**Enhancement of biotic degradation of terrestrial particulate
organic matter in estuarine salinity gradient: Interactive
effects of organic matter pools and change of bacterial
communities**

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ABSTRACT:

Suspended particulate matter (SPM) samples collected in the Rhône River during and after potamoplankton bloom were incubated *in vitro* at different salinities and the degradation of terrestrial vascular plants was monitored using specific lipid tracers (triterpenes, cuticular waxes and phenolic acids). Degradation was observed at a salinity close to that of seawater. Changes in salinity allow for the establishment of specific bacterial communities that seems to harbor bacteria possessing a capacity to degrade algal and vascular plant components more efficiently than these initially present within the river. The presence of potamoplankton (plankton of rivers) clearly enhanced remineralization of vascular plant material. The algal material resulting from the bloom event, which appeared to be intensively degraded during the incubations, could act as a co-substrate or induce the appearance of a bacterial community efficiently degrading terrestrial vascular plants. In SPM samples collected in the salinity gradient of the Rhône Estuary, bacterial degradation of vascular plant material increased with the proportion of algal sterols strongly suggesting the involvement of priming effect. Bacterial diversity analyses showed the dominance of Betaproteobacteria in freshwater and Alphaproteobacteria and Gammaproteobacteria in seawater. These results revealed the importance of potamoplankton and of the structure of bacterial communities during the degradation of terrestrial vascular plant material in estuaries.

INTRODUCTION

The riverine export of organic matter, whether dissolved or particulate is the main land-to-ocean contributor in coastal carbon fluxes. Early estimations ranged that contribution between 0.2 and 1 PgC yr⁻¹ (Kempe 1979, Meybeck 1982), while more recent estimates limited that number to 0.45 PgC yr⁻¹ (Cole et al. 2007) or 0.15 PgC yr⁻¹ (Hedges et al. 1997). Given the current importance of exhaustive and precise carbon budgets and fluxes, it is crucial to look at the behavior of organic matter upon its arrival in coastal ecosystems.

The fate of terrestrial organic matter in the ocean is not fully understood (Handa 1977 ; Hedges et al. 1997) and the importance of this knowledge for global carbon-cycle understanding was evidenced long ago thanks to carbon isotope mass balance models (Berner, 1989). It has long been thought that the fraction of terrestrial vascular plant derived organic matter reaching estuarine areas was refractory to further degradation, mostly due to its intense degradation on land and in the rivers and its high lignin content (Bader 1956, De Leeuw & Baas 1993, Wakeham & Canuel 2006). However, it appears that only a small fraction of the organic matter found in coastal sediments is of terrestrial origin (Hedges et al. 1997), implying that either the estimations of current carbon reservoirs are incorrect, or, more likely, that organic matter undergoes an intense degradation upon reaching seawater (Hedges et al. 1997). Recent estimates of CO₂ efflux from streams and rivers confirmed that terrestrial organic carbon is not as recalcitrant as previously thought (Mayorga et al. 2005). It was recently estimated that over half of the Terrestrial Particulate Organic Matter (TPOM) is remineralized prior to burial (Kandasamy & Nagender Nath 2016).

Over the past decade, a number of studies evidenced that TPOM discharged by rivers was prone to remineralization in coastal areas in polar, temperate and tropical climate zones (Mayer et al. 2008, van Dongen et al. 2008, Vonk et al. 2010, Bourgeois et al. 2011, Rontani et

al. 2014). This efficient mineralization in the marine environment could be attributed to a more efficient use of TPOM by marine bacterial assemblages than by their soil and river counterparts (Garneau et al. 2009). The involvement of ‘priming effects’ (enhanced remineralization of TPOM in the presence of fresh substrates from algal sources; (Steen et al. 2016)) constitutes another possibility (Bianchi 2011, Bianchi et al. 2015, Ward et al. 2016). Indeed, coastal deltaic regions, which are characterized by a high input of TPOM, a high primary production and the presence of microbial communities highly adaptable to physicochemical changes, are generally considered to have a high priming potential (Bianchi 2011). Unfortunately, the priming effect phenomenon has been very poorly studied by the scientific communities working on marine and continental aquatic ecosystems (Guenet et al. 2010) and studies showed contrasting results (Bengtsson et al. 2018). The meta-analysis of results from the literature by (Bengtsson et al. 2018) revealed a weak and non-significant priming effects across fresh water and marine ecosystems. The high photooxidation state of TPOM in some regions could also be at the origin of the intense biodegradation of this material observed in seawater (Rontani et al. 2014). Indeed, solar light-induced fragmentation can reduce structural barriers in higher plant debris and expose new microniches to bacterial colonization (Vähätalo et al. 1998).

The present work is focused on the Rhône Estuary, where only 34% of the POM discharged by the Rhône is deposited (Dufois et al. 2014). We intend to determine if the presence of potamoplankton can enhance bacterial degradation of TPOM. Different hypothesis are tested: i) an interactive effects of OM pools mixing and/or ii) a change in the microbial community to a more adapted one with the increase of salinity. For this purpose, the degradation of suspended particulate matter (SPM) collected in the Rhône River during and after the potamoplankton bloom was studied *in vitro* at different salinities. Due to the overlapping between $\delta^{13}\text{C}$ signatures of potamoplankton and terrestrial higher plants (Tolosa et al. 2013), it was problematic to discern isotopically the sources of organic matter in the SPM samples, the

degradation of TPOM was thus monitored with several lipid tracers specific to terrestrial vascular plants (Table 1). At the same time, changes in the structure of bacterial communities were monitored both in the *in vitro* experiments and in SPM samples collected in the salinity gradient of the Rhône River mouth.

