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1 **Seasonal freshening of NW Mediterranean surface water impacts microbial**
2 **heterotrophic activity and dissolved organic matter**

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17
18 **Key words:** Low salinity waters, microbial heterotrophic activity, dissolved organic matter, NW
19 Mediterranean Sea.

20
21 Declarations of interest: none.

22 **Abstract**

23 The Rhone river represents the most important source of freshwater, nutrients and
24 organic matter to the northwestern (NW) Mediterranean Sea and riverine input markedly affects
25 biogeochemistry and ecosystem functioning in the estuarine and coastal zone. Structures of low
26 salinity waters (LSW) originating near the river plume can also be transported along the
27 continental shelf and offshore. The objective of the present study was to investigate the influence
28 of LSW distant from their source, focusing on dissolved organic matter (DOM) and related
29 microbial processes during two annual cycles (2007 and 2008) at a time series site characterized
30 by the regular occurrence of LSW in spring (Microbial Observatory Laboratoire Arago). We
31 observed enhanced bacterial heterotrophic production and community respiration and specific
32 DOM features within these LSW, concurrently with low net community production. Our results
33 suggest that LSW represent a mechanism of labile DOM supply, thereby sustaining enhanced
34 heterotrophic microbial metabolism.

35

36 **1 Introduction**

37 Riverine input represent approximately 40% of fresh water entering the ocean (Dagg et al.,
38 2004), and thereby plays a key role in linking terrestrial and marine environments. Rivers deliver
39 a large amount of inorganic nutrients to the adjacent coastal area, stimulating rates of primary
40 production and associated food web processes (Frame and Lessard, 2009, Iriarte et al., 2012, Liu
41 et al., 2004). Riverine discharge also transports terrigenous particulate (POM) and dissolved
42 organic matter (DOM), mainly from terrestrial vegetation, to the coastal ocean (Bianchi et al.,
43 2004, Medeiros et al., 2017, Opsahl and Benner, 1997). DOM from freshwater input of the 30
44 major rivers, accounts for 90,2 Tg dissolved organic carbon (DOC) per year (Raymond and

45 Spencer, 2015) which represents ~36% of the global DOC flux to the ocean (250 Tg DOC-C yr⁻¹;
46 ¹; Hedges et al., 1997). The fraction of terrigenous organic matter that is removed in the coastal
47 ocean is not well quantified due to multiple biotic and abiotic processes involved, and the spatio-
48 temporal heterogeneity of river-influenced ocean margins (Bianchi et al. 2011.). In large river
49 systems, such as for example the Mississippi-Atchafalaya river, biological mineralization results
50 in the removal of about 40% of terrestrial DOM in shelf waters (Fichot and Benner 2014).
51 Export of terrigenous DOC from the Amazon river varies on a seasonal scale and accounts for
52 50% to 76% (Medeiros et al. 2015). How riverine input of nutrients and organic matter affect
53 marine ecosystems has been investigated in a range of estuarine and coastal environments (Cole
54 et al., 2007, Dagg et al., 2004 and reference therein). The influence of terrestrial input at varying
55 distances from shore is, however, far less understood.

56 The Mediterranean Sea is a mid-latitude semi-enclosed sea, divided in an eastern extreme
57 oligotrophic basin (<0.1 µg Chlorophyll *a* L⁻¹) and a moderately-oligotrophic western basin (0.1
58 to 3 µg Chlorophyll *a* L⁻¹) (Bosc et al., 2004), mainly due to P-limitation (Krom et al., 1991,
59 McGill, 1965). The Rhone River discharge represents the most important freshwater source to
60 the Mediterranean Sea (Naudin and Cauwet, 1997). The average runoff of 1750 m³ s⁻¹ varies
61 strongly with season and is highest during the frequent precipitation events in spring and autumn
62 (Ludwig et al., 2009, Naudin and Cauwet, 1997). The Rhone River represents an important
63 source of inorganic and organic matter of continental origin to the Mediterranean Sea (de
64 Madron et al., 2011). Increased inorganic and organic nutrient concentrations are reported near
65 the Rhone river freshwater plume in shelf waters (100m depth, up to about 20 nautical miles
66 offshore) in the Gulf of Lion (Diaz et al. 2008, Sempéré et al., 2000) and the associated
67 enhanced biological activity in the estuary and adjacent waters has been documented during

68 several previous field studies (Christaki et al., 2009, Joux et al., 2009, Lefevre et al., 1997, Pujo-
69 Pay et al., 2006).

