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Original Scientific Paper

Fatty Acid Signatures from Bacteria at the Freshwater/Seawater Boundary of the Krka Estuary in Winter, Spring and Autumn*

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Distribution of fatty acids was determined at the halocline and in adjacent brackish and marine waters in the Krka Estuary, on the East coast of the Adriatic Sea, in winter 1987, spring 1988 and fall 1990. Particulate matter (> 0.7 μm) was extracted and analyzed for fatty acids by gas chromatography and gas chromatography/mass spectrometry. Different groups of fatty acids were examined: branched and monounsaturated (vaccenic). Bacterial signatures were highly variable in the estuary, depending on season and the extent of accumulation of organic matter at the halocline. Imprint of branched fatty acids, probably representative for the largest bacterial populations, varied by three orders of magnitude. This imprint was generally low at marine stations with an exception of fall 1990, and high in river/brackish waters. Bacterial signatures were interpreted in terms of relationship between their occurrence and growth conditions expressed as suspended matter, organic carbon and chlorophyll *a* concentrations, as well as nature of organic matter in various water types, fresh, brackish and marine.

INTRODUCTION

Estuaries play an important role in the cycling of numerous elements and chemical species transported from the continent to coastal marine environments *via* river discharge. It is particularly true for organic matter in-

* Dedicated to Marko Branica on the occasion of his 65th birthday.

puts for which the nature, autochthonous *versus* continental, labile *versus* refractory and their fate in estuaries are not well known, due to high complexity of inter-related physical, chemical and biological processes, as well as extreme variability with time.

Present estimation of continentally-derived organic matter inputs to coastal waters is 0.4 Gt C yr^{-1} for both dissolved and particulate organic carbon.[1] The degradability of this organic matter is not very high, compared to algal and phytoplankton derived organic matter.[2] The role of microorganisms is of primary importance for processes governing the recycling of both continentally-derived and autochthonously-produced organic matter in estuaries. The production of bacterial biomass represents a major link between detritus, dissolved organic matter and higher trophic levels.[3]

During the programme devoted to the Krka Estuary, sampling and analytical strategies have been developed to study microorganisms in both fresh and seawaters, and at the halocline by choosing the microbial biomarker approach in the fatty acid series.[4–7]

The purpose of this paper is to improve the detection of bacteria attached to particles, and to interpret the lipid signatures from these various microorganisms, using branched fatty acids and vaccenic acid. Such chemical approaches could contribute to understand the conditions of development of bacteria and their role in complex estuarine environments.

EXPERIMENTAL

Sampling Site : The Krka Estuary

The Krka estuary is an unique model site for studying biogeochemical processes at the halocline.[8,9] The total length of the estuary is ~ 22 km. The estuary is relatively narrow, except for two wider parts: Prokljan Lake and the Sibenik area (Figure 1). Its depth varies between 1–2 m, below the waterfalls, and 42 m in the Sibenik Channel. The mean daily flow of the river varies within the year between 2 and 415 m^3 s^{-1}, with a ten-year mean (from 1981 to 1990) of about 55 m^3 s^{-1}. The estuary is submitted to the influence of a low tide: the maximum tidal range is ~ 0.4 m at the mouth and ~ 0.3 m at the head of the estuary.[10] Because of low terrigenous inputs, organic matter is mainly of phytoplanktonic origin. The estuary is characterized by permanent stratification between the bottom seawater, which enters the estuary at depth, and the surface fresh or brackish waters. The suspended particulate matter load is low in this karstic environment and the freshwater/seawater boundary is highly compressed and stable, and could be easily sampled by divers.[9]

Water samples were collected in winter (March 1987), in spring (May 1988) and in autumn (October 1990). Sampling sites were distributed from the water falls (E0) through the estuary to marine waters off the north coast of Zlarin Island (Figure 1). Water was sampled in surface freshwater (E0), brackish water (P_3, E_3 and E_{4a}), at the boundary layer (P_3, E_3 and E_{4a}) and in bottom seawater layer (P_3, E_3 and E_{4a}). Water was also sampled at station C_1 or C_2 as marine end-members (Table I).

