid deficiency (Montero et al., 2001). In this regard, our results show that, despite the lower EPA proportion in SMA sediment compared to Yuco, higher values of this FA were found in the TL fraction of SMA clam tissue, suggesting a preferential accumulation. These results could explain the overall increase of DHA and EPA observed in bivalves from SMA, suggesting a response or an adaptive mechanism to increase their resistance and immunity under chronic exposure to urban discharges".

Table 2
Major fatty acids of total lipids, neutral lipids and polar lipids of *Diplodon chilensis* collected at (clean area) and SMA sites.

Fatty acid	Total fatty acids		Neutral fatty acids		Polar fatty acids	
	Yuco	SMA	Yuco	SMA	Yuco	SMA
14:0	1.1 ± 0.6	1.2 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	2.2 ± 0.4	2.4 ± 0.6
15:0	1.3 ± 0.3	1.1 ± 0.2	0.8 ± 0.0	0.9 ± 0.1	1.8 ± 0.3	1.2 ± 0.6
16:0	12.7 ± 1.3	10.9 ± 1.4	18.5 ± 2.0	17.6 ± 1.0	8.4 ± 0.7	11.3 ± 1.5*
17:0	0.6 ± 0.1	0.7 ± 0.0	1.3 ± 0.2	1.6 ± 0.4	1.2 ± 0.2	1.4 ± 0.6
18:0	5.9 ± 0.4	6.2 ± 0.3	6.9 ± 1.0	7.1 ± 1.0	6.4 ± 0.7	8.5 ± 1.4*
20:0	8.9 ± 0.7	9.3 ± 0.5	5.0 ± 0.3	5.6 ± 0.4	2.8 ± 0.9	5.7 ± 3.2
i16:0	0.2 ± 0.1	0.2 ± 0.0	1.6 ± 0.3	1.9 ± 0.5	0.4 ± 0.1	0.5 ± 0.1
Pristanic	3.9.2 ± 0.9	5.1 ± 0.9	0.2 ± 0.1	0.3 ± 0.1	1.6 ± 0.6	3.1 ± 0.5*
16:1$n-7$	1.9 ± 0.2	2.0 ± 0.4	3.7 ± 1.0	4.3 ± 1.0	4.5 ± 1.8	1.3 ± 0.2*
16:1$n5$	0.4 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	1.0 ± 0.3	0.2 ± 0.0	0.3 ± 0.0
18:1$n-9$	3.7 ± 0.0	3.8 ± 0.5	5.9 ± 0.2	6.1 ± 0.4	1.9 ± 0.2	1.8 ± 0.1
18:1$n-7$	2.5 ± 0.2	2.5 ± 0.3	5.3 ± 0.4	5.0 ± 0.7	1.3 ± 0.1	1.3 ± 0.3
20:1$n-9$	1.3 ± 0.2	1.1 ± 0.2	2.0 ± 0.4	1.8 ± 0.1	1.0 ± 0.2	0.8 ± 0.0
22:1$n-9$	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	1.3 ± 0.4	0.6 ± 0.3*
24:1	3.9 ± 0.6	3.3 ± 0.4	5.3 ± 0.2	4.4 ± 0.3*	2.8 ± 0.7	3.1 ± 0.3
16:2$n-4$ + phytanic	1.2 ± 0.2	1.0 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
16:3$n-4$	1.1 ± 0.1	1.1 ± 0.1	nd	0.1 ± 0.0	1.0 ± 0.2	0.2 ± 0.0
18:2$n-6$	5.2 ± 0.1	5.6 ± 0.2	6.9 ± 0.7	7.1 ± 0.7	3.1 ± 0.5	3.6 ± 0.5
18:3$n-3$	3.6 ± 0.3	3.5 ± 0.5	5.9 ± 0.9	5.9 ± 1.2	1.3 ± 0.4	1.8 ± 0.6
18:4$n-1$	0.7 ± 0.1	0.7 ± 0.2	1.7 ± 0.7	1.6 ± 0.5	0.2 ± 0.0	0.2 ± 0.0
20:4$n-6$	11.8 ± 0.6	11.7 ± 1.2	5.7 ± 0.9	4.5 ± 0.7	13.7 ± 0.5	11.1 ± 1.5*
20:5$n-3$	6.6 ± 0.3	7.5 ± 0.5*	6.1 ± 0.9	6.6 ± 0.5	5.4 ± 0.7	7.9 ± 0.9*
22:2NMI	7.3 ± 1.2	6.2 ± 0.2	3.1 ± 0.8	2.4 ± 0.1	7.0 ± 1.5	6.1 ± 0.4
22:3$n-6$	1.1 ± 0.0	1.5 ± 0.2	0.7 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	2.2 ± 0.3
22:4$n-6$	1.7 ± 0.3	1.7 ± 0.5	0.6 ± 0.2	0.5 ± 0.1	2.8 ± 0.2	2.1 ± 0.6
22:5$n-3$	1.3 ± 0.2	1.0 ± 0.1	1.1 ± 0.3	0.8 ± 0.1	1.2 ± 0.1	1.0 ± 0.3
22:6$n-3$	1.1 ± 0.1	±0.2	0.6 ± 0.1	0.7 ± 0.3	1.4 ± 0.1	2.2 ± 0.2*
Others[a]	3.7	5.4	5.5	8.8	4.7	7.1
∑SAFA[b]	30.5 ± 1.6	29.7 ± 1.2	33.7 ± 1.7	33.9 ± 1.8	23.3 ± 0.6	30.7 ± 0.9*
∑Branched	4.9 ± 0.3	7.2 ± 0.4*	3.2 ± 0.7	5.1 ± 0.4*	3.1 ± 0.4	4.7 ± 0.5*
∑MUFA	14.4 ± 1.0	14.3 ± 1.1	23.8 ± 1.2	23.6 ± 0.7	14.6 ± 0.4	10.2 ± 0.2*
∑PUFA	43.0 ± 1.4	43.7 ± 1.2	35.7 ± 1.3	35.8 ± 0.9	41.5 ± 1.2	42.3 ± 0.9
∑$n-3$	14.0 ± 0.4	14.5 ± 0.4	14.8 ± 0.8	14.6 ± 1.8	9.3 ± 0.9	14.0 ± 0.6*
∑$n-6$	20.5 ± 0.4	20.7 ± 0.7	15.0 ± 0.5	15.4 ± 0.3	21.2 ± 1.1	20.5 ± 0.9
$n-6/n-3$	1.5	1.5	1.1	1.1	2.2	1.5
∑SAFA/∑MUFA + PUFA	0.5	0.5	0.6	0.6	0.4	0.6
SCFA	1.4 ± 0.4	1.5 ± 0.4	1.6 ± 0.2	1.6 ± 0.3	2.4 ± 0.3	2.6 ± 0.4
LCFA	4.2 ± 0.6	3.3 ± 0.4	5.3 ± 0.2	4.5 ± 0.3*	3.1 ± 0.8	3.1 ± 0.3