70 The hydrodynamics of the Rhone river plume are governed by atmospheric conditions,
71 discharge volume and larger scale water mass circulation. When south-easterly winds
72 predominate, the plume remains close to the coast, while north-westerly winds result in the
73 extension of the Rhone river plume in a south-western direction (Demarcq and Wald, 1984).
74 Intrusions of Rhone river water were detected in 5-20% of a 4-year bi-monthly survey in the Bay
75 of Marseille (Frayse et al., 2014), indicating that these events are frequent phenomena in the
76 coastal Mediterranean Sea. Diluted structures with low salinity waters (LSW) detached from the
77 Rhone river plume can be transferred to open waters (Barrier et al., 2016, Diaz et al., 2008,
78 Nencioli et al., 2011, Petrenko et al., 2017). Under conditions of north-westerly winds and in
79 combination with a persistent slope current along the shelf break of the Gulf of Lion (Northern
80 Current;(Raimbault and de Madron, 2003)), these LSW can be directed south-west (Fig. 1). The
81 transit of these buoyant water masses can last up to 3 weeks, and they can be transported over
82 relatively long distances (Diaz et al., 2008), rendering the Gulf of Lion an appropriate site for the
83 investigation of cross-shelf exchange (Petrenko et al., 2017).

84 The objective of the present study was to investigate whether these structures, referred to
85 as lower salinity waters (LSW), represent a supply of biologically labile DOM to the offshore
86 NW Mediterranean Sea. We addressed this question by observations at the time-series site
87 MOLA (Microbial Observatory of the Laboratoire Arago) that is characterized by the recurrence
88 of LSW at surface in spring (Fig. 1). The combined investigation of the temporal changes in
89 environmental characteristics and microbial processes at Station MOLA provided insight on how

90 freshwater input through these physical features could affect biogeochemistry in the offshore
 91 Mediterranean Sea.