MATERIALS AND METHODS

Sampling

For the biodegradation experiments, fresh SPM samples were collected at the Arles station (43°40'44"N, 4°37'16"E, Fig. 1) on January 24 and March 30, 2017. At these sampling times, particulate organic matter contents were in the same range (31.6 and 36.7 g. Kg⁻¹, respectively). About 1000 l of Rhône River water were sampled at 1 m depth using a teflon-coated high-speed centrifuge (CEPA Z61), 42 and 56 g (wet weight) of SPM were obtained in January and March, respectively which were stored in an ice box until the return to the lab (1 h). After removal of SPM, the remaining freshwater was stored at 4°C. Collected SPM were used as bacterial inoculates and sources of TPOM upon arrival at the laboratory.

Sea water was sampled the 7 February 2017 at the station MESURHO (43° 19' 7"N, 4°51'58"E) near the Rhône River mouth (Fig. 1) in order to be used to adjust salinity for incubation and to gain the inoculum of marine bacteria. At sampling time, the salinity was 20 g kg⁻¹ (Refractometer ATAGO ATC smile).

SPM samples were also collected in February 2012 along a transect following the salinity gradient in the Rhône flow in the framework of the national program MOOSE (Mediterranean Ocean Observing System for the Environment) (Fig. 1). The flow river was 980 m³ s⁻¹. Five subsurface stations were sampled (between 0–3 m depth) with a Niskin bottle according to the salinity gradient: StA, StB, StC, StD and StE with 0, 15, 33, 39 and 40 g kg⁻¹, respectively (Fig.

1). The volume filtered (between 1 and 3 l) depended on SPM concentration at each station. The water was filtered on pre-combusted GF/F filters (Whatman). Filters were immediately frozen at -20°C .

Biodegradation experiments

Two sets of biodegradation experiments were performed. The first one to mimic the effect of the variations of salinity on biodegradation of TPOM with SPM collected during the potamoplankton bloom in January. The second one to compare biodegradation of TPOM in bloom and post-bloom conditions. The experiments were performed in parallel; SPM sample collected on January 24 was stored less than 15 days after its collection. We cannot totally exclude a weak degradation of TPOM during the storage of this sample.

Experimental system were composed by 30 mg (equivalent dry weight) of fresh SPM collected at the Arles station in January or March 2017 and 150 ml of water adjusted at the chosen salinity (0, 10 or 20 g kg^{-1}) in 500 ml Erlenmeyer flasks. Fresh water (obtained from Rhône water after separation of SPM in January) and MESURHO sea water were used for incubations at 0 and 20 g kg^{-1} , respectively. Ten g kg^{-1} salinity was obtained by mixing sea water collected at the MESURHO station and Rhône fresh water. The dissolved organic carbon was in the same range in water from Rhône and MESURHO, it never exceeded $200\text{ }\mu\text{M}$. The lipid content of the dissolved phase was considered as negligible relative to this of SPM.

Aerobic systems were incubated at a temperature close to the *in situ* temperature (15°C). To maintain aerobic conditions, flasks coated with cotton plugs were incubated in the dark on a reciprocal shaker (96 rpm, 5 cm amplitude). At each incubation time (0, 12, 24 and 38 days) three flasks (triplicates) were sampled, one for monitoring changes of bacterial biodiversity and two replicates for lipid analysis. All samples were filtered on pre-combusted GF/F filters (Whatman) and immediately frozen at -20°C until analysis.

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Chemical analysis

139 After defrosted, filters were placed in methanol (20 ml) with excess NaBH₄ (70 mg; 30 min at
140 20 °C). This operation was carried out in order to reduce hydroperoxides present in the samples
141 (Galeron et al. 2015), which could induce autoxidative damages of lipids during the hot
142 saponification step. After NaBH₄ reduction, 20 ml water and 2.8 g KOH were added and the
143 mixture directly saponified by refluxing (75°C) for 2 h. After cooling, samples were acidified
144 with HCl (pH 1) and extracted (3×) with dichloromethane (DCM). The combined DCM extracts
145 were dried, evaporated to dryness and the total lipid extract (TLE) was dissolved in 300 µl of
146 pyridine/N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco; 2:1, v:v) and silylated
147 for 1 h at 50 °C to convert OH-containing compounds to trimethylsilyl -ether or ester
148 derivatives. After solvent removal under a stream of N₂, the derivatized residue was dissolved
149 in 100 µl BSTFA (to avoid desilylation of FAs) and 600 µl of solvent (*n*-hexane).

150

151 *GC–quadrupole time of flight mass spectrometry (GC–QTOF)*

152 Accurate mass measurements were carried out in full scan mode with an Agilent 7890B/7200
153 GC–QTOF System (Agilent Technologies, Parc Technopolis - ZA Courtaboeuf, Les Ulis,
154 France). A cross-linked 5% phenyl-methylpolysiloxane (Agilent; HP-5MS ultra inert) (30 m ×
155 0.25 mm, 0.25 µm film thickness) column was used. Analysis was performed with an injector
156 operating in pulsed splitless mode set at 270 °C and the oven temperature was programmed
157 from 70 °C to 130 °C at 20 °C min⁻¹ and then to 300 °C at 5 °C min⁻¹. The carrier gas (He) was
158 maintained at 0.69 × 10⁵ Pa until the end of the temperature program. Instrument temperature
159 was 300 °C for the transfer line and 230 °C for the ion source. Accurate mass spectra were
160 recorded across the range *m/z* 50–700 at 4 GHz. The instrument provided a typical resolution

ranging from 8009 to 12252 from m/z 68.9955 to 501.9706. Perfluorotributylamine (PFTBA) was utilized for daily MS calibration ($\Delta < 2$ ppm).

Standards

Quantification of lipids was carried out using GC-QTOF with external standards. Sitosterol, sitostanol, α -amyrin, β -amyrin, lupeol, vanillic, syringic and 4-hydroxycoumaric acids were obtained from Sigma-Aldrich. Cutin components (isomeric dihydroxyhexadecanoic acids) were quantified with a standard of 9-10-dihydroxyhexadecanoic acid obtained by oxidation of palmitoleic acid with OsO_4 (McCloskey & McClelland 1965).

