**Impact of environmental conditions on phospholipid fatty acid composition: Implications from two contrasting estuaries**

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**Abstract**

Phospholipid (PL) composition has a tremendous influence on the cell integrity and physiological competency. At the same time plankton PL make important metabolic fuels for higher trophic levels. The goal of this study was to identify environmental control on PL production and their molecular identity of the suspended particles in two different estuaries. We conducted research in subtropical eutrophic Wenchang River Estuary in China and temperate pristine, mesotrophic Krka River Estuary in Croatia. In agreement with the more abundant phytoplankton, PL concentrations were much higher in the Wenchang River Estuary (30.3-178.2 μg L-1) than in the Krka River Estuary (8.4-18.8 μg L-1). Given that six PL classes investigated (phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylserine (PS)) have different roles in the cell, we expected their different fatty acid composition in different environments. We found small differences in the fatty acid composition of PC, PG, and PI between two estuaries. These results suggest that the essential fatty acid compositions of these PL in estuarine plankton are relatively constant in order to preserve membrane functions and/or cell processes in which they are involved regardless of environmental conditions. In contrast, PE, PA and PS fatty acid composition substantially differed between two estuaries as well as throughout the salinity gradient in each estuary. This suggests the adaptability of plankton to remodel these PL depending on the environmental conditions and the plankton community structure. Good environmental conditions (favorable N/P ratio, temperature) are important for increased PL content (% in POC and total lipids) in estuarine plankton and increased essential polyunsaturated fatty acid content in PL which is beneficial to higher trophic levels.

**Keywords** phospholipids ⋅ fatty acids ⋅ estuaries ⋅ temperate ⋅ subtropical ⋅ phytoplankton pigments

**Introduction**

Coastal regions are considered key climate change hot spots worldwide (IPCC 2014). Estuaries are among the most productive environments on Earth. They receive substantial inputs of nutrients and organic matter from the mainland that support high rates of plankton metabolism and primary production (Cloern et al. 2014). This is reflected in higher trophic levels making estuaries a favorable environment for commercially important fish and shellfish farming. In general, estuaries are highly heterogeneous and complex ecosystems characterized by high biodiversity (Cloern et al. 2014; Muylaert et al. 2009).

Among the three major biochemical compounds, lipids, proteins, and carbohydrates, lipids are present in the lowest concentrations, but play disproportional roles in numerous essential biological processes (Arts et al. 2001). They are carbon-rich, with very high energetic value, thus representing important metabolic fuels for higher trophic levels (Lee et al. 1971; Parrish 1998). In addition, the molecular structures of lipids contain important heteroatoms, including phosphorus, nitrogen, sulfur and oxygen. For all organisms’ life and growth, energy, space and nutrients are required. One of the key nutrients is phosphorus. It is assimilated into essential molecules, such as nucleic acids, ATP, and phospholipids (PL). Phospholipids are engaged in (i) establishing the permeability barrier for cells and cell organelles, (ii) providing the matrix for the assembly and function of a wide variety of catalytic processes, (iii) acting as donors in the synthesis of macromolecules, and (iv) actively influencing the functional properties of membrane-associated processes (Dowhan 1997; Dowhan et al. 2008).

Phospholipids, including phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositols (PI) and phosphatidic acids (PA), are predominantly located in extra-chloroplast membranes of the cell. Phosphatidylglycerols (PG) are the exception as they are the only PL located in thylakoid membranes, involved in photosynthetic transport of electrons (Wada and Murata 2007). Phosphatidylcholines and PE are the most abundant structural lipids in membranes, consisting of ~ 68-80% of the total PL (van Meer et al. 2008), while PI, PA and PS are usually minor components of the total PL. They are important signal and regulatory molecules in phytoplankton cells. Phosphatidic acids and PS are also precursors for biosynthesis of other PL (Khozin-Goldberg 2016).

The content of PL in total lipids of selected algal species that belong to Haptophyta, Rodophyta, Chlorophyta and Bacillariophyta range from 1 to 52% (Guschina and Harwood 2009). Investigations of impact of temperature and nutrient availability on growth of diatom *Chaetoceros pseudocurvisetus* revealed that PL share in total lipids increases with temperature both in replete and phosphorus (P)-depleted conditions (Novak et al. 2019). Lipid content of marine bacteria is low, ranging from 1.7 to 7.3% of organic carbon, with PL as main lipids, ranging from 51 to 96% (Goutx et al. 1990a; 1990b). Phospholipids contain FA residues of variable chain lengths and degrees of unsaturation. Lipid FA composition depends on the species and environmental conditions (Li et al. 2005; Guiheneuf et al. 2010; Hixson and Arts 2016; Hernando et al. 2018). Changes in the primary producers' essential polyunsaturated fatty acid content in aquatic environment may be an ecological risk for the higher trophic levels (Müller-Navarra et al. 2000). Omega-3 polyunsaturated fatty acids are essential nutrients with a wide range of health benefits. The most common marine omega-3 polyunsaturated fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), whose primary sources are marine algae and phytoplankton. Their absorption into tissues of organisms of higher trophic levels is more efficient from PL than from triacylglycerols (Schunck et al. 2012).