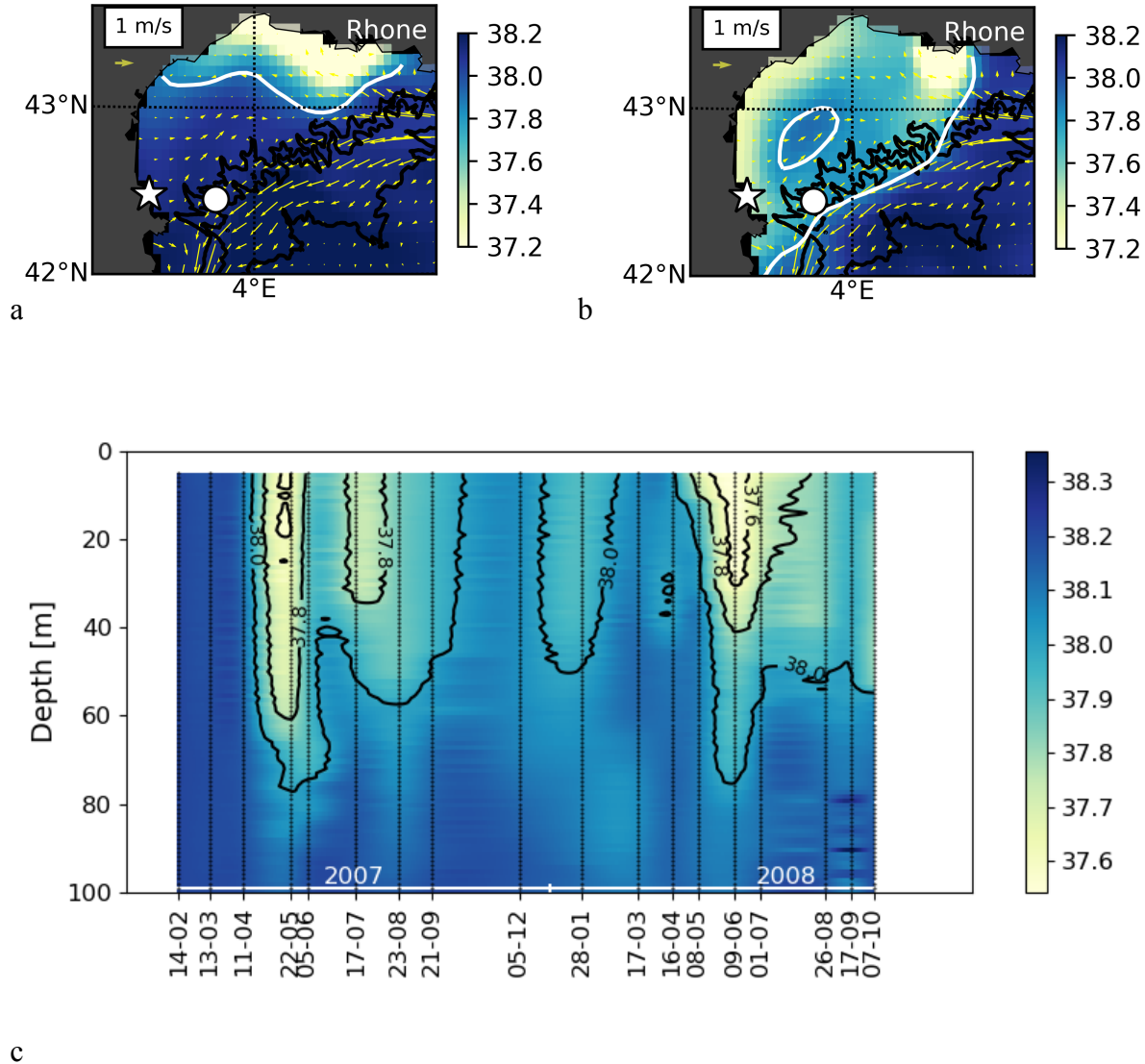


Fig. 1. Spatial and temporal distribution of salinity in the Gulf of Lion (NW Mediterranean Sea). Upper panels: Mean monthly surface salinity (4 km x 4 km) for February 2008 (left panel/a) and June 2008 (right panel/b) (February and May 2007 are shown in S1). Surface salinity distributions are provided by a circulation model overlaid with current speed and directions (yellow arrows), derived from altimetry for the date of sampling at Station MOLA (indicated by a white dot). Black lines represent isobaths (500 m, 1000 m and 2000 m). The white line denotes salinity of 37.85. Lower panel (c). Temporal changes of in situ salinity at Station MOLA

measured during the years 2007 and 2008. Star indicates Banyuls sur mer.

92 **2 Material and Methods**

93

94 2.1. Regional hydrology and seasonality

95 Station MOLA is located on the edge of the continental shelf at the south-western
96 boundary of the Gulf of Lion (42.454°N and 3.688°E) (bottom depth 600m) in the NW
97 Mediterranean Sea (Fig.1). Surface waters of this site are influenced by a northern liguro-
98 provencal current (Raimbault and de Madron, 2003) related to water masses from the Gulf of
99 Lion and the Rhone river. The climatological mean over the past 15 years reveals that the
100 presence of low salinity water (LSW) is a recurrent feature during spring periods at station
101 MOLA (Fig. S2). On a seasonal cycle, Station MOLA is characterized by vertical mixing
102 during winter leading to the input of inorganic nutrients to surface waters ($\sim 1 \mu\text{M NO}_3^- + \text{NO}_2^-$
103 , $\sim 0.05 \mu\text{M PO}_4^{3-}$) (Table S1) that sustain the spring phytoplankton bloom. During the two
104 years considered here, maximum concentrations of Chlorophyll *a* (Chl *a*; 0.75 and $0.85 \mu\text{g L}^{-1}$
105 ¹) (Fig. S3; Table S1) were observed in the upper 40m in March 2007 and February 2008,
106 respectively (Laghdass et al., 2010). During summer, the water column is thermally stratified
107 (Fig. S4) and concentrations of inorganic nutrients are low ($< 0.35 \mu\text{M NO}_3^- + \text{NO}_2^-$, $\sim < 0.03$
108 $\mu\text{M PO}_4^{3-}$) in the surface mixed layer (10 to 30m) and Chl *a* concentrations in surface waters
109 remain $< 0.2 \mu\text{g L}^{-1}$ (Laghdass et al., 2010) (Table S1).