Analysis of the bacterial communities associated to SPM

DNA extraction and PCR amplification

Genomic DNA was obtained from the filters using lysozyme-sodium dodecyl sulfate (SDS) followed by phenol-chloroform extraction and subsequent precipitation using ethanol. DNA concentration was determined spectrophotometrically following treatment with RNase. PCR amplification of bacterial 16S rRNA gene for DGGE analysis was performed using the GML5F – 907MR primer set for Bacterial community as previously described (Bonin et al. 2002, Goregues et al. 2005). For the ribosomal diversity analysis, the V4 region of the bacterial and archaeal 16S rRNA genes were amplified using a universal primer set, 515F-Y (5'-GTGYCAGCMGCCGCGGTAA-3', (Parada et al. 2016) and 806RB (5'-GGACTACNVGGGTWTCTAAT-3', (Apprill et al. 2015). PCR amplification was performed with 50 ng of target DNA and 0,5 μM (1 μL à 10 pmol/ μL) of each primer. The reaction mixture contains 10 μL of GoTaq® PFU (Promega) in a final volume of 50 μL . PCR amplification is done in a minicycle (Biorad T100 Thermal Cycler) for 35 cycles. The matrix DNA is initially denatured for 3 minutes at 94°C. Each cycle includes a step of denaturing the double-stranded

DNA (45 seconds, 94°C), a step oligonucleotide hybridization (60 seconds, 50°C) and an elongation step (90 seconds, 72°C). The amplification ends with a final elongation step at 72°C for 10 minutes. Amplification products are detected by electrophoresis on agarose gel 1.5% (Promega) followed by bromide staining ethidium (colored intercalant) (1µg.ml⁻¹).The 16S rRNA gene amplicons were sequenced by the MiSeq Illumina (paired end 2* 250) platform Get of Genotoul.

Diversity analysis

The analysis of the biodiversity was performed by DGGE for the incubation samples whereas prokaryotic diversity and community structure analysis was based on 16S rDNA- sequencing for the salinity profile samples.

Denaturing Gradient Gel Electrophoresis (DGGE) was performed using a D-code Universal Mutation Detection System (Bio-Rad Laboratories Inc.). Banding patterns across the gels were normalized using a previously constructed ladder composed of seven different bands that was loaded several times on each gel. Fingerprints observed on the gels were analyzed using GelCompar II (Applied Maths, St-Martens-Latem, Be). After normalization, automatic band detection was performed using a minimum profiling level of 5% relative to maximum. Band comparison between different profiles was settled with a 2% position tolerance and a 1% optimization to allow for tolerance of bands shifts within and among lanes. For DGGE patterns, calculation of the pair-wise similarities of densitometric profiles was based on Pearson's correlation coefficient (Michener & Sokal 1957) to construct dendrograms using the Unweighted Pair-Group Method Analysis (GelCompar II software, Applied Maths, St-Martens-Latem, Be).

The 16S amplicons were sequenced by the MiSeq Illumina (paired end 2 * 250) platform Get of Genotoul. The paired-end raw reads were firstly overlapped and merged by platform GeT of

Genotoul. The analysis of the 16S-assembled data was relying on the use of QIIME 1.91 (Kuczynski et al. 2012). The removal of low quality bases and chimera sequences were performed by QIIME 1.91 script and UCHIME (Edgar et al., 2011), respectively. The high-quality sequences were next clustered into Operational Taxonomic Unit (OTU) using the UCLUST algorithm and a threshold of 97% of sequence identity. The taxonomic assignment of representative sequences of OTUs was performed using the green genes database (Edgar, 2010). The OTU table was filtered for low abundance OTUs (Bokulich et al., 2013). Finally, sub-sampling normalization, alpha and beta diversity were characterized by Phyloseq, an open-source software package project for R (www.r-project.org) (McMurdie and Holmes, 2013; R Core Team, 2008).

Statistical analyses

The results were analyzed using the R® (GNU) software. Multivariate analyses (main components (PCAs) and multiple factorials (MFAs)) were carried out in order to combine the results of chemical and microbiological extractions. The influence of the different conditions and incubation times were verified by mean (ANOVA) comparisons and Pearson or Spearman correlations (normal data or not).

RESULTS AND DISCUSSION

Lipid composition of SPM samples collected at the Arles station

In SPM samples from the Rhône River (Fig. 1, StA), fatty acid (FA) profiles are generally dominated by saturated FA and these of sterols by cholest-5-en-3 β -ol (cholesterol) and 24-ethylcholest-5-en-3 β -ol (sitosterol) (Galeron et al. 2015) (See appendix). In contrast, the sample collected in January appeared to contain high proportions of polyunsaturated FA (C_{16:3},

235 C_{16:4}, C_{18:4}, C_{20:3} and C_{20:5}), cholesta-5,24(25)-dien-3 β -ol (desmosterol), 24-methylcholesta-
 236 5,22*E*-dien-3 β -ol (brassicasterol) and 24-methylcholesta-5,24(28)-dien-3 β -ol (24-
 237 methylenecholesterol) (Fig. S1A) (See appendix). This typical algal lipid composition
 238 confirmed the presence of the yearly potamoplanktonic bloom event, which was previously
 239 observed in the Rhône River (Galeron et al. 2015). The timing (ranging from January to March)
 240 and the intensity of this event seem to be strongly dependent on meteorological conditions
 241 (temperature, rainfall intensity and cloud cover thickness). On the basis of the high proportions
 242 of desmosterol and 24-methylenecholesterol detected, this bloom was attributed to freshwater
 243 diatoms. Indeed, 24-methylenecholesterol is the most common sterol in diatoms (Volkman
 244 2003, Rampen et al. 2010) and desmosterol was proposed as a taxonomic marker for some
 245 species of diatoms, particularly for *Rhizosolenia*, where it is the dominant sterol (Barrett et al.
 246 1995). This assumption is well supported by the drop of silica concentration observed at the
 247 same period (MOOSE data 2017, unpublished data).
 248 In contrast, the SPM sample collected on March 7, 2017 exhibited a more classical lipid profile
 249 with very small proportions of PUFA, desmosterol and 24-methylenecholesterol attesting to the
 250 disappearance of the bloom (Fig. S1B).
 251 The two samples (January and March) also contained: (i) isomeric dihydroxyhexadecanoic
 252 acids arising from the hydrolysis of cuticular waxes (von Wettstein-Knowles 1993, Otto et al.
 253 2005), (ii) a variety of pentacyclic triterpenoids with structures based on the ursene (α -amyrin
 254 and ursolic acid), oleanene (β -amyrin and oleanolic acid) or lupene (lupeol) hydrocarbon
 255 skeletons common in higher plant materials such as leaves, bark, roots and wood (Pancost &
 256 Boot 2004, Otto et al. 2005) and (iii) phenolic acids (vanillic, syringic, ferulic, 4-
 257 hydroxycoumaric acids) (Fig. S1). These last acids are generally considered as characteristic of
 258 lignin from non-woody vascular plant tissues (e.g., grass leaves, conifer needles) (Hedges &
 259 Mann 1979). They may also constitute ester-bound aromatic constituents of suberin