Samples are expressed as means ± SD as % w/w ($n = 5$).
* Significant differences between Yuco and SMA are indicated by asterisks ($P < 0.05$). Samples were obtained during winter. I = iso; AI = anteiso; pristanic = 2,6,10,14 tetramethylpentadecanoic; phytanic = 3,7,11,15-tetramethyldecanoic; NMI = non-methylene interrupted.
[a] Amount of fatty acids <1%.
[b] Total include minor fatty acids, listed as supplementary material. Branched = i13:0 + i14:0 + i15:0 + i16:0 + i17:0 + ai15:0 + ai17:0 + 7Me 16:0 + pristanic. Short chain fatty acids (SCFA) = C10 + C12 + C13 + C14. Long chain fatty acids (LCFA) ⩾ C24:0.

On the other hand, a recent study on the influence of anthropogenic pollution in a river ecosystem, shows that PUFA, DHA and EPA contents decrease in gammarids collected from a region influenced by industrial and commercial sewage discharges, characterized by the increased levels of several nutrients and also some metals (Gladyshev et al., 2012). A decrease in PUFA levels could respond to oxidative damage to unsaturated FA, which can be produced by increased metal levels in the aquatic system (Di Salvatore et al., 2013; Rocchetta et al., 2006). In our polluted site (SMA), no evidence of the increase of metals has been found, in coincidence with the absence of industrial activity in the Lacar lake area (Rocchetta et al., 2014; Sabatini et al., 2011).