110

111 2.2. Sample collection

112 Seawater was sampled between February 2007 and October 2008 on a monthly time
113 scale except for the autumn-winter period when the sampling frequency was reduced due to

114 bad weather conditions. Samples were collected in the upper 150m water column with 12 liter
115 Niskin bottles mounted on a rosette equipped with a SeaBird SBE 19 CTD. For bacterial
116 heterotrophic production, and the characterization of dissolved organic matter 8 depths in the
117 upper 150m were sampled. For the determination of microbial community respiration and net
118 community production 6 depths in the euphotic layer were sampled (upper 80m).

119

120 2.3. Bacterial heterotrophic production, microbial community respiration and net community 121 production

122 We used (^3H) leucine incorporation and the centrifugation method to determine bacterial
123 heterotrophic production (BP) (Smith & Azam, 1992). The detailed protocol and data are
124 reported in (Obernosterer et al., 2008; Laghdass et al., 2010). Bacterial growth rates (BGR)
125 were determined by dividing BP by cell biomass derived from bacterial abundance. To
126 convert abundance to biomass, we applied a conversion factor of $12.4 \text{ fg C cell}^{-1}$ (Fukuda et
127 al. 1998). We measured microbial community respiration (CR) and net community production
128 (NCP) from changes in the concentration of dissolved oxygen (O_2) in 24h light and dark
129 incubations as described in (Lefèvre et al. 2008). Incubations were performed at
130 Photosynthetically Active Radiation (PAR) levels of 1%, 4%, 8%, 25%, 50% and 100% of
131 surface values, using optical density filters (Nickel screens). The outdoor incubators were
132 connected with a running seawater system to maintain the incubation bottles at the
133 temperature of the mixed layer. The 1% light depth was below the mixed layer depth during
134 spring and summer with differences in temperature ranging between 2°C in May-June and up
135 to 5° in August (Table S1). The measurements at time zero and all incubations were done in 8
136 replicate 125 mL borosilicate glass bottles. Dissolved O_2 concentration was determined by

137 spectrophotometric detection of iodine following the Winkler reaction, using a Hitachi U-
138 3010 Spectrophotometer equipped with a sipper system (Labasque et al., 2004).

139

140 2.4. Dissolved organic carbon and total hydrolysable amino acids

141 Samples for dissolved organic carbon (DOC) and dissolved total hydrolysable amino
142 acids (THAA) were filtered through two pre-combusted glass fiber filters (Whatman GF/F
143 25mm, 0.7 μm nominal pore size). Samples for DOC were acidified (H_3PO_4 , pH 2) and stored
144 in pre-combusted glass tubes at room temperature in the dark until analysis. DOC was
145 analyzed by high temperature catalytic oxidation (HTCO) technique (Benner and Strom,
146 1993) using a Shimadzu TOC-5000 analyzer.