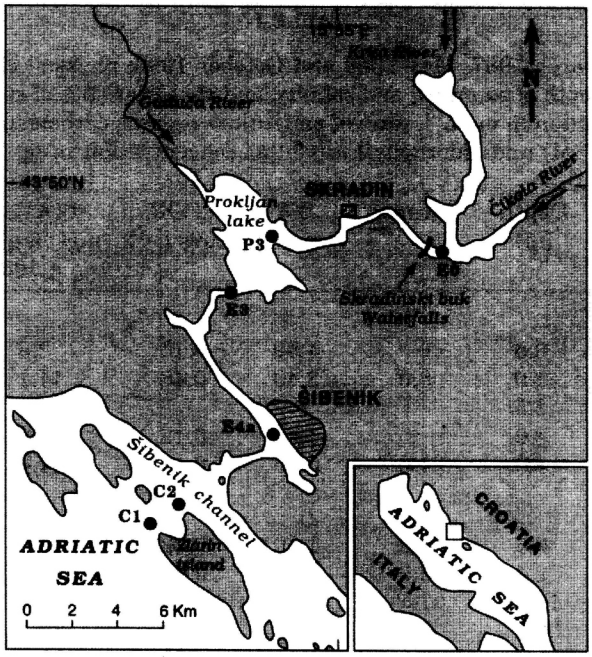

Figure 1. Sampling sites occupied in the Krka Estuary, in March 1987, May 1988 and October 1990.

Sampling and Filtration

Water samples (20 L) were collected by *in situ* pumping with the help of a diver to control with precision the position of the halocline (March 1987 and October 1990) or by using a cleaned 5 L Niskin bottle, closed horizontally by a diver at the halocline (May 1988). A few hours after sampling, water was filtered under low pressure, using a stainless steel filter holder and glass fibre filters (Whatman GF/F, 0.7 μm pore size, 142 mm diameter). After collection, filters were kept frozen at < – 20 °C until analysis.

Analysis of Fatty Acids

Extraction of the filters was performed by two methods. The first classical method used for 1987 samples consisted of an extraction performed in a Soxhlet (2 × 12 h) with a mixture of methylene chloride-methanol (3:1, *v/v*). For samples of 1988 and 1990, the extraction with the one-phase methylene chloride-methanol-water Bligh and Dyer procedure[11] was selected in order to avoid any risk of degradation of polyunsaturated compounds. Each extract was spiked with a known amount of deuterated tricosanoate as internal standard for gas chromatography (GC) quantitation. Extracts dissolved in a mixture of methanol-toluene (1:1, *v/v*) were saponified during 2 h in a solution of KOH 1 N under argon. After cooling, addition of distilled water and acidification to pH 2 with HCl (4 N), lipid fractions were extracted three times with a hexane-ether mixture (9:1, *v/v*). The extracts were

TABLE I

Krka Estuary, March 1987, May 1988 and October 1990; physical characteristics of water samples, depth of sampling and salinity, and characteristics of suspended organic matter : concentration in mg L^{-1}, ratio of particulate organic carbon/suspended matter (POC/SM) and chlorophyll *a* (Chl *a*) concentration in μg L^{-1}.

Station	Depth (m)	Salinity	SM (mg L^{-1})	POC (mg L^{-1})	POC/SM mgC/mg SM × 100	Chl *a* μg l^{-1}
March 1987						
Eo surf	0.5	0	4.00	0.15	3.8	0.53[a]
E4a surf	1.0	8.0	3.40	0.48	14.1	3.11[a]
E4a int	3.5					3.51[a]
E4a bot	4.0		1.40	0.12	8.6	0.93[a]
C2	4.0	34.0	0.90	0.13	14.4	0.47[a]
May 1988						
E3 surf	0.5	2.0	1.60	0.42	26.0	1.14[a]
E3 int	2.4	9.7	2.40	0.69	28.8	1.43[a]
E3 bot	10.0	37.4	3.45	0.22	6.4	0.52[a]
E4a surf	0.2	6.0	1.60	0.63	39.4	2.05[a]
E4a int	1.8	13.0	4.57	1.68	36.9	26.34[a]
E4a bot	30	38.0	0.67	0.09	13.3	0.18[a]
C2	6.0	37.4	1.07	0.20	18.3	0.24[a]
October 1990						
Eo	0.2	0	2.60	0.66	25.4	5.72[b]
P3 surf	0.2	16.0	1.94	0.43	22.2	
P3 int	0.7	22.0	7.60	0.48	6.3	
P3 bot	12.7	38.0	1.20	0.17	14.2	
C1	0.2	38.0	0.36	0.04	11.1	0.13[b]