In this study, we conducted an in-depth investigation of the PL in two contrasted estuaries. We combined those results with diverse environmental data. The Krka River Estuary (KRE) in Croatia and the Wenchang River Estuary (KRE) in China substantially differed in temperature and nutrient status, and consequently phytoplankton community structure. Using high-performance liquid chromatography (HPLC)/electrospray ionization (ESI) tandem mass spectrometry (MS/MS), we performed phospholipid fatty acid (PLFA) profiling of six most abundant phospholipids: PC, PG, PE, PA, PI and PS. This study aimed to address forcing variables responsible for PL abundance and to define the influence of different environmental conditions on PL composition. To the best of our knowledge, this is the first study that provides complete fatty acid profiling of the main six phospholipid classes in estuaries.

**Methods**

Study sites and sample collection

The Wenchang and Wenijao Rivers enter the WRE (Fig. 1a). It is a shallow system with a water depth of max 3 m (Liu et al. 2011). It is characterized by a tropical monsoonal climate. Its temperature is lower in the dry season (November-April) (23.3–28.7 °C) than in the wet season (May-October) (27.0–33.6 °C) (Li et al. 2014). Riverine input, groundwater discharge and aquaculture effluents are the major source of nutrients entering into the WRE (Liu et al. 2011). The concentrations of inorganic nutrients vary along salinity gradient and seasonally, being ~0-100 µmol L-1 NO3¯, ~0-5.5 µmol L-1 NO2¯,~0-70 µmol L-1 NH4+, ~0-1.3 µmol L-1 PO43¯, and ~5-150 µmol L-1 SiO44¯ (Liu et al. 2011). Chl *a* concentrations in WRE are tide and season (dry/wet) dependant, ranging from 0 to 27 µg L-1 (Herbect et al. 2011). Dissolved organic carbon (DOC) content in the WRE reaches the values up to 20 mg L-1 (Herbeck et al. 2013), indicating its highly eutrophic character.

The Krka River Estuary is a 25 km long estuary that spreads from the Skradinski Buk waterfalls to the Šibenik Channel (Fig. 1b). The water depth gradually increases from 5 m below the waterfalls to 43 m at the mouth. It is temperate estuary with annual temperature variations between 4.5 and 28.8 °C (Gržetić et al., 1991; Cetinić et al. 2006). The main sources of nutrients in this estuary are the Krka River, city of Šibenik (Gržetić et al., 1991), and numerous submarine groundwater discharges connected to the karst aquifer (Liu et al. 2019). Measured nutrient concentrations vary within ranges of 0-59.2 µmol L-1 NO3¯, 0-1.1 µmol L-1 NO2¯, 0-13.2 µmol L-1 NH4+, 0-1.73 µmol L-1 PO43¯, and 0-65.8 µmol L-1 SiO44¯ (Gržetić et al., 1991; Svensen et al. 2007). The KRE is highly stratified as a result of its sheltered position and low tidal movements (0.2–0.5 m) (Gržetić et al., 1991). The boundary layer between fresh and salty waters is characterized by a steep halocline that varies in thickness and depth, dependent on freshwater inflow and wind. Most of the primary production takes place in the 0.2-4 m brackish layer above the sharp halocline (Svensen et al. 2007). In situ primary production (measured in winter, autumn and late summer) vary mostly from 1 to 30 mg C m-3 h-l (Gržetić et al. 1991). Seasonal distribution of phytoplankton biomass is characterized with highest biomass in spring and autumn-winter period and lowest during summer stratification, with Chl *a* ranging from 0.07 to 4.73 µg L-1 (Bužančić et al. 2012). Krka is a pristine river with DOC of only 0.5 mg L-1 (Louis et al. 2009), while DOC concentration in KRE is on average 1 mg L-1 (Lechtenfeld et al. 2013).

Samples were collected using 5-liter Niskin bottles from the surface water (depth of 0.5 m) following the salinity (S) gradient from riverine end-member (S = 0) to marine end-member (S = 37.9 in the KRE and S = 33.3 in the WRE) (Fig. 1). Water sampling was performed from September 4th to 9th 2014 in the KRE and from May 8th to 10th 2015 in the WRE.

In this paper our main focus was on estuaries, i.e. brackish waters with variable salinities (S) and inhabited by freshwater, estuarine, and marine water phytoplankton. Therefore, data obtained from the estuaries were exclusively discussed. Data obtained from freshwater (S = 0) and marine water (S = 33.3 and 37.9 in the WRE and KRE, respectively) end-member are also shown in the figures for comparisons, but are omitted from the discussion.

Basic environmental analysis

Temperature (T), salinity and pH of the KRE and WRE water samples were measured in situ by multiparameter probes HQ40D (Hach Lange, Germany) and Multi 350i (WTW, Geotech Environmental Equipment, Denver, USA), respectively.

Samples (50 mL) for the analysis of ammonium (NH4+) were stabilized by addition of 2 mL of phenol solution (1 mol L-1; 95 % ethanol) (Ivančić and Degobbis 1984) and stored in the dark at 4 °C. Samples (500 mL) for all other nutrients were stored at -20 °C. The concentrations of total inorganic nitrogen (TIN) (TIN = nitrate (NO3¯), nitrite (NO2¯), and NH4+), and orthophosphate (PO43¯) were determined by spectrophotometric methods following Strickland and Parsons (1972).