147 THAA were analyzed by high performance liquid chromatography (HPLC) after liquid-
148 phase acid hydrolysis according to a recent protocol described by (Escoubeyrou and
149 Tremblay, 2014). This protocol uses a high volume (100 μL) of undiluted samples injected on
150 a robust hybrid C18 column. HPLC analyses were performed on an Ultimate 3000 Dionex
151 system equipped with an autosampler and a fluorescence detector (excitation at 335 nm,
152 emission at 450 nm). The separation was done with a Phenomenex Gemini C18 column
153 (250x4.6 mm, 5 μm , 110 \AA) and a Security Guard column (4 x 3 mm). We used the Dauwe
154 Degradation Index (DI, (Dauwe and Middelburg, 1998)) calculated based on the relative
155 distribution of individual AA and adapted for DOM (as reported in Peter et al., 2012, Davis
156 et al 2009; Kaiser and Benner 2009) as an indicator for the degradation state of DOM. The
157 original DI was calculated from Principal Component Analysis (PCA) applied to diagenetic
158 altered sediments were $\text{DI} = \sum ((\text{mol}\% \text{ amino acid} - \text{average mol \% amino acid})/\text{standard}$

159 deviation mol%)* PCA amino acid score) .

160

161 **3 Results and Discussion**

162

163 To investigate whether LSW revealed a specific biogeochemical signature at Station
164 MOLA, we compared the depth profiles of microbial heterotrophic activity and of the DOM
165 characteristics at the dates of the most pronounced salinity anomalies during the observation
166 period (May 22 2007 and June 9 2008) with those of the annual medians (Fig. 2).

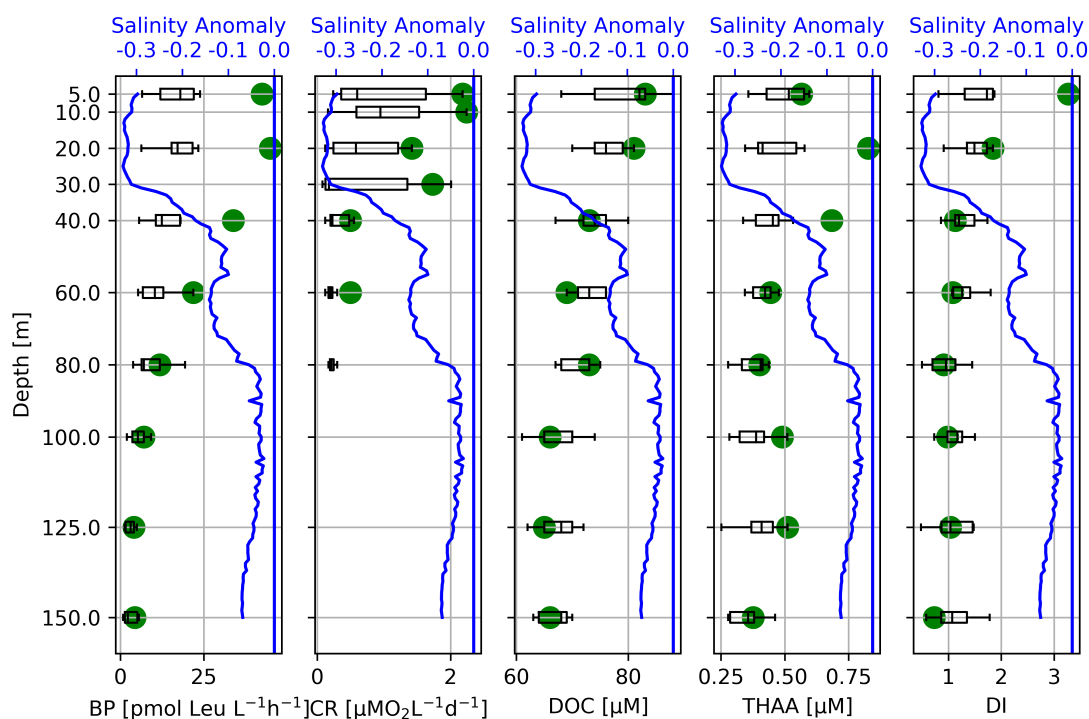


Fig. 2. Depth profiles of salinity anomaly (blue line), microbial activity and DOM characteristics (box plots) at Station MOLA for the year 2008. The black lines across the boxes represent the median annual values. The ends of the boxes define the 25th and 75th percentile and the error bars represent the 10th and 90th percentiles. Dots represent the individual value for the date for which the salinity anomaly is shown (June 9 2008). Results for 2007 are shown in Fig. S5. BP: Bacterial heterotrophic production ($\text{pmol Leu L}^{-1} \text{h}^{-1}$), CR: Community Respiration ($\mu\text{M O}_2 \text{L}^{-1} \text{d}^{-1}$), DOC: Dissolved Organic Carbon (μM), THAA: Total Hydrolyzable Amino Acids (μM), DI: Dauwe Degradation Index.