(Kolattukudy et al. 1981, Riederer et al. 1993, Kögel-Knabner 2002). It may be noted that ferulic acid and 4-hydroxycoumaric acid were also identified as components of ligno-cellulose complexes of grasses linking carbohydrates and lignin via ester bonds (Lam et al. 2001). Since the applied alkaline hydrolysis treatment cleaves esters, but not ethers, the phenolic acids detected are likely derived from ester-bound moieties of suberin and ligno-cellulose rather than from the ether-linked lignin macromolecule (Otto et al. 2005). 4-Hydroxycoumaric and ferulic acids (C) are only present in non-woody tissues of vascular plants, while syringic acid (S) is unique to angiosperms and vanillic acid (V) exists in all vascular plants (Table 1) (Sun et al., 2016). On the basis of the ratio C/V and S/V (Hedges and Mann, 1979) observed, TPOM carried by the Rhône River seems dominated by debris of angiosperm and grass leaves.

Isomeric dihydroxyhexadecanoic acids, α - and β -amyrins, lupeol, vanillic, syringic and 4-hydroxycoumaric acids were thus selected as tracers (Table 1) to monitor the degradation of TPOM during the different incubations. Sitosterol is also commonly associated with terrestrial higher plant inputs (Lütjohann 2004), but some microalgae (and notably diatoms) are also known to produce this sterol (Volkman 2003, Rampen et al. 2010). Due to the expected presence of high proportions of diatoms during the bloom event, a significant part of this sterol could not be traced back to higher plant material in SPM samples collected the 24th January, so we thus discarded the use of this widespread and ambiguous tracer during our *in vitro* studies of TPOM degradation.

Ex situ experiments to mimic variation of salinity and fresh algae input on bacterial degradation of TPOM

The bacterial degradation of TPOM was monitored with selected specific lipid tracers (Table 1) during the incubation of SPM collected in the Rhône River in January (during the bloom event) at different salinities (0, 10 and 20 g kg⁻¹) and at 15 °C. The decreasing concentrations

of 3 β -hydroxy-urs-12-en-11-one and 3 β -hydroxy-olean-12-en-11-one (specific tracers of α - and β -amyrin autoxidation, respectively) ((Galeron et al. 2015, Galeron et al. 2016) observed during these incubations allowed us to exclude the involvement of autoxidative artifacts. This demonstrates that changes in the composition of these lipids observed during our incubations were microbially mediated.

Cuticular wax components (isomeric dihydroxyhexadecanoic acids) and specific triterpenes of vascular plants (α - and β -amyrins and lupeol) appeared to be degraded more efficiently at the highest salinity (Table S1, Fig. 2A-C). It is interesting to note that this degradation took place without accumulation of intermediate catabolites likely due to rapid turnover. In the case of phenolic acids (vanillic, syringic and 4-hydroxycoumaric acids), a decrease of their concentrations was observed at all salinities towards the end of the incubation (Fig. 2D-F). However, in the case of the highest salinity an increase in the concentration could be observed at the end of the incubation (Fig. 2D-F). This increase can be attributed to the bacterial degradation of lignin, suberin or ligno-cellulose, well known to produce these acids (Otto *et al.*, 2005). Indeed, in the literature there are a number of reports of bacteria (*Streptomyces*, *Nocardia*, *Sphingomonas*, *Pseudomonas*, *Rhodococcus* ...) that can break down lignin structures and produce phenolic acids (for a review see (Bugg et al. 2011).

During the incubations, bacterial degradation also affected lipids of the algal components of the bloom event. As in the case of TPOM, the extent of degradation of these algal lipids increased with salinity (C_{20:5} FA, desmosterol and phytol degradation percentages ranging from 90, 70 and 40% at 0 g kg⁻¹ to 95, 90 and 70% at 20 g kg⁻¹, respectively (data not shown)).

A global analysis of the evolution of the structure of the bacterial community associated to Rhône SPM by DGGE clearly shows the impact of salinity on bacterial communities. Changes in salinity allow for the establishment of specific communities for each salinity,

dominated by different phylotypes (Fig. 3). The communities observed after incubation of Rhône SPM at salinity 20 g kg⁻¹ (T12, T24, T38) are close to those observed in situ at the same salinity (greater than 50% similarity) (Fig. S2). This community seems to harbor bacteria possessing higher capacities to degrade algal and vascular plant components than these initially present within the river.

Interactive effects of the presence of freshwater algae on the bacterial degradation of TPOM

Comparison of the bacterial TPOM biodegradation after incubation with the two kinds of SPM (collected in January during the potamoplankton bloom or in March after this event) clearly showed a faster degradation of cuticular wax components and triterpenes in the presence of potamoplankton after incubation at the same salinity (20 g kg⁻¹) (Table S1, Fig. 4A-C). Interestingly, the increase in phenolic acid concentration (attributed to lignin or suberin degradation) observed at the end of the incubations with January SPM was not observed in the case of incubations with March SPM (Table S2, Fig. 4D-F). The presence of freshwater phytoplankton (dominated by diatoms) thus enhances TPOM remineralization. TPOM was more intensively degraded with January SPM in the presence of high level of potamoplankton, which could have a beneficial interactive effect on these processes or induce a change in microbial communities.