(a) Chl *a* measured by HPLC, from Denant *et al.*[12]
(b) Chl *a* measured following the method of Strickland and Parsons,[23] by Moriera-Turcq[24]

evaporated to dryness under a stream of pure nitrogen. Then, fatty acids were isolated by adsorption chromatography on a small column (4 mm i.d.) filled with 2 g of SiO_2 (Merck G_{60}, 5 % water deactivated). The first two fractions, eluted by 6 mL of hexane, and 6 mL of hexane-ethyl acetate (50:1, *v/v*), contained aliphatic and aromatic hydrocarbons, respectively ; the third fraction, eluted by 20 ml of ethyl acetate, contained fatty acids, sterols and alcohols. The fatty acids thus obtained were converted into methyl esters by a solution of 14% BF_3 in methanol and the esters purified by adsorption chromatography on $Si0_2$ under the same conditions as described for the isolation of fatty acids. Methyl esters (FAME) were found in the second fraction. Samples collected in 1990 have been analyzed using a different procedure, involving a separation of lipid classes, before analysis of fatty acids. Thus bacterial compound amounts have been added from different lipid classes (in prep.).

Gas Chromatography Analysis (GC)

GC analyses of FAME were performed with a Girdel 3000 gas chromatograph and a Varian 6000 gas chromatograph equipped with a flame ionization detector (FID) and a Ross-type injector. Samples were analyzed using a non-polar fused silica capillary column (30 m length, 0.25 mm i.d.) coated with DB5 (Chromoptic, France). The oven temperature was programmed from 100 to 300 °C, at a rate of 2 °C min^{-1}. A polar fused silica capillary column (25 m × 0.32 mm i.d.) coated with Silar 5CP (Chrompack, France) was also used for fatty acid identification. The oven temperature was programmed from 100 to 195 °C, at a rate of 2 °C min^{-1}. Helium was used as carrier gas (flow rate 2 mL min^{-1}). The detector temperature was 320 °C. Components were quantitated by using the FID response and calibration with respect to the internal standard.

Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

GC-MS analyses were performed with a R10-10C Nermag quadrupole spectrometer coupled to a Girdel 32 gas chromatograph. The chromatographic conditions (non-polar column, injector, oven temperature, carrier gas) were as described above. Operating conditions for the mass spectrometer were : transfer line temperature 310 °C; electron energy 70 eV, 1 scan per second, mass range 31–550 a.m.u. Electron impact mass spectra were acquired and processed using an on-line PDP 11/23 computer with a Sidar 111 data system.Fatty acid identifications were confirmed by comparing mass spectra and retention times with those obtained from commercial standards.

Fatty acids are designated for example as 18:1ω7, where 18 is the total number of carbon atoms, 1 is the number of double bonds and 7 its position in the carbon chain from the terminal methyl group as indicated by the greek letter omega.

RESULTS

The sampling strategy was designed to collect waters from the riverine station E0 to the marine end-member stations C1 or C2 and at intermediate stations with a stratified and stable brackishwater-seawater interface (P3, E3 and E4a)[12,13,14] (Figure 1). The general parameters salinity, suspended particulate matter, particulate organic carbon and chlorophyll-*a* concentrations, are presented in Table I for samples collected during the 3 cruises. Table II gives, for the different stations, the concentrations of : total fatty acids (FA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), odd carbon numbered branched fatty acids (BrFA),15:0 iso and anteiso + 17:0 iso and anteiso) and vaccenic acid, 18:1ω7. These two signatures, odd carbon-chain branched compounds and vaccenic acid, as well as others such as total odd- and even-chain branched compounds have been recognized as bacterial markers in sediments[15–19] and in estuarine particles.[6,14]

Table II

Fatty acid concentrations (ng L^{-1}) measured in the Krka Estuary in winter 1987, spring 1988 and autumn 1990, for the following species : total fatty acids (FA), monounsaturated (MUFA), polyunsaturated (PUFA), branched-chain iso and anteiso 15:0 and 17:0 (BrFA), and vaccenic acid 18:1ω7.

Station	$\sum$ total FA	$\sum$ MUFA	$\sum$ PUFA	$\sum$ Br FA	18:1ω7
March 1987					
Eo surf	7 000	2 422	784	252	357
E4a surf	75 900	25 123	16 318	1 594	3 036
E4a inter	28 400	7 554	7 753	483	1 022
E4a bot	9 400	2 782	1 589	150	385
C2	2 600	824	330	36	107
May 1988					
Eo surf	6 600	904	1 296	150	100
E3 surf	3 600	911	724	119	169
E3 int	11 200	2 789	2 912	202	526
E3 bot	1 900	526	274	57	87
E4a surf	10 600	1 863	3 100	331	850
E4a int	26 600	6 437	5 054	293	771
E4a bot	1 900	484	106	47	49
C2	4 900	1 891	799	78	225
October 1990					
Eo surf	4 578	1 126	993	183	92
P3 surf	13 601	5 137	699	486	1 300
P3 int	11 520	2 821	505	217	722
P3 bot	9 443	1 999	242	266	498
C1	11 431	3 734	3 127	783	415