Pigment analysis

While generally chlorophyll *a* (Chl *a*) is used as a convenient proxy of phytoplankton biomass, many other phytoplankton pigments exhibit chemotaxonomic associations that might be used in the characterization of phytoplankton assemblages (Gibb et al. 2000). We have detected fucoxanthin (*fuco*, diatoms) and peridinin (*perid*, dinophytes), two marker pigments mostly associated to microphytoplankton. We found also marker pigments more typical of nanophytoplankton: chlorophyll c3 (*chl c3*, prymnesiophytes and chrysophytes), butanoyloxyfucoxanthin (*but*, chrysophytes), 19’–hexanoyloxyfucoxanthin (*he*x, prymnesiophytes), chlorophyll *b* (*chl* *b*, chlorophytes and prasinophytes), violaxanthin (*viola*, chlorophytes and prasinophytes), alloxanthin (*allo*, chrysophytes), and lutein (*lut*, chlorophytes and prasinophytes). Finally, we found also a typical picophytoplankton marker pigment zeaxanthin (*zea*, cyanobacteria) (Jeffrey and Vesk 1997).

For the pigment determination, 1 L of seawater was filtered through 0.7 μm Whatman GF/F filters pre-burned at 450 °C for 5h and preserved in –80 °C liquid nitrogen until the analysis. The extraction in 4 ml of cold 90% acetone was performed by sonication, and the extracts were collected by centrifugation. The composition of phytoplankton pigments, soluble in organic solvents, were analyzed by HPLC following the method by Barlow et al. (1997). Acetone extracts were mixed 1:1 (v/v) with 1 M ammonium acetate and injected into the HPLC system with 3-mm Thermo Hypersil-Keystone column MOS2, C-8, 120 A pore size, 150×4.6 mm (Thermo Hypersil-Keystone, Bellefonte, PA, USA). Pigments were separated at the flow rate of 1 mL min–1 using a linear gradient program with duration of 40 min by using solvent A and B. Solvent A consisted of 70:30 (v/v) methanol: 1 M ammonium acetate, while solvent B was 100% methanol. Chlorophylls and carotenoids were detected by the absorbance at 440 nm (SpectraSYSTEM, Model UV 2000, Thermo Fischer Scientific, USA). Qualitative and quantitative analyses of individual pigments were performed by the external standard calibration using authentic pigment standards (VKI, Denmark).

Particulate organic carbon (POC)

For the POC determination, 0.12-1 L of estuarine water were filtered through 0.7 µm Whatman GF/F filters pre-burned at 450 °C for 5h. A solid sample module SSM-5000A connected to a Shimadzu TOC-VCPH carbon analyzer calibrated with glucose was used for POC analysis. Concentrations of POC were corrected based on blank filter measurements. The average filter blank, including the instrument blank, corresponded to 5 µg C L-1. The reproducibility for the glucose standard was 3%.

Lipid analysis

For the lipid class determination, 0.5-3 L of riverine/estuarine/seawater were collected in glass containers and passed through the 200 µm stainless steel screen to remove zooplankton and larger particles. It was followed by filtration through 0.7 µm Whatman GF/F filters pre-burned at 450 °C for 5 h. Particulate lipids were extracted by a modified one-phase solvent mixture of dichloromethane-methanol-water (Bligh and Dyer 1959). N-hexadecanone was added as internal standard to each sample to estimate the lipid recoveries in the subsequent steps of the sample analysis. The extracts were evaporated to dryness under nitrogen gas, stored at -20 °C for one day and dissolved in 20 µL dichloromethane immediately before analysis.

Lipid classes were determined by thin-layer chromatography with flame ionization detection (TLC-FID) (Iatroscan MK-VI, Iatron, Japan). The classes were separated on Chromarods SIII and quantified by external calibration with standard lipid mixture, with a hydrogen flow of 160 mL min-1 and air flow of 2000 mL min-1. The standard deviation determined from duplicate runs accounted for 1-14 % of the lipid classes′ relative abundance. Eighteen lipid classes were detected by this technique (including hydrocarbons, wax and steryl esters, fatty acid methyl esters, ketone (standard hexadecanone), triacylglycerols, free fatty acids, alcohols, 1,3-and 1,2-diacylglycerols, sterols, pigments, monoacylglycerols, monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulfoquinovosyldiacylglycerols, mono- and di-phosphatidylglycerols, phosphatidylethanolamines and phosphatidylcholines). The separation scheme involved subsequent elution steps in solvent systems of increasing polarity followed by a subsequent partial burn of Chromarods. Total lipid concentrations were obtained by summing all lipid classes quantified by TLC-FID. Detailed procedures are described in Gašparović et al. (2015; 2017).

Separation of PL present in sample mixture was carried out using UltiMate 3000 Rapid Separation HPLC (RSLC) (Dionex, Germany) system. Acquity UPLC BEH C18 (2.1 × 100 mm with 1.7 µm particles) (Waters, Milford, Massachusetts, USA) column was maintained at 50 ºC while gradient elution was employed. The solvent system included solution A: LC-MS grade methanol: ultrapure water (1:1, v:v; 10 mM NH4-acetate, 0.1% formic acid) and solution B: LC-MS-grade isopropanol (10 mM ammonium acetate, 0.1% formic acid). The gradient started from 55% A / 45% B, reached 90% B in 40 min, 99% B in 2 min, and remained there for 10 min, then to 45% B in 1 min, followed by equilibration for 22 min. The flow rate was 0.15 mL min-1 and injected volume of sample mixture was 10 µL. Immediately before analysis dichloromethane was evaporated and sample was redissolved in a solution of methanol : chloroform (1:2, v:v). HPLC system was online with amaZon ETD ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) for fatty acid composition analysis. The mass spectrometer was equipped with the standard ESI ion source (nebulizer pressure: 8 psi; drying gas flow rate 5 L min-1; drying gas temperature 250 ºC; the potential on the capillary –/+ 4500 V). The lipid profiling was performed in both positive and negative ion modes. The data were collected at a mass range of m/z = 100-1200. The ESI MS/MS was performed using collision energy of 1 eV. For PC species, the positive ionization mode was used ([M+H]+), while for PE, PS, PI, PG and PA species negative ionization mode was used ([M-H]-). For the discovery, annotation, and putative identification of phospholipids, we used an in-house assembled lipid library derived from LIPID MAPS ([http://www.lipidmaps.org/)](http://www.lipidmaps.org/). Here, composition of PL classes is discussed in terms of variety of PL molecular species. We present phospholipid fatty acid profiling, ranging from 14 to 22 carbon atoms (C14–C22).