In situ behavior of TPOM in the salinity gradient of the Rhône River

The stable carbon isotopic ($\delta^{13}\text{C}$) signatures of the main lipids (fatty acids and sterols) were measured previously in the SPM samples collected along the salinity gradient of the Rhône

River (Galeron et al. 2018). Sitosterol (-27.1‰) was depleted in ^{13}C relative to 24-methylenecholesterol (-21.6‰) indicating a mainly terrestrial origin. We therefore used the percentage of sitostanol relative to the parent sitosterol for *in situ* TPOM bacterial degradation estimates. Indeed, stanols serve as useful indicators of bacterial degradation of Δ^5 -sterols (Gagosian et al. 1982, Wakeham 1989, De Leeuw & Baas 1993). In the SPM samples collected in the Rhône Estuary, the percentage of sitostanol appeared to increase logarithmically from 0.4 to 15.4 % (Table S3) with the proportion of algal sterols (brassicasterol and 24-methylenecholesterol) relative to sitosterol (Fig. 5). The plateau observed at high proportions of algal material may be attributed to the well-known inhibition effect observed when co-substrates (algal sterols) and substrate (sitosterol) compete for the same enzymes (Dalton, 1982). Indeed, reduction of brassicasterol, 24-methylenecholesterol and sitosterol to the corresponding stanols involves the same enzymatic sequence. This enhancement of the biodegradation of sitosterol (arising mainly from terrestrial vascular plants in the Rhône River) (Galeron et al., 2015) with an increasing proportion of algal material (co-substrate) suggests that the presence of algal material favors TPOM degradation in the Rhône Estuary. Concerning the origin of the algal material present in these SPM samples, the relatively enriched $\delta^{13}\text{C}$ signature of 24-methylenecholesterol (a well-known diatom marker) (Volkman 2003, Rampen et al. 2010) allowed to exclude the presence of freshwater phytoplankton, which typically exhibits more depleted $\delta^{13}\text{C}$ values (-34‰ to -36‰) (Tolosa et al. 2013). The enhancement of the biotic degradation of TPOM in the salinity gradient of the Rhône River seems thus to result from the presence of marine diatoms.

The bacterial degradation of vascular plant material in the Rhône Estuary is well supported by: (i) the depleted carbon isotopic ($\delta^{13}\text{C}$) signatures (ranging from -30.9‰ to -27.8‰) of palmitoleic acid mainly arising from bacteria in the SPM samples investigated and (ii) the $\delta^{13}\text{C}$ signatures of the specifically bacterial vaccenic (-30‰) and branched

pentadecanoic (-29‰) acids previously measured by (Bourgeois et al. 2011) in sediments from the same deltaic region of the Rhône River. These different isotopic data confirm that bacteria present in the Rhône Estuary grew on ^{13}C depleted C_3 terrestrial plant material and not ^{13}C enriched marine algae.

In situ biodiversity along the salinity gradient

The prokaryotic biodiversity was analyzed at different sampling sites along the salinity gradient in the Rhône River mouth. Five subsurface stations were sampled StA, StB, StC, StD and StE corresponding to the following salinities: 0, 15, 33, 38 and 39 g kg^{-1} . From the five libraries, 342123 sequences of 16S rRNA gene were analyzed (Table 2). Good's index showed that the sequencing depths were sufficient to cover 72.86 to 85.70 % of the microbial diversity (Table 2). The subdivision into operational taxonomic units (OTUs) sharing >97 % and after discarding low-quality read identity allowed the detection of the different OTUs (Table 2). A drastic decrease of species diversity was observed at the boundary between the two water masses (StA in the Rhône River and sea water, StB salinity 15 g kg^{-1}) since both Shannon's diversity (H') and Simpson's diversity ($D1$) indexes ($H' = 5.87$, $D1 = 0.93$) were lower than in Rhône water ($H' = 7.75$, $D1 = 0.98$) (Table 2). For higher salinities (StC, StD, StE) the diversity increases again, corresponding to a change in communities.

Rhône River communities was dominated by Proteobacteria (Alphaproteobacteria, Gammaproteobacteria and Betaproteobacteria). At intermediate salinity (15 g kg^{-1}), Betaproteobacteria strongly increased and new taxonomic classes were observed (Cytophagia, Flavobacteria). This community is also characterized by the presence of Actinobacteria. At salinities close to that of seawater Flavobacteria, Alphaproteobacteria, Gammaproteobacteria and Thaumarchaeota dominated, while Betaproteobacteria were not detected (Figs 6 and 7).

Within the Proteobacteria classes, different families were observed within the salinity gradient. Alphaproteobacteria are dominated by Rhodobacteraceae in freshwater and then Pelagibacteraceae (at StB, 15 g kg⁻¹) and finally Rhodospirillaceae was detected as the salinity increased (at StC, 33 g kg⁻¹). Among Gammaproteobacteria, Sinobacteraceae dominated in freshwater, while Halomonadaceae and Alteromonadaceae were detected at salinities over 15 g kg⁻¹. Within Betaproteobacteria at StB, Rhodocyclaceae present in freshwater are substituted by Comamonadaceae and Moraxellaceae, which then was progressively not detected with increasing salinities (Fig. 6). Results indicated a significant change in the composition of the microbial population all along the salinity gradient. In freshwater, the communities are dominated by Betaproteobacteria; at StB, the communities are characterized by the presence of Actinobacteria, whereas at the highest salinities Flavobacteria, Alphaproteobacteria and Gammaproteobacteria were to be the most abundant. The dominance of Gamma and Alphaproteobacteria in seawater and that of Betaproteobacteria in freshwater is in concordance with the observations of (Newton et al. 2011) previously reported.