DISCUSSION

Bacterial signatures will be first discussed using the data of odd-chain branched fatty acids, iso and anteiso 15:0 and 17:0, and 18:1ω7, which are considered as largely representative of bacteria and whose concentrations have been determined for all three cruises, using comparable methods (Table II). The sum of all branched compounds in the carbon range 14–17 is also used as a criterion of bacterial signature in the branched fatty acid series. No significant differences could be observed between the two criteria base on odd-chain and all branched fatty acids as a good relationship exists for the two series of fatty acids ($r^2 = 0.994$; $n = 17$). An exception must be noted at station E4a at the interface, where the concentration of 16:0 iso is very high,[14] 1729 ng L^{-1}, and could sign the presence of a specific bacterial population accumulated at the interface.

Although the material analyzed is composed of particles > 0.7 μm, lipid signatures will be expressed and discussed as volume concentrations of specific fatty acids, in order to compare this information directly with chlorophyll *a*, a parameter generally expressed per liter of water.

Bacterial signatures were highly variable in the estuary similar to those observed from direct countings and determinations of bacterial activity.[20] Marine waters showed the lowest signature of branched fatty acids in winter and spring : 36 ng L^{-1} at the end-member C2 station in winter, < 100 ng L^{-1} at C2 and in bottom sea water at stations E3 and E4a in spring. A completely opposite situation existed in fall 1990 where highest signature of branched fatty acids was observed at the marine end-member station C1, with a concentration of 783 ng L^{-1}. Highest signature of the branched fatty acids, > 1000 ng L^{-1}, was encountered in winter, in surface brackish water, at station E4a.

Trends of the 18:1ω7 were generally similar, with highest concentrations observed in winter. However maxima of the two signatures where not always simultaneously encountered, as for example in October, when maxima of the branched acids and 18:1ω7 signatures occurred at the marine station C1 and at P3 surface, respectively.

Each season corresponded to different situations about water stratification, primary production or chlorophyll-*a* level and accumulation of suspended matter occurring at the halocline. In spring, the gradient of salinity was highly marked at the halocline, with increased amounts of suspended matter, particulate carbon, chlorophyll *a* (Table I) and total fatty acids, MUFA and PUFA (Table II). At station E3, a significant enrichment of microbial signatures (branched fatty acids and 18:1ω7) was observed at the halocline, whereas at station E4a, concentrations of branched fatty acids and 18:1ω7 at the halocline were intermediate between surface and bottom waters. Recent studies showed that accumulation of biologically-derived organic matter at the halocline induce the formation of a thin film that can be identified by direct visual observation, as well as by measurements of surface-active material, both dissolved and particulate.[9,21] This film contains a potential food supply for heterotrophic organisms. This condition existed undoubtedly in spring at E3 int and E4a int with indications of freshly synthesized organic matter given by concentrations of chlorophyll *a*, 1.43 and 26.34 μg L^{-1}, respectively, and concentrations of PUFA, 2.9 and 5.0 μg L^{-1}, respectively, higher than in adjacent waters. Thus, the corresponding detrital matter would be fresh, leading to a high microbiological activity in the film with regard to adjacent waters. Of note is that, at station E4a, the bacterial signature, as that of Chl *a*, is undoubtedly associated with sewage effluents and high inputs of nutrients from the city and harbour of Sibenik, whereas station E3 can be considered as a rather pristine environment.

Winter and fall showed different situations. In winter, the interface is slightly enriched in chlorophyll *a* with regard to adjacent waters, but presented an intermediate concentration in branched fatty acids and 18:1ω7,