167

168 The extent of the salinity anomalies of about -0.3 was similar in both years, but lower
 169 salinity waters were present to 60m in 2007 and constrained to the upper 30m in 2008. The
 170 annual median values of BP varied between 10-20 $\text{pmol Leu L}^{-1} \text{h}^{-1}$ in the upper 60 m water
 171 column and they were below 5 $\text{pmol Leu L}^{-1} \text{h}^{-1}$ ≥ 100 m for both years. During LSW, rates of
 172 BP were 2-4 times higher than the annual medians in the upper 40 m water column, a pattern

173 that was particularly pronounced in 2008. Annual medians of CR varied between 0.5 and 1 μmol
174 $\text{O}_2 \text{ L}^{-1} \text{ d}^{-1}$ in the upper 40 m water column and they were $< 0.5 \mu\text{mol O}_2 \text{ L}^{-1} \text{ d}^{-1}$ below this depth.
175 During LSW in 2008, rates of CR were up to 3-fold higher than the annual median, while
176 differences were minor in 2007. In addition, bacterial growth rates revealed highest values (0.17
177 to 0.26 d^{-1}) during the LSW of both years (Table S1). These observations indicate a signature of
178 enhanced microbial heterotrophic activity within LSW that was more pronounced in 2008 than
179 in 2007, possibly due to a stronger dilution with surrounding water masses during our first year
180 of observation.

181 To link these observations to the characteristics of DOM, we considered bulk DOC, the
182 concentration of dissolved THAA and the DI (Fig.2). Annual medians of bulk DOC
183 concentrations varied between $60 \mu\text{M}$ and $80 \mu\text{M}$ in the upper 150 m water column. In surface
184 waters, we did not observed any pronounced differences between the annual medians and LSW.
185 This is most likely due to the high DOC concentrations in surface waters during summer
186 observed at our study site (Laghdass et al., 2010). The accumulation of DOC in stratified surface
187 waters during summer is a recurrent phenomenon reported for Mediterranean waters (Avril,
188 2002, Romera-Castillo et al., 2010, Sánchez-Pérez et al., 2016) and other oligotrophic oceans
189 (for BATS- (Carlson et al., 2002); for HOTS- (Mopper and Schultz, 1993)). Inorganic nutrient
190 limitation of heterotrophic microbes has been suggested as the underlying mechanism for the
191 observed build-up of DOC in stratified surface waters (Thingstad and Rassoulzadegan, 1999).
192 This idea is supported by short-term (24h) incubation experiments performed at our study site
193 revealing that labile organic carbon and phosphorus were the main limiting factors of BP during
194 summer (Laghdass et al. 2012).