Data analysis

The principal component analysis (PCA) was performed in order to determine influence of environmental parameters on fatty acid composition of six targeted PL and to find out PL classes′ markers for phytoplankton groups revealed by pigment analysis. The PCA was performed using Statistica Release 7 software. PCA was carried out after log-transformation of the data to reduce the influence of extreme values or outliers. Due to the large number of PLFA variables (>100), after preliminary PCAs that included all PLFA, the significantly correlated variables (factor loadings ≥ 0.5) were selected for further PCA. Variables that were discarded are listed in the Supplementary Tables S1a and b.

For the calculation of lipid contribution to POC, we assumed that carbon content of lipids was 70%.

**Results**

Environmental conditions

Temperature variations in the WRE were within a 3-4 °C range (28.1 to 31.5 °C) with a decreasing trend towards marine end-member (Fig. 2a). The KRE was characterized by temperature ranging from 21.9 to 26.2 °C, with a T increase towards marine end-member (Fig. 2b). Concentrations of TIN were significantly higher in the WRE (4.0-154.9 µmol L-1) than in the KRE (1.9-5.8 µmol L-1). The concentrations of PO43¯ varied within 0.48-1.98 µmol L-1 and 0.21-0.69 µmol L-1 in the WRE and KRE, respectively (Figs. 2a and b). As a result, ratio of N/P was much higher, with wider range as well, in the WRE (2.2-232.5, average 77.6) than in the KRE (7.6-24.4, average 12.0).

 Autotrophic plankton community was much more abundant in the WRE in comparison to the KRE (Figs. 3a and b). Concentrations of Chl *a* ranged from 2.45 to 87.71 µg L-1 (average 30.10 µg L-1), and from 0.28 to 1.31 µg L-1 (average 0.85 µg L-1) in the WRE and KRE, respectively.

The relative abundance of autotrophic plankton biomarker pigments differed for the two estuaries (Figs. 3a and b). Diatoms (pigment *fuco*) were the dominant phytoplankton group in both estuaries. In the WRE, besides diatoms, cyanobacteria (*zea*) dominated the community. The phytoplankton community substantially differed in the KRE. A significant contribution of the pigment *chl c3* suggested an important abundance of prymnesiophytes and chrysophytes. Among these two groups, chrysophytes were more abundant in less saline water according to pigment *lut*, while the substantial contribution of the pigment *hex* indicated important involvement of prymnesiophytes in the more saline waters.

POC and lipids

The concentrations of POC in the WRE and KRE varied between 1015 and 5363 µg L-1 (average 2869 µg L-1) and 140 and 441 µg L-1 (average 274 µg L-1), respectively (Figs. 4a and b). The total lipid (TL) concentrations varied between 128.6 and 661.0 µg L-1 (average 328.1 µg L-1) and 27.7 and 49.3 µg L-1 (average 39.4 µg L-1) in the WRE and KRE, respectively (Figs. 3a and b). Within the TL, the concentrations of PL ranged from 30.3 to 178.2 µg L-1 (average 89.4 µg L-1) and from 8.4 to 18.8 µg L-1 (average 11.9 µg L-1) in the WRE and KRE, respectively (Figs. 4a and b). Total lipid and PL carbon contributions to the POC content (TLC and PLC) in the WRE ranged from 5.4 to 12.1% (average 8.8%) and from 1.3 to 3.7% (average 2.4%), respectively. In the KRE, TLC and PLC ranged from 6.9 to 16.2% (average 11.1%) and from 1.7to 6.5% (average 3.5%), respectively (Figs. 4a and b). Phospholipids contributed more to TL in the KRE than in the WRE. Phospholipids constituted 22.4-31.0% (average 27.5%) and 17.0-39.9% (average 30.8%) of TL in the WRE and KRE, respectively.

Phospholipidomics

Phospholipid molecular diversity

Greater molecular diversity (Figs. 5a and d) was found in the WRE. For both estuaries molecular diversity decreased in the order of PC > PG > PA > PE > PI > PS, being on average 107 (110) PC species, 50 (31) PG species, 39 (16) PA species, 30 (15) PE species, 9 (11) PI species and 1 (2) PS species in the WRE (in parentheses for the KRE). Molecular diversity generally decreased towards the marine end-member, with the exception of PA and PE in the WRE.