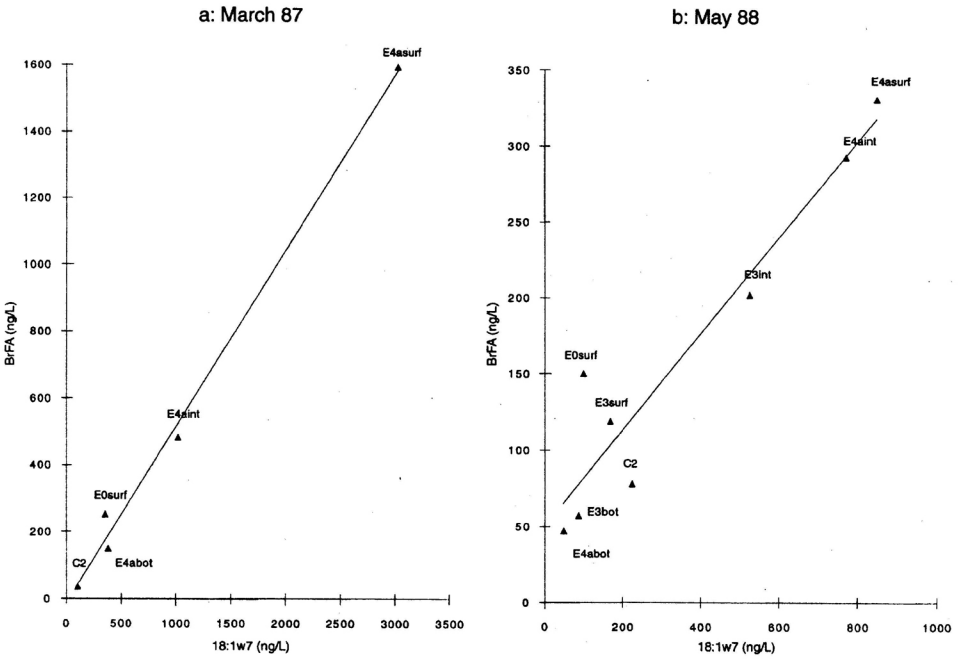

Figure 2. Relationship between bacterial signatures expressed as concentrations of odd carbon-chain 15:0 and 17:0 iso and anteiso fatty acids and vaccenic acid, 18:1ω7, in a: winter 1987; b: spring 1988.

like for total FA, MUFA and PUFA, lower than in surface waters, but higher than in bottom waters. The situation encountered in fall 1990 was completely different; the halocline was characterized by a depleted bacterial signature, although suspended matter was highly enriched. A strong difference appeared in the nature of organic matter accumulated at the halocline at this season. At P3 int a low organic carbon content of the suspended matter, 6.3%, and a low concentration of PUFA, 0.5 μg L^{-1} together with a low value of the ratio PUFA/total FA, 0.04, suggested that the organic matter was highly degraded. As a consequence, bacteria would find a poor food supply on the particles, leading to the drastic change observed for their signature.

Within varying seasonal conditions and large range of water types (riverine, brackish, mixed at the halocline and marine), we tentatively compared different patterns described by relationships between the two signatures provided by branched fatty acids and 18:1ω7.

Figure 2a shows that a good correlation exists between the two bacterial signatures (r^2 = 0.994; slope value: 0.526) in winter. The same tendency is observed in spring with a r^2 value of 0.902 (Figure 2b), although with a different value of the slope: 0.316.

This shows that the two types of compounds are closely associated in the lipid signatures of bacteria. Additionally these two profiles suggest that the bacterial signatures exhibit no discrimination according to salinity and type of water.

No clear relation is found in autumn 1990; the interface is undoubtedly disturbed with a low amount of PUFA. Moreover, station C1 exhibits very different characteristics: low concentration in POC and chlorophyll *a*, high level of PUFA.

If we consider the two winter and spring situations, differences of values of slope in the relation BrFA/18:1ω7 suggest that the two signatures reflect different populations and/or various physiological status according to the environmental conditions.[22] Among various factors, the abundance of organic carbon, much more marked in spring than in winter (Table I), could play a role in the control of bacterial assemblages.

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SAŽETAK

Odzivi bakterijskih masnih kiselina na granici riječna/morska voda ušća rijeke Krke zimi, ljeti i u jesen

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Određena ja raspodjela masnih kiselina na haloklini i uzorcima obližnje morske i bočate vode u ušću rijeke Krke zimi 1987, u proljeće 1988, i u jesen 1990. Masne kiseline analizirane su plinskom kromatografijom i plinskom/masenom kromatografijom nakon ekstrakcije partikularne tvari (> 0.7 μm). Ispitane su skupine razgranatih i mononezasićenih masnih kiselina. Bakterijske naznake su bile vrlo promjenjive ovisno o sezoni i stupnju akumulacije organske tvari na haloklini. Koncentracije tragova razgranatih masnih kiselina, koje najvjerojatnije potječu od najzastupljenije populacije bakterija, variraju u rasponu od tri reda veličine. Te su naznake općenito niske na morskim postajama, osim u jesen 1990, i visoke u riječnim i bočatim vodama. Izmjerene vrijednosti interpretirane su kao rezultat rasprostranjenosti bakterija i uvjeta rasta izraženih standardnim parametrima: suspendirane tvari, organski ugljik i koncentracije klorofila *a*, te prirodom organske tvari u različitim tipovima riječne, bočate i morske vode.