To unravel the relationships, the composition of the microbial community at the class level taxonomy and the percentage of TPOM biodegradation, estimated from the ratio sitostanol/sitosterol and the ratio (brassicasterol and 24-methylene cholesterol)/sitosterol reflecting the amount of algal in the salinity gradient between, we performed a bi-plot MFA (Fig. 7). The proportion of algal material strongly influences the biodegradation of sitosterol (Fig. 7), which derives mainly from terrestrial plants in this area (Galeron et al. 2015). This phenomenon seems to be of more relevance in seawater (at salinities of 39-40 g kg⁻¹) (Fig. 7). The assimilative capacity of this recalcitrant organic material is therefore different according to the communities present in the environment (Bianchi 2011). Interestingly, Crenarchaeota, more abundant at intermediate salinities (33 g kg⁻¹) and well-known to degrade proteins and products released by diatoms (co-substrate) more efficiently than bacteria (Kirchman et al. 2007) could

be also responsible for the enhancement of the degradation of TPOM. According to these nutritional strategies, bacteria more efficient at degrading recalcitrant organic matter would be located in the estuarine zone. Indeed, on the basis of the competitive exclusion principle, generalist species (degrading recalcitrant compounds through priming and tolerant to changes) should dominate diversified habitats (sea), while specialized species (in the degradation of the TPOM) should live in a simplified environment (river) (Pimm et al. 1991, Kassen 2002).

CONCLUSIONS

In order to determine what phenomena are at the origin of the increase TPOM biodegradation in seawater, SPM samples collected in the Rhône River during and after a bloom of potamoplankton were incubated at different salinities (0, 15 and 20 g kg⁻¹). The degradation of TPOM was monitored with specific lipid tracers (triterpenes, phenolic acids, cuticular wax components). It appeared that TPOM was degraded more efficiently at the highest salinity and in the presence of potamoplankton. The communities observed after incubation at the highest salinity (20 g kg⁻¹), which are close to those observed *in situ* at the same salinity, degraded algal and vascular plant components more efficiently than that present within the river. The enhanced degradation of TPOM observed in the presence of potamoplankton (dominated by diatoms) is likely due to the presence of an algal co-substrate or/and a more efficient bacterial community during the bloom period. The bacterial consumption of TPOM observed in SPM samples collected in 2012 in the salinity gradient of the Rhône River was attributed to: (i) the involvement of priming effect, this process being likely induced by the presence of important proportions of marine diatoms, or (ii) a more efficient use of TPOM by marine bacterial assemblages than by their river counterparts. Indeed, the increase of salinity in the river mouth results in an enrichment in Gammaproteobacteria, which seem to be more efficient degraders of TPOM than Betaproteobacteria dominating the communities in the river.

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Figure captions

Figure 1: Summary map showing sampling locations.

Figure 2: Concentrations (ng mg^{-1}) of isomeric dihydroxyhexadecanoic acids (cuticular wax components) (A), β -amyrin (B), lupeol (C), vanillic acid (D), syringic acid (E) and 4-hydroxycoumaric acid (F) measured during incubations of Rhône SPM collected in January after incubation at different salinities.

Figure 3: Multiple Factor Analysis (MFA), correlation biplot showing 16 samples (individual factor map) and the correlation circle with the 8 most representative phylotypes (pink arrows, migration distances observed in DGGE gels, Fig S2), geochemical tracers (phytol, waxes, β -amyrin, α -amyrin, ursolic acid, blue arrow) and salinity (red arrows). The PCA accounts for 58.6 % of the total variation incubation conditions (Axis 1:35.77% and Axis 2:22.77 %). Samples were identified as their incubation time (T0, T12, T24 or T38 incubation days) followed by their salinity (0 g kg^{-1} (S0), 10 g kg^{-1} (S10) or 20 g kg^{-1} (S20)).

Figure 4: Concentrations (ng mg^{-1}) of isomeric dihydroxyhexadecanoic acids (cuticular wax components) (A), β -amyrin (B), lupeol (C), vanillic acid (D), syringic acid (E) and 4-hydroxycoumaric acid (F) measured during incubations of Rhône SPM collected in January and March at the salinity of 20 g kg^{-1} .

Figure 5: Biodegradation percentage of TPOM (estimated with the ratio sitostanol / sitosterol) in relation to the proportion of algal sterols (estimated with the ratio (brassicasterol + 24-methylenecholesterol) / sitosterol) in SPM samples collected in 2012 in the Rhône River and Mouth (salinity gradient).

Figure 6: Percentages of abundance of OTUs, classed at family level and ranked according to their class. Samples collected in 2012 at the Rhône (Arles) station (StA) and in the salinity gradient (StB, StC, StD and StE at 15, 33, 39 and 40 g kg⁻¹, respectively).

Figure 7: Multiple Factor Analysis (MFA), correlation biplot showing 5 samples (collected in the salinity gradient of the Rhône River plum in 2012) and the correlation circle with the 9 most representative class (pink arrows, see figure 6), Biodegradation rate (blue arrow), amount of co substrate (red arrow) and salinity (green arrows), The MFA accounts for 75.4 % of the total variation incubation conditions (Axis 1:49.99% and Axis 2:25.41%).

Figure S1: TOF mass chromatograms of the silylated total lipid extract of SPM collected in the Rhône River at the Arles station during (the 24/01/2017) (A) and after (the 30/03/2017) (B) the potamoplankton bloom event.

Figure S2: Dendrogram from DGGE analyses of the evolution of the bacterial community according to Jaccard's indexes and the classification algorithm hierarchy UPGMA (Unweighted Pair Group Method with Arithmetic mean) samples according to time incubation (0 (T0), 12 (T1), 24 (T2) or 38 (T3) days of incubation) and the water salinity (0 g kg⁻¹ (S0), 10 g kg⁻¹ (S10) or 20 g kg⁻¹ (S20)).