195 Amino acids represent a quantitatively significant and biologically labile fraction of
196 DOM, and temporal or spatial changes of this pool can therefore provide insights to
197 modifications of DOM characteristics (Davis et al., 2009, Kaiser and Benner, 2012). The
198 concentrations of dissolved THAA varied between 262 and 1027 nM, with higher concentrations
199 in the upper 40m. Within LSW in 2008, THAA concentrations were up to 2-fold higher than the
200 annual median, a pattern that was absent in 2007 (Fig. 2 and Fig S5). The contribution of
201 dissolved THAA to DOC, referred to as the amino acid yield, varied between 1.4-5.6% in the
202 upper 150 m water column. The depth profiles of the amino acid yields during the LSW events
203 were similar to those observed for dissolved THAA (data not shown). The DI is a chemical
204 based indicator of the degradation state of organic matter, based on the relative distribution of
205 individual amino acids (Dauwe and Middelburg 1998). Over the course of organic matter
206 degradation, certain amino acids are selectively utilized resulting in a decrease of the DI (Dauwe
207 et al. 1999). Based on the amino acid composition of marine plankton and sediment organic
208 matter from varying depth layers, the DI was initially proposed to characterize the degradation
209 stage of particulate organic matter. Its application has been extended to DOM in a range of
210 marine (Amon et al., 2001, Davis et al., 2009, Tremblay et al., 2015; Kaiser and Benner 2009)
211 and freshwater environments (Ylla et al. 2011, Peter et al. 2012), with values ranging from -3 to
212 +5. The observed decreases in the DI over the course of biodegradation experiments further
213 suggest this index to reflect changes in DOM bioavailability (Amon et al. 2001; Rochelle-
214 Newall et al. 2004). At Station MOLA, the annual median DI varied between 1 and 1.5 and no
215 pronounced depth profile was detectable over the 150 m water column. These positive values are
216 indicative of overall diagenetically fresh DOM (Davis et al., 2009, Kaiser and Benner, 2009,
217 Peter et al., 2012). In LSW, the DI was noticeably higher in the upper 20m as compared to the

218 respective average median values in both years (Fig. 2 and Fig S5). Thus, DOM in the
219 uppermost water layer of LSW had a different chemical signature that is indicative of more
220 bioavailable DOM. Complementary to the analyses illustrated in Fig. 2, we carried out a
221 Principal Component Analysis (PCA) with the combined biogeochemical and microbial
222 parameters of the two years from surface waters (5m) (Fig. S6). The PCA analysis revealed one
223 group consisting of samples from May and June from both years being explained by variables of
224 microbial activity (BP, $r = 0.85$; CR, $r = 0.77$; BGR, $r = 0.88$) and features of DOM (dissolved
225 THAA concentration, $r = 0.48$; amino acid yields, $r = 0.21$; DI, $r = 0.52$) that were positively
226 correlated with PC1. By contrast, this group was also explained by salinity, which was
227 negatively correlated with PC1 ($r = -0.81$). Taken together, these results suggest that LSW
228 contains bioavailable DOM that sustains enhanced microbial heterotrophic activity in the
229 offshore NW Mediterranean Sea.

230 This observation raises the question of the origin of the DOM within LSW. The LSW
231 that we observe at Station MOLA most likely originates from the Rhone River and its plume
232 (Fig. 1). The pronounced pattern observed within LSW could be due to an enrichment in
233 compounds from recent primary production occurring in the vicinity of the estuary (Diaz et al.,
234 2008) and in small amounts of riverine, terrigenous DOM. Besides these sources that can be
235 considered allochthonous to our study site, *in situ* primary production within the LSW could fuel
236 the observed enhanced microbial activity. To address this question we followed the pattern in
237 euphotic depth integrated fluxes of net community production (NCP) and CR. Highest fluxes of
238 NCP were observed in March and April in both years, corresponding to the spring bloom period
239 (Fig. 3). During the LSW in May and June 2007, NCP was negative and associated with highest
240 depth-integrated fluxes of CR, illustrating a switch from net autotrophy in March and April to

241 net heterotrophy in May and June 2007. During the LSW event in 2008, euphotic layer
 242 integrated fluxes of CR were again enhanced and associated with low positive (June 2008) and
 243 negative (July 2008) fluxes of NCP (Fig. 3). These NCP fluxes indicate low *in situ* primary
 244 production in LSW. The depletion in nitrate and phosphate in LSW (Table S1) could be the
 245 limiting factor of autotrophic activity, while heterotrophs can utilize these elements in organic
 246 form. These results support the idea that heterotrophic microbial processes are favored as
 247 compared to autotrophic processes within these buoyant water masses, suggesting that external
 248 rather than *in situ* produced DOM sustains heterotrophic activity.