Phospholipid saturation/unsaturation

The highest degree of unsaturation was observed for PG in both estuaries: double bonds 1.50-2.57 (average 2.09) and 1.57-2.35 (average 2.07) for the WRE and KRE, respectively (Figs. 5b and e). The greatest double bond variability was observed for PS (0-1.25 in the WRE and 0.50-2.50 in the KRE) and for PI (0.79-1.88 in the WRE and 0.79-3.00 in the KRE). The lowest double bond variability in both estuaries was observed within PC, 1.53-2.05 (average 1.70) in the WRE and 1.56-1.79 (average 1.66) in the KRE. Unsaturation of PE and PA in the WRE was characterized with double bonds 0.74-1.45 (average 1.10), and 1.35-2.26 (average 1.66), respectively. In the KRE unsaturation of PE and PA included double bonds 1.00-2.00 (average 1.49), and 1.15-1.85 (average 1.59), respectively. On average, PL species unsaturation in the WRE (average double bonds 1.2-1.9) was lower than that in the KRE (average double bonds 1.4-2.4). The average polyunsaturated fatty acid relative content (%) (Supplementary Fig. S1) was highest for PG and PC in both estuaries, being 44.1% and 44.7% in the WRE, and 47.0% and 43.5% in the KRE, respectively. In comparison, the relative content of polyunsaturated fatty acids in PE was 19.1% in the WRE, and was much higher, 32.3%, in the KRE. In addition, average polyunsaturated fatty acid content in PA was 34.3% and 30.6% in the WRE and KRE, respectively. Their average relative contents in PI was 40.8% and 30.0%, whereas in a few detected PS it was 4.2% and 40.5% in the WRE and KRE, respectively.

The average relative distributions of double bonds in PL are shown in Figs. 6a and b. Detailed double bond relative distributions (all samples) are shown in Supplementary Fig. S2 for the WRE and KRE. Due to the few PS found in both estuaries, PS is omitted from Figs. 6-9, and Supplementary Figs. S5 and S6. Common features for all samples of both estuaries are observed. Most common fatty acids in all PL were saturated and those with one double bond. Phosphatidylcholines contained more fatty acid acyl chains with three and four double bonds with respect to other PL, whereas PG contained predominantly FA acyl chains with one double bond.

Phospholipid acyl chain length

Phospholipid fatty acyl chain lengths (acyl carbon number) varied among stations and different PL in the WRE with no apparent patterns (Figs. 5c and f). Phospholipid PI were characterized by the average longest fatty acyl chain lengths in both estuaries (18.8 and 19.0 in the WRE and KRE, respectively). In comparison, average fatty acyl chain lengths of PC, PG and PA were 18.0, 17.9, and 17.8, respectively in the WRE, and 17.7, 18.2 and 17.6, respectively, in the KRE. The average fatty acyl chain lengths of a few PS detected were 16.7 and 17.0 in the WRE and KRE, respectively. The regularity and general trend were observed for the KRE: the longest fatty acyl chain lengths were detected in freshwater and marine end-members, while fatty acyl chain lengths were on average shorter in estuarine waters.

The relative distributions of PL average number of fatty acyl chain lengths are shown in Figs. 6c and d. Detailed PL fatty acyl chain lengths and relative distributions (all samples) are shown in Supplementary Fig. S3 for the WRE and KRE. Generally, fatty acids with 18 carbon atoms (C18) were most common in PG and C22 in PI.

Phospholipid fatty acid composition

The relative distribution of identified FA (%) within each PL class is presented in Fig. 7 and in Supplementary Tables S2-S7 for the WRE and KRE. The average relative distributions are shown in Supplementary Fig. S4 and Supplementary Table S8. The composition of fatty acids in PC (Fig. 7a, Supplementary Table S2) was similar for the two estuaries and deviation in the content of individual fatty acid did not affect the uniformity of the general pattern. The most common fatty acids in PC were 16:0, 16:1, 18:1, 18:2, 18:3 and 18:4, while PG (Fig. 7b, Supplementary Table S3) were characterized by fatty acid 18:1 with the substantial contribution of 16:1 and 20:5. A pattern appeared also for the PE, PA and PI (Figs. 7c, d and e, respectively, and Supplementary Tables S4-S6). Fatty acids 16:0, 16:1, 18:0 and 18:1 were most common in PE, while 16:0, 16:1 and 18:1 were most common in PA. Phosphatidylinositol was found to be enriched with fatty acids 22:0 and 22:6 with respect to other PL. The fatty acid relative distributions were mainly retained from the river across the estuaries to the nearby sea, particularly for PC and PG.

The average contribution of odd-chain fatty acids, which are bacterial biomarkers (Dalsgaard et al. 2003), was 8.7% in both estuaries (PS is omitted from the calculation) (Supplementary Fig. S4 and Supplementary Table S8). The content of odd-chain fatty acid varied in individual PL; it was the lowest in PG (average 8.3 and 1.7%), average 15.2% and 7.4% in PE, and average 10.4% and 11.3% in PA for the WRE and KRE, respectively. Their content was on average 8.3 % in PC for both estuaries. The contribution of odd-chain fatty acids to total fatty acids fluctuated considerably in PI and PS, from 0% to 29.5% in PI and from 0 to 50% in PS for both estuaries.

We were interested whether any PL contained higher percentages of eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Fig. 7 and Supplementary Fig. S5). EPA contributed to fatty acid composition on average 3.3%, 9.2%, 4.1% and 3.0% in PC, PG, PE and PA in the WRE and on average 2.4%, 7.8%, 5.5% and 4.8% in PC, PG, PE and PA in the KRE, respectively. In comparison, the contribution of DHA was 2.2%, 6.0%, 2.8% and 6.4% in PC, PG, PE and PA, respectively, in the WRE, and 2.0%, 6.5%, 4.3% and 6.6% in PC, PG, PE and PA, respectively, in the KRE. EPA was the dominant polyunsaturated fatty acid in PG with respect to other PL in the eutrophic WRE samples and was often the dominant polyunsaturated fatty acid in PG in the mesotrophic KRE samples. Occasionally, PI could be a source of DHA.