APPENDIX

Cholesterol

Brassicasterol

24-Methylenecholesterol

Sitosterol

Desmosterol

Betulin

β -amyrin

11-Oxo- β -amyrin

Oleanolic acid

α -amyrin

11-Oxo- α -amyrin

Ursolic acid

Lupeol

Vanillic acid

Syringic acid

Ferulic acid

4-Hydroxy cinnamic acid

**Isomeric dihydroxyhexadecanoic acids
(Cutin components)**

	sampling date	longitude	latitude	salinity
St A	22/02/2012	4.37.16	43. 40.44	0
St B	23/02/2012	4.51.64	43.18.77	15
St C	24/02/2012	4.51.64	43.18.57	33
St D	25/02/2012	4.52.91	43.18.43	39.3
St E	22/02/2012	5.02.57	43.18.89	40
Mesurho		4.51.58	43.19.2	20

Individual factor map

Correlation circle

Order	Family		Order	Family	
Actinobacteria	ACK-M1		Flavobacteriia	Cryomorphaceae	
Alphaproteobacteria	Pelagibacteraceae			Flavobacteriaceae	
	Rhodospirillaceae		Gammaproteobacteria	Alteromonadaceae	
	Rhodobacteraceae			Colwelliaceae	
Bacteroidia	Porphyromonadaceae			Halomonadaceae	
Betaproteobacteria	Comamonadaceae			Oceanospirillaceae	
	Methylophilaceae			Pseudoalteromonadaceae	
	Rhodocyclaceae			Sinobacteraceae	
Cytophagia	Cytophagaceae		Thaumarchaeota	Cenarchaeaceae

Individual factor map

Correlation circle

Table 1

Origin of the main lipid tracers (See appendix) employed during the present work.

Lipid tracers	Origin	References
9,16- and 10,16-dihydroxyhexadecanoic acids	Cuticular waxes of higher plants	Von Wettstein-Knowles, 1993
α -Amyrin	Angiosperms	Pancost and Boot, 2004; Otto et al., 2005
β -Amyrin	Angiosperms	Pancost and Boot, 2004; Otto et al., 2005
Lupeol	Angiosperms	Pancost and Boot, 2004; Otto et al., 2005
Ursolic acid	Angiosperms	Pancost and Boot, 2004; Otto et al., 2005
Oleanolic acid	Angiosperms	Pancost and Boot, 2004; Otto et al., 2005
Vanillic acid	Vascular plants	Hedges and Mann, 1979
Syringic acid	Angiosperms	Hedges and Mann, 1979
Ferulic acid	Vascular plants (non woody)	Hedges and Mann, 1979
4-Hydroxycoumaric acid	Vascular plants (non woody)	Hedges and Mann, 1979
Brassicasterol	Phytoplankton	Volkman, 2003; Rampen et al., 2010
24-Methylenecholesterol	Diatoms	Volkman, 2003; Rampen et al., 2010
Desmosterol	Diatoms	Volkman, 2003; Rampen et al., 2010
Sitosterol	Phytoplankton and vascular plants	Volkman, 2003
Sitostanol	Biodegradation of sterols	Wakeham, 1989

Table 2 : Phylum-level taxonomic distribution of bacterial and archeal assemblages from sampling stations along salinity gradient at the Rhône river mouth

	salinity	Number of trimmed sequences	Number of observed OTU (97%)	Good's coverage (%)	shannon index	Simpson index
StA	0	74780	52161	85.35	7.75	0.98
StB	15	69753	57801	71.53	5.87	0.93
StC	33	73376	68842	85.70	7.02	0.97
StD	39	55065	42430	72.86	6.39	0.96
StE	40	69146	54486	82.75	6.83	0.97

A **B**

Table S1

Evolution of the concentration (ng mg⁻¹) of vascular plant components during incubations at different salinity conditions

	Sample ^a	α -Amyrin	β -Amyrin	Lupeol	Isomeric dihydroxy C _{16:0} acids ^b
Rhône SPM (Bloom samples)	T0S0	0.7	0.8	0.8	30.1
	T0S0	0.5	0.9	1.0	44.6
	T12S0	0.3	0.5	0.9	24.5
	T12S0	0.2	0.3	0.3	48.5
	T24S0	0.4	0.3	0.8	75.3
	T24S0	0.2	0.2	0.2	11.7
	T38S0	0.2	0.2	0.3	15.8
	T38S0	0.6	0.7	0.8	4.1
	T0S10	0.5	0.6	0.5	24.6
	T0S10	0.2	0.3	0.3	15.5
	T12S10	0.5	0.5	0.6	30.3
	T12S10	0.5	0.6	0.6	10.5
	T24S10	0.2	0.2	0.3	5.2
	T24S10	0.5	0.6	0.7	16.1
	T38S10	0.4	0.6	0.8	4.0
	T38S10	0.4	0.5	0.7	4.9
	T0S20	1.1	1.4	1.3	22.0
	T0S20	1.1	1.3	1.5	63.2
	T12S20	1.0	1.1	1.0	36.8
	T12S20	3.7	1.5	1.9	17.2
	T24S20	0.4	0.4	0.4	8.3
	T24S20	0.2	0.3	0.2	12.6
	T38S20	0.3	0.4	0.4	8.9
	T38S20	0.3	0.5	0.5	11.4
Rhône SPM (Post-bloom samples)	T0S20	0.1	0.2	0.2	7.3
	T0S20	0.3	0.3	0.6	7.3
	T12S20	0.2	0.4	0.3	8.6
	T12S20	0.3	0.5	0.5	1.5
	T24S20	0.2	0.3	0.3	6.4
	T24S20	0.2	0.3	0.3	5.4
	T38S20	0.1	0.2	0.3	4.7
	T38S20	0.1	0.2	0.3	6.2

^a T = incubation time (0 ; 12 ; 24 or 38 days), S = Salinity (0 ; 10 or 20 g kg⁻¹)

^b Cuticular wax components

Table S2

Evolution of the concentration (ng mg⁻¹) of phenolic acids during the different incubations