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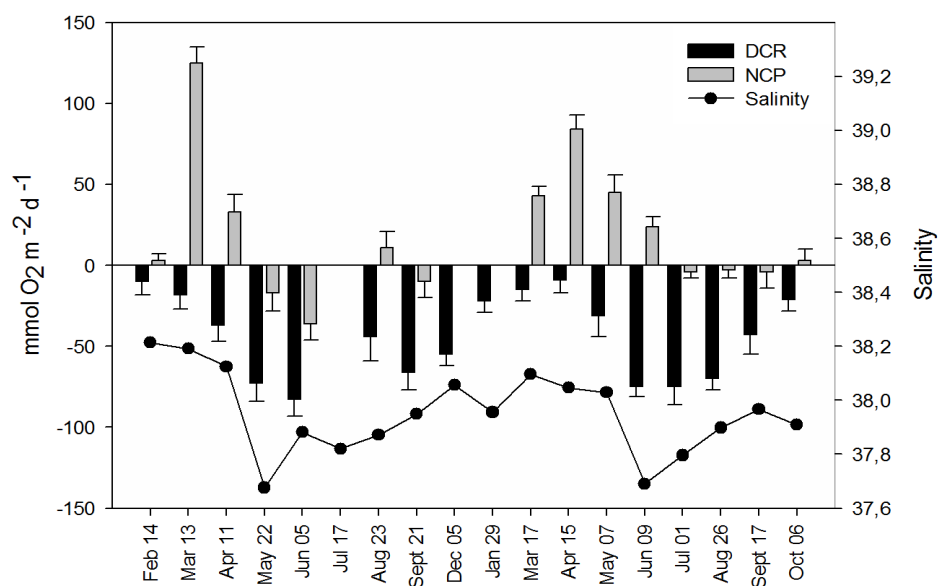


Fig. 3. Euphotic depth integrated fluxes of Net Community Production (NCP) and Community Respiration (CR) and salinity at 5m depth. Results are shown for 2007 (February 14 to December 5) and 2008 (January 29 to October 6).

251

252 Our observations from a time-series site in the NW Mediterranean Sea extend previous studies
253 within LSW lenses in close vicinity to the Rhone river estuary and they provide insights on how
254 biological activity in these water masses could change with time and distance from its origin.
255 Direct measurements and satellite observations report increased autotrophic productivity in
256 coastal LSW lenses as compared to surrounding marine waters (Auger et al., 2011, Diaz et al.,
257 2008), and the high NCP determined *in situ* (Joux et al., 2009) suggests net autotrophy of the
258 system in the vicinity of the estuary. With increasing distance from the plume and time since
259 formation, inorganic nutrients are depleted and primary production is based on regenerated
260 nutrients (Diaz et al., 2008). This suggests a scenario where autotrophic processes dominate in
261 early stages of LSW and heterotrophic processes become more important with distance and time.

262 Primary production in coastal marine environments and export of terrigenous DOC are
263 potential sources of organic carbon for the open oligotrophic ocean (Smith and MacKenzie
264 1987, Bauer and Druffel 1998; Medeiros et al. 2015) and proposed transport mechanisms are
265 physical features such as for example mesoscale eddies, internal waves or currents. The resulting
266 spatio-temporal decoupling between the origin of organic matter in the coastal zone and its
267 respiration in open waters was suggested to explain in part the imbalance between primary
268 production and respiration in the oligotrophic ocean (Duarte et al. 2013; Williams et al. 2013).
269 The LSW could represent this linkage in the NW Mediterranean Sea. In addition to recent and *in*
270 *situ* primary production, photochemical transformations of DOM could be an important
271 mechanism in providing bioavailable substrates in these buoyant surface water masses in
272 particular when compounds of terrigenous origin are present (Abboudi et al., 2008, Amon and
273 Benner, 1996, Obernosterer and Herndl, 2000). The combination of a suite of tools including

274 satellite-based and autonomous instrumentation offers the possibility for a more detailed
275 characterization and adapted sampling strategy of these mesoscale features at different locations
276 in the open ocean. Taken together, our study illustrates the potential of water masses of estuarine
277 origin to affect biological activity in the offshore oligotrophic ocean. The extension, lifetime and
278 occurrence of these types of buoyant water masses will govern their impact on marine
279 biogeochemistry and ecosystem functioning at different scales and distances from shore.

280

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294

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