The average relative distribution combination of double bonds in two fatty acyl chains and the combination of two fatty acyl chain lengths are presented in Figs. 8 and 9 and Supplementary Tables S9 and S10 for the WRE and KRE. Details for all samples are given in Supplementary Figs. S8 and S9 and Supplementary Tables S11-S22. Again, common features were noticed. The dominant double bond combination of two fatty acid chains in PC, PE, PA and PI was one saturated and one unsaturated fatty acid with one double bond (0+1), while PG was characterized by double bond combination 1+1 being the most common species (Fig. 8 and Supplementary Tables S11-S16).

The relative distribution of the fatty acyl chain length combination (%) exhibited similarities between two estuarine systems (Fig. 9, Supplementary Fig. S9 and Supplementary Tables S17-S22). This is especially evident in PC for which the combination of fatty acyl chain lengths C16 and C18 (16+18) was the most common. PG was characterized by the predominance of fatty acid combination 16+18 and 18+18. Combinations of longer fatty acid chains dominated within PI, whereas fewer similarities were evident for PE and PA.

Influence of environmental conditions on phospholipid fatty acid composition

The PCA incorporating all PLFA data and variables including temperature, salinity, pH, oxygen, Chl *a*, TIN and PO43¯ concentrations were performed to define possible influence of environmental conditions on fatty acid composition of six targeted PL. The analyses showed the different effects of environmental parameters on the fatty acid composition of PL for the two estuaries (Supplementary Fig. S6). The greatest positive PC1 loadings for the WRE samples had temperature, TIN, Chl *a* and O2 with fatty acid 18:3 in PC, fatty acids 18:3, 18:4, 20:3 and 22:2 in PG, fatty acids 16:0 and 20:2 in PE and 18:2, 18:3, and 22:4 in PA. For the KRE samples, the greatest positive PC1 loadings had TIN, Chl *a* and O2 with fatty acids 14:0, 15:1, 16:2, 17:0 and 22:2 in PC, fatty acids 18:3 and 22:6 in PG, fatty acid 16:0, 18:1 and 20:4 in PE, fatty acids 16:1 and 18:1 in PA. The greatest negative PC1 loadings for the WRE samples had salinity and fatty acids 15:0, 22:3, 22:4 and 22:6 in PC, fatty acid 18:1 in PG, fatty acid 15:0 in PE, fatty acid 20:0 in PA. The greatest negative PC1 loadings for the KRE samples had salinity and fatty acids 15:0, 20:3, 20:4, 20:5, 22:0 and 22:4 in PC, fatty acids 14:0, 20:2, 20:4 and 20:5 in PG, fatty acids 16:1, 18:0, 20:3, 20:4, 22:0 and 22:4 in PE and fatty acids 16:0 and 20:1 in PA. The greatest positive PC2 loadings for the WRE samples had PO43¯, fatty acid 22:2 in PC, fatty acids 14:0 and 16:1 in PG, fatty acids 15:1 and 17:1 in PE, fatty acids 14:0, 15:0 and 20:1 in PA. For the KRE samples, the greatest negative PC2 loadings had fatty acids 14:1, 15:1, 16:4 and 18:3 in PC, fatty acid 18:0 in PG, fatty acid 22:4 in PE. These analyses suggest that only common feature for the two estuaries was increased contribution of longer chain fatty acids (C20 and C22) at higher salinities.

Analysis of possible phospholipid fatty acids as specific phytoplankton group(s) markers

Intending to define possible PC, PG, PE and/or PA fatty acids as specific markers for phytoplankton group(s), we performed PCA considering estuarine phytoplankton marker pigments and PLFA variables. After preliminary PCA, the significantly correlated variables (factor loadings ≥ 0.5) were selected for further PCA (Supplementary Fig. S7). For both estuaries correlations of PLFA with some pigment were observed. At the same time, many pigments did not show any significant correlation with PLFA, including *fuco*, *allo*, *hex* and *viola* in the WRE and *allo* and *zea* in the KRE. Different PLFA correlated with the particular pigment for the two estuaries. For example, *perid* was grouped with PC22:2 in the WRE, while *perid* was grouped with PC14:1, PA18:0 and PA18:2 in the KRE.

**Discussion**

Phytoplankton play central roles in food webs and global cycling of elements (C, P, N). Changes in their cellular compositions affect organic matter flux both to higher trophic levels and to deep waters (Falkowski et al. 2004). In this paper, we focused on the plankton PLFA composition of two very different estuaries with regards to temperature, nutrient loads, and consequently different phytoplankton communities. Data on the total lipid-derived fatty acid compositions in the estuaries, seas/oceans and phytoplankton monocultures are relatively abundant (e.g. Scribe et al. 1991; Galois et al. 1996; Derieux et al. 1998; Canuel 2001; Pedrosa-Pamies et al. 2018). However, data on total PLFA are scarce (Table 1a). Here, we took the step forward of carrying out a more detailed PLFA characterization in two estuaries by analyzing the fatty acid composition of individual PL including PC, PG, PE, PA, PI and PS.