Bivalves have very limited or no capability to synthesize PUFA (Chu and Greaves, 1991; De Moreno et al., 1976, 1977). However they are capable of *de novo* synthesis of a particular kind of FA called non-methylene interrupted fatty acids (NMI) (Zhukova, 1986, 1991) which are very abundant among bivalve polar lipids (Zhukova and Svetashev, 1986). In a recent study, (Munro and Blier, 2012) have shown that marine bivalves, especially long-lived species, can replace DHA (PUFA most highly sensitive to oxidation) for less peroxidizable FA like NMI and monene FA. The higher proportion of these FA reduces the susceptibility to lipid peroxidation while maintaining proper membrane fluidity. In our study, no difference in the proportion of NMI between clams from both sites is evident, while DHA and EPA are significantly increased in SMA, at the expense of MUFA. This implies a higher susceptibility to membrane lipid oxidation, which is only partially compensated by the reduction in AA and, indeed, we have observed higher lipid oxidation levels in SMA than in Yuco. The high concentration of these PUFA in phospholipids makes them prime targets for oxidative damage, which could trigger peroxidation cascades (Pamplona 2008). The increased GSH content in SMA clams is probably helping to avoid oxidative damage to proteins, by neutralizing carbonyl compounds generated in the lipid peroxidation cascade (Aldini et al., 2007).

It is well established that the biosynthesis of $n-3$ PUFA in animals starts with C18$n-3$ and can proceed until it yields 22-carbon PUFA (22:6$n-3$) (Sprecher, 2000). In our study, not only higher proportions of C18-$n3$ are present in PL of SMA clams but also higher proportions of EPA and DHA, as we discuss above. The higher proportion of FA-intermediates in the C-22 PUFA biosynthesis (e.g. 18:4$n-3$, 20:4$n-3$, 20:5$n-3$) is probably a reflection of

Table 3
Fatty acid and fatty acid ratios used as biomarkers for different food sources. Biomarkers (expressed as ratios) indicate the relative importance of one food source over another. These ratios were calculated for *Diplodon chilensis* tissue fatty acids from the different lipid fractions (total (TL) and neutral (NL)), collected either from Yuco (clean area) or from SMA sites. Sediment samples collected from both study sites were also analyzed using total lipid fraction (TL).