		Sample ^a	Vanillic acid	Syringic acid	4-Hydroxycoumaric acid
Rhône SPM (Bloom samples)		T0S0	3.5	2.4	6.6
		T0S0	4.9	3.1	8.4
		T12S0	2.5	2.5	6.6
		T12S0	1.6	1.6	5.5
		T24S0	2.5	2.0	5.3
		T24S0	1.6	1.0	2.9
		T38S0	1.5	1.0	4.8
		T38S0	1.3	0.9	1.9
		T0S10	4.4	1.9	4.8
		T0S10	2.7	1.7	4.0
		T12S10	1.2	1.0	4.2
		T12S10	0.5	0.7	1.6
		T24S10	1.5	0.9	3.3
		T24S10	1.8	1.7	3.3
		T38S10	1.1	1.3	2.2
		T38S10	2.1	1.2	6.6
		T0S20	3.9	2.1	6.6
		T0S20	3.6	2.1	6.8
		T12S20	1.9	1.7	5.1
		T12S20	1.1	1.2	2.9
		T24S20	1.1	0.9	2.3
		T24S20	1.2	0.8	4.6
		T38S20	1.9	1.2	4.5
		T38S20	2.3	1.6	5.2
Rhône SPM (Post-bloom samples)		T0S20	2.9	0.8	2.9
		T0S20	4.0	2.3	7.1
		T12S20	1.5	0.9	3.4
		T12S20	1.1	0.9	2.1
		T24S20	1.4	1.2	3.2
		T24S20	1.3	0.9	2.7
		T38S20	1.2	1.0	3.8
		T38S20	1.1	0.9	2.5

^a T = incubation time (0, 12, 24 or 38 day), S = Salinity (0, 10 or 20 g kg⁻¹)

Table S3 :

(Brassicasterol + 24-methylenecholesterol) / sitosterol	Sitostanol (%)
0.012	0.432
0.054	1.697
0.167	5.978
0.121	4.734
0.114	3.573
0.411	6.656
0.135	3.183
0.722	6.652
0.492	10.025
0.078	6.951
0.601	10.128
0.579	11.803
0.548	11.619
1.947	8.133
2.486	13.043
2.994	12.149
1.746	9.017
2.926	15.393
2.250	11.376

Table S4

Phylum	StA	StB	StC	StD	StrE
Other	0.082	0.085	1.343	0.930	0.467
Crenarchaeota	0.045	0.209	1.519	0.992	3.838
Euryarchaeota	0.004	0.068	1.184	0.577	3.467
[Parvarchaeota]	0.000	0.000	0.000	0.000	0.000
Acidobacteria	3.272	0.029	0.144	0.011	0.000
Actinobacteria	3.920	10.050	6.766	3.182	5.261
Armatimonadetes	0.074	0.000	0.000	0.000	0.000
BRC1	0.078	0.000	0.000	0.000	0.000
Bacteroidetes	6.664	24.997	20.023	23.266	15.031
Chlamydiae	0.000	0.005	0.000	0.000	0.000
Chlorobi	0.349	0.017	0.000	0.003	0.000
Chloroflexi	0.472	0.201	0.539	0.304	1.004
Cyanobacteria	0.000	0.000	0.000	0.000	0.000
Elusimicrobia	0.008	0.000	0.000	0.000	0.000
FCPU426	0.000	0.000	0.000	0.000	0.000
Fibrobacteres	0.000	0.000	0.079	0.000	0.000
Firmicutes	2.122	0.121	1.364	0.057	0.146
Fusobacteria	0.161	0.024	0.053	0.022	0.000
GN04	0.000	0.000	0.000	0.000	0.000
Gemmatimonadetes	0.377	0.041	0.238	0.121	0.326
Lentisphaerae	0.000	0.000	0.036	0.005	0.020
NC10	0.008	0.000	0.000	0.000	0.000
Nitrospirae	0.511	0.012	0.098	0.000	0.000
OP3	0.000	0.000	0.000	0.000	0.000
PAUC34f	0.000	0.000	0.089	0.019	0.080
Planctomycetes	0.348	0.073	0.136	0.084	0.600
Proteobacteria	77.619	63.525	63.894	69.312	65.715
SAR406	0.000	0.085	0.851	0.571	1.915
SBR1093	0.004	0.058	0.717	0.396	1.401
Spirochaetes	0.012	0.000	0.070	0.000	0.000
TM6	0.000	0.000	0.000	0.000	0.000
TM7	0.072	0.000	0.000	0.000	0.000
Tenericutes	0.006	0.000	0.000	0.000	0.000
Verrucomicrobia	3.668	0.398	0.660	0.143	0.718
WS3	0.128	0.002	0.079	0.003	0.000
ZB3	0.000	0.000	0.119	0.005	0.010

Table S5 :

Phylum	Class	Order	Family	StA	StB	StC	StD	StE
Actinobacteria	Actinobacteria	Actinomycetales	ACK-M1	0,000	3,309	1,484	0,136	0,000
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	0,359	0,048	0,231	0,005	0,000
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	0,148	7,343	3,217	0,313	0,000
Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	0,000	0,445	3,052	9,539	0,255
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	1,751	15,935	12,657	11,783	12,877
Crenarchaeota	Thaumarchaeota	Cenarchaeales	Cenarchaeaceae	0,011	0,153	1,903	1,028	6,780
Proteobacteria	Alphaproteobacteria	Rickettsiales	Pelagibacteraceae	0,179	2,240	20,627	11,960	60,728
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	29,662	3,405	17,895	31,328	3,772
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	16,487	58,260	20,236	3,734	0,082
Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	2,015	6,142	3,999	0,369	0,016
Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	2,384	0,423	0,212	0,033	0,049
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	14,283	0,044	0,240	0,056	0,000
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	0,000	1,393	9,153	23,855	1,496
Proteobacteria	Gammaproteobacteria	Alteromonadales	Colwelliaceae	0,021	0,170	0,113	0,023	0,000
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	0,021	0,432	4,546	5,674	13,888
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	0,000	0,052	0,000	0,000	0,000
Proteobacteria	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	0,169	0,065	0,188	0,070	0,058
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	32,511	0,140	0,245	0,093	0,000