Consistent with significantly higher nutrient concentrations, autotrophic plankton abundance, according to the Chl *a* content, was much higher in the eutrophic WRE than in the KRE. In general, eutrophic coastal regions in the tropics are sites of high phytoplankton biomass (Cotovicz et al. 2018). In line with very different environmental conditions, the phytoplankton community differed between the estuaries, but also across salinity gradient in each estuary. However, it is not surprising that we found dominance of diatoms in both estuaries knowing that coastal river plumes are in general the places of diatom growth owing to the continuous nutrient inputs (Wawrik and Paul 2004). The abundance of cyanobacteria in the WRE reflects influence of high temperature on their dominance (Mesquita et al. 2020).

Here we assumed that PLFA were mainly of autotrophic plankton origin (Gašparović et al. 2014). However, the contribution of heterotrophic bacteria cannot be neglected. The average contribution of bacterial fatty acid markers (odd chain fatty acids) to total fatty acids was 8.7% in both estuaries, which was considerable. Total PL content is dependent on plankton biomass, consequently higher concentrations of PL were detected in the eutrophic WRE (30.3-178.2 μg L-1) than in the mesotrophic KRE (8.4-18.8 μg L-1). Literature and our data on PL concentrations (Table 1b) expectedly show that total PL content increases from the oligotrophic to eutrophic aquatic environment.

The PL relative content (%) in plankton is influenced by environmental conditions and is species-specific (Guschina and Harwood 2009). The PL content in TL in different aquatic environments spans across a wide range, 6-55% (Table 1c). Here, PL relative content (averages 27.5% and 30.1% in TL in the WRE and KRE, respectively) is in accordance with earlier findings. Higher PL relative content (%) in both POC and TL was obtained in the KRE than in the WRE (i.e. more PL synthesized per Chl *a* in the KRE than in the WRE). These results indicate importance of more favorable N/P ratio in the KRE than in the WRE. Unfavorable environmental conditions lead to phytoplankton lipid remodeling. Phytoplankton development under oligotrophic conditions, especially during nitrogen deficiency leads to the triacylglycerol accumulation at the expense of other lipid classes, including PL (Parrish and Wangersky 1987; Bourguet et al. 2009; Novak et al. 2019). Phosphorus limitation and high seawater temperatures lead to enhanced glycolipid instead of phospholipid accumulation (Gašparović et al. 2013).

Here found large PL molecular diversity, especially within PC, possibly reflects a complex community (freshwater, estuarine and marine), as well as their responses to fluctuations in environmental conditions, e.g. salinity, nutrient concentrations, light intensity, temperature, ....

Although we investigated two notably different estuaries, similarities in the PLFA composition, number of double bonds and fatty acyl chain lengths were observed, particularly for PC, PG and PI. We assume that plankton maintains basic PC, PG and PI fatty acid composition to preserve the roles they play in the cell. At the same time, the composition of other, less common, fatty acids in PC, PG and PI differed between stations and the estuaries. The main PC features that were essential for optimal function in estuarine plankton membranes were: 1) fatty acids 16:0, 16:1, 18:1, 18:2, 18:3 and 18:4, 2) higher content of unsaturated fatty acids with three and four double bonds with respect to other PL, 3) dominance of double bond combination in two fatty acid chains 0+1, and 4) relative invariability of total unsaturation with respect to other PL. These could indicate the conservatism of cellular PC synthesis in terms of preserving the integrity of the cell itself and maintaining the physicochemical properties of membranes, at least for the environmental conditions in estuaries covered by this study and phytoplankton groups detected.

The only PL present at measurable level in thylakoid membranes are PG (Wada and Murata 2007). Higher fatty acid unsaturation in PG, with respect to other investigated PL, can be expected given the role of PG in photosynthetic electron transport in thylakoids (Wada and Murata 2007), knowing that fatty acid unsaturation improves photosynthetic electron flux across more liquid thylakoid membrane (Siegenthaler and Murata 2004). The most important PG fatty acids 18:1, 16:1 and to a lesser extent 20:5 were probably favorable for maintaining thylakoid membrane function. It seems that double bond combination 1+1 and fatty acyl chain length combinations 16+18 and 18+18 were basic in PG and important for PG proper functioning.

Long-chain fatty acids C20 and C22, were probably important for PI to successfully conduct particular cellular function(s), including the role in cell growth, signal transduction processes and membrane anchoring of proteins in plants (Riekhof and Benning 2009).

The great variability in fatty acid saturation/unsaturation and chain lengths of PE, PA, and particularly PS indicate that fatty acid composition of those PL is species-specific, dependent on the plankton growth phase and/or a response to diverse environmental conditions.

Two investigated estuaries differed, among others, in water temperature. Since unsaturated fatty acids are important in adjusting membrane fluidity, it is suggested that the degree of unsaturation of the membrane lipid fatty acids increases with temperature decrease (Murata and Los 1997). Our data on fatty acid unsaturation in the two estuaries, i.e. higher polyunsaturated fatty acid contribution to total fatty acids detected in the colder KRE, are in the agreement with previous reports. However, the fatty acid composition is influenced by season, as well. Connelly et al. (2016) found more saturated fatty acids during winter in the Beaufort Sea shelf and explained by fatty acid cycling and/or fatty acids from heterotrophs.

Statistical PCA indicated that the increase in salinity possibly influenced the increased proportion of long chain fatty acids in PL. Fatty acid remodeling is continuous process that is triggered by changing environmental conditions (e.g. Urzica et al. 2013), and depends on the phytoplankton growth phase (e.g. Boelen et al. 2001). At the same time, fatty acid remodeling can be accomplished very fast, within an hour or two (Urzica et al. 2013, Rai and Gaur 2001). Therefore, we concluded that due to the complex and different environmental conditions and plankton community structures in the WRE and KRE we did not identify particular environmental parameters responsible for the synthesis of specific fatty acid(s) of six PL classes investigated. Also, as indicated by the PCA analysis (Fig. S7), we could not define particular PLFA marker(s) for phytoplankton group(s), most likely due to complex phytoplankton composition and different environmental conditions in the WRE and KRE.