Taxa	Biomarker	Tissue				Sediment	
		TL		NL		TL	
		Yuco	SMA	Yuco	SMA	Yuco	SMA
Diatoms[a]	∑16 specific	4.2 ± 0.5	4.2 ± 0.7	4.1 ± 0.5	4.7 ± 0.8	34.0 ± 2.0	25.8 ± 1.2*
	16:1*n*7	1.9 ± 0.2	2.0 ± 0.4	3.7 ± 1.0	4.3 ± 1.0	30.6 ± 1.1	17.4 ± 0.9*
	20:5*n*3	6.6 ± 0.3	7.5 ± 0.2*	6.1 ± 0.9	6.6 ± 0.5	4.4 ± 0.1	2.8 ± 0.1*
	16:1/16:0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	2.13 ± 0.6	1.0 ± 0.2*
Green algae[b]	∑16,18,20 − *n*3	3.7 ± 0.4	3.5 ± 0.7	6.1 ± 0.8	6.2 ± 0.9	3.4 ± 0.2	3.6 ± 0.2
	∑PUFAs 18	8.9 ± 0.5	9.3 ± 0.8	13.1 ± 1.4	13.3 ± 1.0	3.7 ± 0.5	4.6 ± 0.8
	18:3*n*3	3.6 ± 0.3	3.5 ± 0.5	5.9 ± 0.9	5.9 ± 1.2	1.1 ± 0.1	0.4 ± 0.1*
Dinophyta[c]	22:6*n*3	1.1 ± 0.1	1.4 ± 0.2	0.6 ± 0.1	0.7 ± 0.3	nd	0.6 ± 0.3
	Specific ratio FA	2.8 ± 0.5	2.9 ± 0.2	3.5 ± 0.1	3.4 ± 0.3	4.0 ± 0.9	2.1 ± 0.5*
Zooplancton[d]	20:1 + 22:1	1.7 ± 0.5	1.6 ± 0.8	2.8 ± 0.7	2.6 ± 0.6	0.8 ± 0.2	1.4 ± 0.6
Bacteria[e]	∑Branched	4.9 ± 1.0	7.1 ± 1.0*	3.4 ± 0.4	5.3 ± 0.3*	6.6 ± 1.1	12.9 ± 1.8*
	∑Branched/15:0	3.9 ± 0.7	6.5 ± 0.5*	4.0 ± 0.4	5.8 ± 0.3*	10.7 ± 0.9	21.2 ± 1.7*
	18:1*n*7	2.5 ± 0.2	2.5 ± 0.3	5.0 ± 0.6	5.3 ± 0.7	2.8 ± 0.2	3.7 ± 0.3*
Detritus[f]	PUFAs 22 − *n*6	2.8 ± 0.3	3.2 ± 0.5	1.3 ± 0.7	2.2 ± 0.3	0.4 ± 0.3	0.9 ± 0.2
	HUFAs *n*6 + *n*3	23.9 ± 1.7	25.1 ± 1.0	16.2 ± 1.2	16.5 ± 1.1	5.4 ± 0.7	5.3 ± 0.9
	HUFAs *n*6	14.7 ± 0.4	14.9 ± 0.8	7.9 ± 1.1	8.1 ± 0.5	0.9 ± 0.4	1.6 ± 0.7*
	HUFAs *n*3	9.5 ± 0.4	10.2 ± 0.7	8.3 ± 0.9	8.5 ± 1.1	4.5 ± 0.4	3.7 ± 0.2*
Urban discharges[g]	18:1*n*9 + 18:1*n*7	6.2 ± 0.4	6.3 ± 0.6	11.2 ± 0.8	11.9 ± 0.8	5.0 ± 0.9	7.2 ± 0.7*
	18:1*n*9 + 18:2*n*6	8.9 ± 0.9	9.3 ± 0.4	12.7 ± 0.6	13.9 ± 0.4*	3.5 ± 0.2	7.1 ± 0.1*
Terrestrial plants[h]	24:0	0.2 ± 0.1	0.3 ± 0.1	nd	nd	0.1 ± 0.1	0.1 ± 0.1
	24:1	3.9 ± 0.4	3.3 ± 0.6	5.3 ± 0.2	4.4 ± 0.3*	2.6 ± 0.3	2.8 ± 0.4

[a] Diatoms: ∑16 specific diatom marker FA (16:1*n*7 + 16:2*n*4 + 16:3*n*4 + 16:4*n*1) (Navarrete et al., 2000), 16:1*n*7, 20:5*n*3 (Napolitano, 1999; Perga et al., 2009; Gomes et al., 2010), 16:1/16:0 (Leveille et al., 1997, Napolitano et al., 1997).
[b] Green algae: 16:3*n*3 + 18:3*n*3 + 20:4*n*3 (Leveille et al., 1997), ∑C18 PUFAs (18:2*n*6 + 18:3*n*6 + 18:3*n*3) (Volkman et al., 1998), 18:3*n*3 (Gomes et al 2010).
[c] Dinophyta: Specific ratio FA (16:0 + 18:4*n*3 + 20:5*n*3 + 22:3*n*6)/(18:3*n*3 + ∑C16 PUFAs) (Leveille et al., 1997), 22:6*n*3 (Lau et al., 2009; Gomes et al., 2010).
[d] Zooplancton: 20:1 + 22:1 (Gomes et al. 2010; Parrish et al, 2000).
[e] Bacteria: 18:1*n*7, Branch-chain odd FA (i13:0 + i14:0 + i15:0 + ai15:0 + i17:0 + ai17:0 + 7Me 16:0 + 2, 6, 10, 14 Methyl-C15:0) (Sushchik et al., 2003; Perga et al., 2009; Carreira et al., 2011).
[f] Detritus (particulated organic matter): PUFAs 22:2*n*6 + 22:3*n*6 + 22:4*n*6 (Desvilettes et al., 1994), HUFAs *n*3 (20:3*n*3 + 20:4*n*3 + 20:5*n*3 + 21:5*n*3 + 22:5*n*3 + 22:6*n*3), HUFAs *n*6 (20:2*n*6 + 20:3*n*6 + 20:4*n*6 + 22:2*n*6 + 22:3*n*6 + 22:4*n*6) , HUFAs *n*3 + *n*6.
[g] Urban discharge: 18:1*n*9 + 18:2*n*6, 18:1*n*9 + 18:1*n*7 (Sakdullah and Tsuchiya, 2009).
[h] Terrestrial: 24:0 (Perga et al., 2009, Lau et al., 2009), 24:1 (Napolitano et al., 1997), 20:3*n*6 (Volk and Kiffney, 2012). (nd = not detectable).

an increase of this biosynthetic pathway compared to Yuco (Sushchik et al., 2003).

4.2. FA as trophic markers

FA markers for potential food sources show a relationship between sediments and *D. chilensis* tissues. SMA sediments contain lower proportions of diatom, green algae and Dinophyta markers than those of Yuco (Table 3), suggesting that phyto-plankton/benthos biodiversity is affected by urban pollution (Edelberg, 2004; Sabatini et al., 2011).

Previous reports have shown that the phytoplankton of Patagonian oligotrophic lakes is typically dominated by diatoms (Diaz et al., 1998; Temporetti et al., 2009). Digestive content studies have also indicated that diatoms are the main algal food source chosen by *D. chilensis* (Acevedo et al., 1993). Accordingly, diatom biomarkers are the most abundant in sediments of both sites, followed by those of bacteria and detritus. EPA proportions found in the sediments are not in concordance with the results found in clam tissue, probably due to food particle selection or metabolism by clams.

Both sites are next to an autochthonous forest with similar species composition, which provides plenty of vegetal material to the lake. It has been previously suggested by Pollero et al. (1981) that terrestrial plant detritus has great influence on *D. chilensis* diet. This is in agreement with our results showing higher levels of $n-6$ than $n-3$ PUFA in both sediment and clams. This vegetal detritus is colonized and partially degraded by fungi and bacteria and, then transported to the lake by the runoff. The SMA site is placed near the Pocahullo river mouth, which could contribute to increase vegetal detritus content. In fact, detritus FA markers show a higher terrestrial organic matter input in SMA sediments ($n-6$ HUFA) and a lower plankton detritus ($n-3$ HUFA) influence, compared to Yuco (Alfaro et al., 2006; Desvilettes et al., 1994; Kiffney and Richardson, 2010). Both *n*6-and *n*3-HUFA are important components of TL and NL of *D. chilensis*, presenting the TL fraction higher content of *n*6-HUFA. This reflects an important energetic contribution of terrestrial plant detritus.

In addition to forest detritus, SMA receives urban effluent inputs. Accordingly, higher levels of urban discharges and bacteria markers, namely 18:1$n-9$, 18:2$n-6$ and 18:1$n-7$ (Sakdullah and Tsuchiya, 2009) are present in SMA sediments, together with specific bacteria biomarkers (branched FA), which were two-fold higher in SMA compared to Yuco. In accordance, SMA *D. chilensis* tissues show increased proportion of branched FA and 18:1$n-9$ + 18:2$n-6$ in SMA clams.

We can conclude that (1) chronic exposure of *D. chilensis* to the increasing eutrophic process observed during last decade in SMA, has affected its lipid composition increasing the ratio between storage lipids and membrane structure lipids but without altering the total lipid content. FA composition was most affected in the PL fraction, but PUFA content and the SAFA/PUFA ratio do not vary.

The higher levels of DHA and EPA found in clam PL of SMA could be involved in increased stress resistance and immunity in SMA bivalves as an adaptive response to the chronic exposure to sewage

discharges. However, these higher levels could also involve higher susceptibility to lipid peroxidation in these clams. This seems to be reflected by the increased lipid peroxidation and lipofuscins levels and the activation of the GSH antioxidant system, to avoid the damage produced by lipid peroxidation cascades. In a previous study (Sabatini et al., 2011), an increase in GST activity was shown in samples from the polluted area (SMA), which is not observed in this study, probably in relation with the differences in the tissue used between both works.(2) FA biomarkers in clam reflect the main sediment composition in both sites. Specific markers show that terrestrial plant detritus is very important for *D. chilensis* energetic budget and that bacteria also constitute an important dietary item, especially in the polluted area.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.marpolbul.2013.12.011.

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