Autotrophic plankton is the origin of the long-chain omega-3 polyunsaturated fatty acids EPA and DHA to higher trophic levels. They have key roles in the marine species growth and are critical to their survival (Jónasdóttir 2019). We found that there was no significant difference between two estuaries regarding EPA and DHA proportion in PL. Eventually, PG and PI might be an important source of EPA and DHA, respectively.

**Conclusions**

Herein, we present the comprehensive analysis of six main phospholipids (PC, PG, PE, PA, PI and PS) at the level of individual lipid species, for the two notably different estuaries, the pristine and temperate Krka River Estuary and the eutrophic, subtropical Wenchang River Estuary. Favorable nutrient conditions, as found in the KRE, lead to enhanced PL content of plankton, which are at the same time richer in polyunsaturated fatty acids that consequently benefit higher trophic levels.

Estuarine plankton maintains favorable PC, PG, and PI fatty acid composition for optimal membrane function(s) in which these PL are involved, irrelevant of the environmental conditions and plankton community structure. This suggests that mechanisms of preserving essential fatty acid composition is universal, despite probable energy investment by the cell due to the advantages of maintaining fine control of cell functioning. It is indicated that several features are preferable for the role of PG, including higher acyl chain unsaturation with respect to other PL. Regarding PC and their functions, important are C16 and C18 fatty acids. PC are characterized by lower variability of total unsaturation with respect to other PL. In comparison, long-chain fatty acids (C22 and C20) seem to be important for the roles of PI in the estuarine plankton cells. The fatty acid composition of PE, PA, and PS differed between the estuaries as well as throughout the salinity gradient in each estuary. This suggests the adaptability of plankton to remodel these PL depending on the environmental conditions as well as difference in the plankton community structure.

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**Declarations**

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**Conflicts of interest** The authors declare that they have no conflict of interest.

**Availability of data and material** Data are available from the corresponding author on reasonable request.

**Code availability** Not applicable

**Authors' contributions** IVŠ and SK did HPLC/MS/MS lipid and data analysis, TN analyzed and processed TLC-FID lipid data, MČ did environmental data analysis, ZLj analyzed pigments, EH and RZ performed nutrient analysis. BG, MM and ZZ conceived, planned and initiated the study; TN, MČ, JD, RZ, ZZ and BG performed field sampling, BG and IVŠ wrote the first manuscript draft. All authors discussed the results, edited the manuscript and approved the final submitted manuscript.

**Figure Captions**

**Fig. 1** Sampling stations named after corresponding salinities; a) the Wenchang River Estuary (WRE) and b) the Krka River Estuary (KRE)

**Fig. 2** Environmental properties in the a) WRE and b) KRE and their freshwater and marine water end-members: PO43¯ (black column), TIN (grey column) and T (circles)

**Fig. 3** Chlorophyll *a* content and the relative abundance of pigments of the major autotrophic plankton groups (%) in the a) WRE and b) KRE and their freshwater and marine water end-members. Note different scales for Chl *a* in two estuaries

**Fig. 4** Organic matter in the a) WRE and b) KRE and their freshwater and marine water end-members: particulate organic carbon (POC), total lipids (TL), phospholipids (PL), the contribution of TL carbon to POC (TLC) and the contribution of PL carbon to POC (PLC)

**Fig. 5** The average phospholipid characteristics in the WRE (a-c) and KRE (d-f) and their freshwater and marine water end-members. The number of molecular species (diversity) (a and d), fatty acyl double bond (number of DB) (b and e) and fatty acyl chain length (number of carbon atoms) (c and f) of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylserines (PS). Due to very few PS species, it is omitted from the Figs. 5b,c,e and f

**Fig. 6** The average relative distribution of double bonds (%) (a and b) and fatty acyl chain length (number of carbon atoms) (%) (c and d) of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) (a and c) and phosphatidylethanolamine (PE) and phosphatidic acid (PA) (b and d) in the WRE (a and c) and KRE (b and d)

**Fig. 7** Fig. 7 Phospholipid fatty acid relative distribution (%) in the Wenchang River Estuary (WRE) and the Krka River Estuary (KRE), and their freshwater and marine water end-members. Data are presented for phosphatidylcholine (PC) (a), phosphatidylglycerol (PG) (b), phosphatidylethanolamine (PE) (c), phosphatidic acid (PA) (d), phosphatidylinositol (PI) (e), and phosphatidylserine (PS) (f)

**Fig. 8** The average relative distribution of fatty acids' double bond combinations (%) of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) (a and c) and phosphatidylethanolamine (PE) and phosphatidic acid (PA) (b and d) in the WRE (a and b) and KRE (c and d)

**Fig. 9** The average relative distribution of fatty acyl chain length (number of C atoms) combination (%) of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) (a and c) and phosphatidylethanolamine (PE) and phosphatidic acid (PA) (b and d) in the WRE (a and b) and KRE (c and d)

**Fig. 1**



**Fig. 2**



**Fig. 3**



**Fig. 4**



**Fig. 5**



**Fig. 6**



**Fig. 7**







**Fig. 8**



**Fig. 9**

